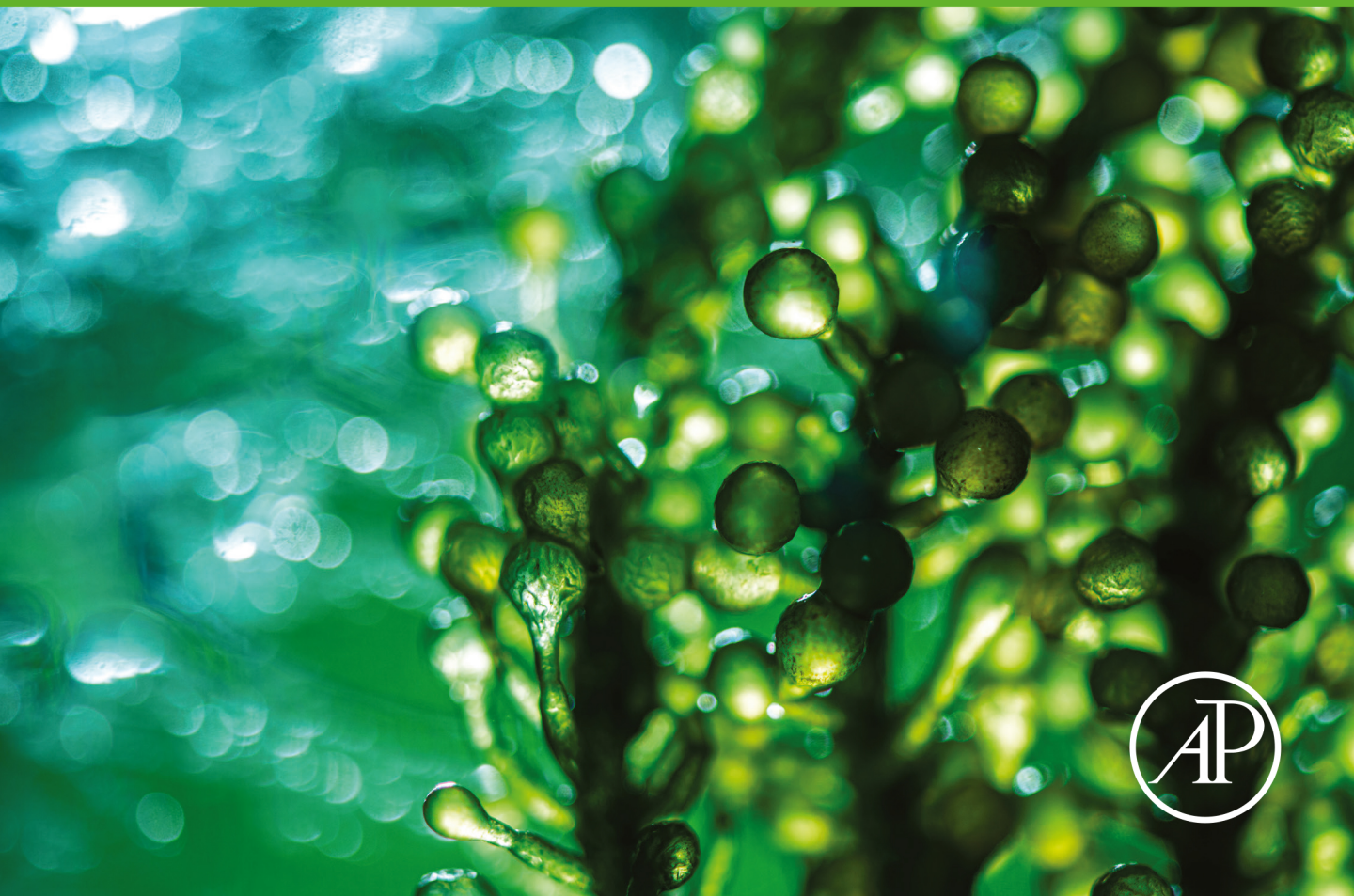
Edited by
Eduardo Jacob-Lopes | Mariana Manzoni Maroneze
Maria Isabel Queiroz | Leila Queiroz Zepka

Handbook of Microalgae-Based Processes and Products

Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds



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Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds

Edited by

Eduardo Jacob-Lopes
Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Mariana Manzoni Maroneze
Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Maria Isabel Queiroz
School of Chemistry and Food, Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil

Leila Queiroz Zepka
Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

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This book is dedicated to the memory of Eduardo Rodrigues Lopes.

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Contents

Contributors ..... *xxi*
Foreword by Michael A. Borowitzka ..... *xxvii*

PART 1 FUNDAMENTALS

Chapter 1: Microalgae biotechnology: A brief introduction *3*
Yusuf Chisti
1.1 Introduction 3
1.2 A brief history of everything 4
1.3 Algae as human food 7
1.3.1 Microalgae and seafood 10
1.4 Nitrogen for sustainable agriculture 13
1.5 Potential biofuels and other organic chemicals 15
1.6 Carbon sequestration by microalgae 15
1.7 Algae-based treatment of wastewaters 17
1.8 Harmful algae 18
1.9 Commercial production and processing of microalgal biomass 19
1.10 Concluding remarks 20
References 20

Chapter 2: Morphophysiological, structural, and metabolic aspects of microalgae *25*
Maria Isabel Queiroz, Juliana Guerra Vieira, Mariana Manzoni Maroneze
2.1 Introduction 25
2.2 Morphological aspects of microalgae 26
2.3 Cell ultrastructure of microalgae 27
2.3.1 Prokaryotic cells 29
2.3.2 Eukaryotic cells 29
2.4 General considerations on microalgae 30
2.4.1 Prokaryotic microalgae (cyanobacteria) 31
2.4.2 Eukaryotic microalgae 33
2.5 Metabolic aspects of microalgae 36
2.5.1 Photosynthetic metabolism 36
2.6 Concluding remarks 44
References 44

Chapter 3: Microalgae culture collections, strain maintenance, and propagation *49*
Sergio de Oliveira Lourenço
3.1 Living biological collections 49
3.2 The operation of microalgae culture collections 50
3.2.1 Basic laboratory infrastructure 52
3.2.2 Identification of cultures 61
3.2.3 Propagation of cultures 63
3.2.4 Conditions of keeping microalgae in culture collections 66
3.2.5 Strain quality control and routine problems 68
3.2.6 Cryopreservation of microalgae in culture collections 70
3.2.7 Microalgae as models of organisms in genomics, proteomics, and GMO studies—The role of culture collections 77
3.3 Some microalgae culture collections across the world 78
3.4 Concluding remarks 83
References 83

Chapter 4: Synthetic biology applied to microalgae-based processes and products *85*
Dinesh Kumar Saini, Sunil Pabbi, Anil Prakash, Pratyoosh Shukla
4.1 Introduction 85
4.1.1 Aspects of synthetic biology 86
4.2 Feasibility of microalgae for synthetic biology to produce various products 88
4.2.1 Biofuels 88
4.2.2 Various other bioactive products 91
4.3 Challenges to developing microalgae as a chassis for synthetic biology 92
4.4 Future perspective 94
4.5 Conclusion 94
Acknowledgments 95
References 95

PART 2 MICROALGAE-BASED PROCESSES

Chapter 5: Photobioreactor design *101*
Jose C. Merchuk
5.1 Chemical reactors versus photobioreactors 101
5.1.1 The chemical reactor 101
5.1.2 The photobioreactor 103
5.2 Kinetics of photo-biosynthesis 106
5.2.1 *P-I* charts 106
5.2.2 Other kinetic models 106
5.2.3 PSF 106

5.3 Fluid dynamics relevance 108
5.3.1 The effects of fluid dynamics on photosynthesis 108
5.3.2 Photosynthesis and ordered mixing 110
5.4 Ingenuity and inventiveness 116
5.5 Advanced models 122
5.6 Conclusions 124
Acknowledgment 124
References 125

Chapter 6: Microalgae production systems 127
Francisco Gabriel Acién Fernández, José María Fernández-Sevilla, Bernardo Llamas Moya, Emilio Molina Grima
6.1 Introduction 127
6.2 Major factors on microalgae production 129
6.2.1 Microalgae requirements 130
6.2.2 Photobioreactor capacity 135
6.3 Photobioreactor types 146
6.3.1 Raceway reactors 146
6.3.2 Tubular reactors 150
6.3.3 Other designs 154
6.3.4 Thin-layer reactors 156
6.4 Future trends 158
References 158

Chapter 7: Wastewater treatment based in microalgae 165
Alfredo de Jesús Martínez-Roldán, Rosa Olivia Cañizares-Villanueva
7.1 Introduction 165
7.2 Wastewater treatment 166
7.3 Microalgae for WW treatment 168
7.3.1 Removal of nitrogen and phosphorus 169
7.3.2 Heavy metal removal 172
7.3.3 Elimination of colorants 176
7.3.4 Elimination of emerging pollutants 177
7.4 Conclusion 178
References 179
Further reading 184

Chapter 8: Carbon dioxide capture and utilization using microalgae 185
Patricia Ruiz-Ruiz, Adrián Estrada, Marcia Morales
8.1 Introduction 185
8.2 Photosynthesis and carbon dioxide fixation by microalgae 186
8.2.1 The light reactions of photosynthesis 186
8.2.2 CO_2 fixation: The Calvin-Benson cycle and the CO_2 concentrating mechanism 187
8.3 Cultivation systems for CO_2 capture using microalgae 189
8.3.1 CO_2 sources and physicochemical properties 189

8.3.2 Strains 190
8.3.3 Physicochemical aspects involved in CO_2 capture by microalgae 191
8.3.4 Culture systems for CO_2 capture with microalgae 192
8.3.5 Carbon balances for evaluation of microalgae-based CO_2 capture systems 194
8.4 Strategies for CO_2 capture improvement 197
8.4.1 Genetic engineering and metabolic modifications for the improvement of CO_2 capture 197
8.4.2 Photobioreactors: Enhancing design for CO_2 capture improvement 199
8.5 Remarks and conclusion 201
Acknowledgments 202
References 202

Chapter 9: Dewatering and drying of algal cultures 207

Jaison Jeevanandam, Michael Kobina Danquah

9.1 Introduction 207
9.2 Historic timeline of algal biomass production 208
9.3 Conventional dewatering methods of algae processing 209
9.3.1 Flocculation 209
9.3.2 Centrifugation 210
9.3.3 Filtration 211
9.3.4 Gravity sedimentation 211
9.3.5 Flotation 212
9.3.6 Electrophoresis methods 213
9.4 Conventional drying methods for algae processing 213
9.4.1 Solar drying 213
9.4.2 Convective drying 214
9.4.3 Spray-drying 214
9.4.4 Freeze-drying 215
9.4.5 Other drying methods 215
9.5 Latest trends in dewatering and drying of algae for commercialization 216
9.6 Future perspective 217
9.7 Conclusion 218
References 218

Chapter 10: Microalgae harvesting techniques 225

Ana Filipa Esteves, Cíntia Jesus Almeida, Ana Luísa Gonçalves, José Carlos Pires

10.1 Introduction 225
10.2 Challenges in microalgal biomass harvesting 228
10.3 Effective harvesting techniques for microalgal biomass recovery 229
10.4 Harvesting methods 231
10.4.1 Gravity sedimentation 232
10.4.2 Flocculation 235
10.4.3 Flotation 253
10.4.4 Centrifugation 259
10.4.5 Filtration methods 261

10.5 Selection of the most appropriate harvesting technique 268
10.6 Research needs 270
10.7 Conclusions 271
Acknowledgments 271
References 272

Chapter 11: Extraction of biomolecules from microalgae 283
Yulin Hu, Amarjeet Bassi
11.1 Introduction 283
11.2 Extraction approaches for carotenoids 285
11.2.1 Cell disruption 285
11.2.2 Extraction methods 290
11.2.3 Purification 292
11.3 Biofuels production 293
11.3.1 Biodiesel 293
11.3.2 Bioethanol 298
11.3.3 Bio-crude oil 299
11.4 Future perspectives and conclusions 302
Acknowledgment 303
References 303

PART 3 MICROALGAE-BASED PRODUCTS

Chapter 12: Biogas from microalgae 311
Bárbara Rincón
12.1 Introduction 311
12.2 Anaerobic digestion of microalgae 312
12.3 Bacteria and archaea in the AD of microalgae 315
12.4 Main drawbacks affecting AD of microalgae 315
12.4.1 Complex cell walls 315
12.4.2 Low C/N ratios 317
12.4.3 Humidity of microalgal biomass 319
12.4.4 Salinity 319
12.5 AD of spent microalgae 320
12.6 Nutrient and CO_2 recycling: Toward a sustainable closed loop 321
12.7 Biorefinery concept 323
12.8 Conclusions 323
References 323

Chapter 13: Biodiesel from microalgae 329
Zheting Bi, Brian He
13.1 Introduction 329
13.2 Strains of microalgae for biodiesel production 331
13.2.1 Lipid-rich microalgae as a biodiesel feedstock 331
13.2.2 Cultivation of high lipid bearing microalgae 332
13.2.3 Process parameters affecting microalgae cultivation 335

13.2.4 Practical techniques for stimulating lipid production in microalgae 338
13.2.5 Genetically engineered strains of microalgae for biodiesel production 342
13.3 Microalgae preparation for biodiesel production 343
13.3.1 Harvesting microalgae 343
13.3.2 Drying microalgae 345
13.3.3 Lipid extraction 348
13.4 Microalgal biodiesel production 350
13.4.1 Transesterification of microalgal lipids 350
13.4.2 Supercritical alcohol transesterification for algal biodiesel production 352
13.4.3 Process parameters of in situ supercritical transesterification of microalgal lipids 354
13.5 Summary and perspectives 358
References 361

Chapter 14: Bioethanol production from microalgae 373
Didem Özçimen, Anıl Tevfik Koçer, Benan İnan, Tugba Özer
14.1 Introduction 373
14.2 Microalgae 374
14.3 Bioethanol 376
14.4 Bioethanol production from microalgal biomass 379
14.4.1 Microalgae cultivation 379
14.4.2 Harvesting and drying 381
14.4.3 Pretreatments and saccharification 383
14.4.4 Fermentation and distillation 384
14.5 Conclusion 385
References 386

Chapter 15: Biohydrogen from microalgae 391
Harshita Singh, Debabrata Das
15.1 Introduction 391
15.2 Pathways of hydrogen production from microalgae 392
15.2.1 Biophotolysis 393
15.2.2 Biohydrogen production using microalgal biomass as a feedstock in dark fermentation 402
15.3 Challenges and future perspectives of biohydrogen production from microalgae 409
15.4 Conclusion 413
Acknowledgment 413
References 413
Further reading 418

Chapter 16: Microalgae single cell oil 419
Yusuf Nazir, Hafiy Halim, Pranesha Prabhakaran, Aidil Abdul Hamid, Yuanda Song
16.1 Introduction 419
16.1.1 Term definition 420
16.1.2 A brief history of MSCO development 420
16.2 Microalgae regarded as MSCOPS, their major fatty acid compositions and potential applications 424
16.2.1 Potential application of MSCO as biofuel feedstock 424
16.2.2 Potential application of MSCO in PUFA production 426
16.3 Biosynthetic pathway for MSCO biosynthesis and its regulation 427
16.3.1 Biosynthetic pathway for MSCO biosynthesis 427
16.3.2 Regulation of fatty acid biosynthetic pathway for improved MSCO production by manipulating the environmental stresses and metabolic engineering 431
16.4 Cultivation system of microalgae for MSCO production 435
16.5 Current market, challenges, and future prospects of MSCO 437
16.6 Conclusions 439
References 439

Chapter 17: Microalgal bio-fertilizers 445
Arun Kumar, Jay Shankar Singh
17.1 Introduction 445
17.2 Microalgae as bio-fertilizer 447
17.2.1 Nitrogen fixation 447
17.2.2 Improving availability of phosphorus 449
17.2.3 Improving physical properties of soil 450
17.2.4 Reclamation of saline soil 450
17.2.5 Plant growth promoters 451
17.3 Microalgae-based bio-fertilizer 452
17.3.1 Cultivation 452
17.3.2 Harvesting 454
17.3.3 Biomass valorization 455
17.4 Microalgae cultivation in wastewater 455
17.5 Integration of microalgae biomass production with wastewater treatment 457
17.6 Conclusions 457
References 459

Chapter 18: Pigments from microalgae 465
Fernando Pagels, Daniel Salvaterra, Helena Melo Amaro, Ana Catarina Guedes
18.1 Introduction 465
18.2 Industrial application of microalgal pigments 468
18.2.1 Pharmaceutical use and prospecting 468
18.2.2 Food and nutraceuticals 470
18.2.3 Feed 471
18.2.4 Other applications 472

18.3 Pigments bioprocesses 472
18.3.1 Phycocyanin 472
18.3.2 β-Carotene 475
18.3.3 Astaxanthin 477
18.3.4 Lutein 477
18.4 Economical interest and challenges 479
18.4.1 Market size 480
18.4.2 Constraints 482
18.4.3 Prospects and solutions 484
Acknowledgments 485
References 485

Chapter 19: Nutritional quality and bioactive properties of proteins and peptides from microalgae 493

Caleb Acquah, Sean M. Tibbetts, Sharadwata Pan, Chibuike Udenigwe

19.1 Introduction 493
19.2 Microalgae proteins 495
19.2.1 Microalgae protein extraction 497
19.3 Bioactive peptides from microalgae 498
19.4 Nutritional quality of microalgae proteins 500
19.4.1 Microalgae amino acid profile 505
19.4.2 Digestibility of microalgae proteins 506
19.4.3 Protein utilization parameters 507
19.5 Challenges and opportunities 518
References 520

Chapter 20: Bioactive polysaccharides from microalgae 533

Nastasia Prybylski, Claire Toucheteau, Hicham El Alaoui, Nicolas Bridiau, Thierry Maugard, Slim Abdelkafi, Imen Fendri, Cédric Delattre, Pascal Dubessay, Guillaume Pierre, Philippe Michaud

20.1 Introduction 533
20.2 Structural diversity of polysaccharides from microalgae 535
20.3 Processes production, extraction, and purification of EPS from microalgae and cyanobacteria 540
20.4 Polysaccharides from microalgae as antiinfectious agents 551
20.4.1 Antiviral activity of PS from microalgae and cyanobacteria 551
20.4.2 Antibacterial activity of PS from microalgae and cyanobacteria 553
20.4.3 Antifungal activities of PS from microalgae and cyanobacteria 554
20.5 Bioactivity and structure-activity relationships 555
20.5.1 Antioxidant activity and free radical scavenging 556
20.5.2 Anticancer activity 557
20.5.3 Immunomodulatory activity 558
20.5.4 Antiinflammatory activity 559
20.5.5 Anticoagulant activity 560
20.5.6 Antitussive activity 561

20.5.7 Antilipidemic and antiglycemic activities 561
20.5.8 Antiaging activity 562
20.6 Future perspectives 562
References 563

Chapter 21: Sterols from microalgae 573
Mariane Bittencourt Fagundes, Raquel Guidetti Vendruscolo, Roger Wagner
21.1 Introduction 573
21.2 Structural aspects of sterols 574
21.3 Sterol biosynthesis 576
21.4 Sterols 581
21.5 Sterols in cyanobacteria 582
21.6 Environmental influence on microalgae sterols production 583
21.7 Sterols analysis 584
21.8 Bioactivity of sterols 587
21.9 Industrial sterol production 589
21.10 Conclusion 590
References 591

Chapter 22: "Bioplastics from microalgae"—Polyhydroxyalkanoate production by cyanobacteria 597
Martin Koller
22.1 Introduction 598
22.2 Polyhydroxyalkanoate (PHA) biopolyesters as a potential remedy of today's plastic scenario 600
22.2.1 General aspects about PHA biopolyesters 600
22.2.2 Raw materials available for economic and sustainable PHA biopolyester production 603
22.3 Cyanobacteria as PHA-producing cell factories 606
22.3.1 General aspects of PHA biosynthesis by cyanobacteria 606
22.3.2 Enzymatic background of PHA biosynthesis by cyanobacteria 610
22.4 Factors affecting cyanobacterial PHA composition, production, and other storage compounds 613
22.4.1 Composition of PHA produced by cyanobacteria 613
22.4.2 Production and intracellular degradation conditions for PHA in cyanobacteria 614
22.4.3 Other storage compounds produced by cyanobacteria in parallel with PHA biosynthesis 616
22.5 Comparison of autotrophic and mixotrophic cultivation of cyanobacteria 618
22.5.1 *Spirulina* sp. under different carbon-supply regimes 618
22.5.2 *Nostoc* sp. under different carbon-supply regimes 619
22.5.3 *Anabaena* sp. under different carbon-supply regimes 620
22.5.4 *Synechocystis* sp. under different carbon-supply regimes 620
22.5.5 *Aulosira* sp. 622

22.6 Extremophilic cyanobacterial PHA producers 623
22.6.1 PHA production by thermophilic cyanobacteria 623
22.6.2 PHA production by halophilic cyanobacteria 624
22.6.3 Cyanobacteria cultivated at extreme pH conditions 625
22.7 Mixed microbial culture cultivations 625
22.8 Genetic engineering—A tool to boost PHA biosynthesis by cyanobacteria 627
22.9 Production scales and production techniques for cyanobacterial PHA production 632
22.10 Conclusion 636
References 636

Chapter 23: UV-screening from microalgae 647

Ana Lucía Morocho-Jácome, Eleane de Almeida Cezare-Gomes, João Carlos Monteiro de Carvalho, Rafael Sauce, Catarina Rosado, Maria Valéria Robles Velasco, André Rolim Baby

23.1 Introduction 647
23.2 UV-induced skin problems 648
23.3 UV-absorbing compounds from microalgae 649
23.3.1 Carotenoids 649
23.3.2 Mycosporine-like amino acids 651
23.3.3 Scytonemin 653
23.4 Industrial applications of UV screening from microalgae 653
23.5 Conclusions 653
References 654

Chapter 24: Volatile organic compounds from microalgae 659

Karem Rodrigues Vieira, Pricila Nass Pinheiro, Leila Queiroz Zepka

24.1 Introduction 659
24.2 Biosynthesis mechanism of volatile organic compounds in microalgae 660
24.3 Environmental factors affecting VOCs production from microalgae 666
24.4 Application of VOCs from microalgae 668
24.5 Techniques for VOCs recovery 678
24.6 Conclusions and future perspectives 681
References 682

Chapter 25: Microalgae as enzymes biofactories 687

Michele Rigon Spier, Bianca Peron-Schlosser, Luana Cristina Paludo, Luis Alberto Gallo-García, Cristina Maria Zanette

25.1 Introduction 687
25.2 Microalgal enzymes 689
25.2.1 Amylases 689
25.2.2 Peroxidases 693
25.2.3 Proteases 694
25.2.4 Lipases 694
25.2.5 Phytases 695

25.2.6 Superoxide dismutases (SODs) 695
25.2.7 Carbonic anhydrase 696
25.2.8 Laccases 698
25.2.9 Galactosidases 698
25.2.10 L-asparaginase 699
25.3 Final considerations 700
References 701

PART 4 ENGINEERING APPROACHES APPLIED TO MICROALGAL PROCESSES AND PRODUCTS

Chapter 26: Process integration applied to microalgae-based systems 709
Ihana Aguiar Severo, Mariany Costa Deprá, Rosangela Rodrigues Dias, Eduardo Jacob-Lopes
26.1 Introduction 709
26.2 A general overview of microalgae-based processes and products 710
26.3 Process integration fundamentals 714
26.3.1 Energy integration 715
26.3.2 Mass integration 718
26.3.3 Water integration 720
26.4 Environmental and economic indicators of microalgae integrated systems 723
26.4.1 Life cycle assessment 723
26.4.2 Economic outlooks 726
26.5 Concluding remarks 728
References 730
Further reading 735

Chapter 27: Process intensification applied to microalgae-based processes and products 737
Rajshree Amrut Patil, Shankar Balajirao Kausley, Saurabh Milind Joshi, Aniruddha Bhalchandra Pandit
27.1 Introduction 737
27.2 Process intensification at different phases of bulk production of microalgae 738
27.2.1 Selection of microalgae strain 738
27.2.2 Cultivation 740
27.2.3 Harvesting 741
27.2.4 Cell drying 748
27.2.5 Cell disruption methods 749
27.3 Process intensification methods applied to different microalgae-based products 752
27.3.1 Biodiesel 752
27.3.2 Bioethanol 756
27.3.3 Biohydrogen/biogas 759

27.3.4 Process intensification in wastewater treatment using microalgae 760
27.3.5 Process intensification in carbon dioxide capture using microalgae 761
27.3.6 Process intensification in microalgal pigments extraction 762
27.3.7 Single-cell protein for food application 763
27.4 Conclusions 763
Acknowledgments 765
References 765

Chapter 28: Microalgal biorefineries 771

Alice Ferreira, Luisa Gouveia

28.1 Introduction 771
28.2 Biorefinery concept 772
28.3 Microalgae-based biorefinery 773
28.3.1 Wastewater treatment 774
28.3.2 Carbon dioxide mitigation 774
28.3.3 Biofuel generation 775
28.3.4 Valuable products obtained from microalgae 777
28.3.5 Microalgae-based bioplastics 781
28.3.6 Microalgae-based biofertilizers 781
28.4 Examples of microalgae-based biorefinery 782
28.4.1 *Nannochloropsis* sp. biorefinery 782
28.4.2 *Anabaena* sp. biorefinery 783
28.4.3 *Chlorella vulgaris* biorefineries 784
28.4.4 *Chlorella protothecoides* biorefinery 786
28.4.5 *Chlamydomonas reinhardtii* biorefinery 786
28.4.6 *Dunaliella salina* biorefinery 787
28.4.7 *Dunaliella tertiolecta* biorefinery 787
28.4.8 *Arthrospira* (*Spirulina*) biorefinery 788
28.4.9 *Spirogyra* sp. biorefinery 788
28.4.10 *Scenedesmus obliquus* biorefinery 789
28.4.11 *Tetraselmis* sp. biorefinery 790
28.5 Conclusions 791
Acknowledgments 791
References 791

Chapter 29: The bioeconomy of microalgae-based processes and products 799

Mauro Vigani

29.1 Introduction 799
29.2 Algae bioeconomy and sustainability 800
29.3 Algae products and circularity 803
29.4 Supply chain characteristics 807
29.5 Algae production and trade 808
29.6 Conclusions 819
References 820

Chapter 30: Life cycle assessment of microalgae-based processes and products 823
Lucas Reijnders
30.1 Introduction 823
30.2 What is life cycle assessment (LCA) and in which respects can LCA be useful? 823
30.3 Application of peer-reviewed life cycle assessment to microalgae-based products and processes 827
30.3.1 Biofuels 828
30.3.2 Applications in the food and feed sector 833
30.3.3 Other applications 835
30.4 Conclusions 836
References 836

Chapter 31: Exergy analysis applied to microalgae-based processes and products 841
Karina A. Ojeda, Eduardo Sánchez-Tuirán, Jose Gonzalez-Diaz, Manuel Gomez-Ochoa, Vyacheslav Kafarov
31.1 Introduction 842
31.1.1 Benefits of algae biofuel 842
31.1.2 Basic algae biology 842
31.2 Cultivation and harvesting technologies 843
31.2.1 Cultivation systems 843
31.2.2 Harvesting technologies 844
31.3 Extraction technologies 844
31.4 Current state of microalgae-feedstock biorefineries 845
31.4.1 Products from algae 845
31.5 Exergy analysis in microalgae-feedstock biorefineries 846
31.5.1 Main concepts of exergy 846
31.5.2 Exergy formulation 846
31.5.3 Exergy applied to biomass processes 847
31.5.4 Exergy analysis in a microalgae-feedstock biorefinery 848
31.6 Case studies 848
31.6.1 Case 1: Biodiesel production from *Chlorella vulgaris* microalgae through acid esterification and basic transesterification using methanol 849
31.6.2 Case 2: Biodiesel production from *C. vulgaris* microalgae through a pretreatment with $ZnCl_2$ and basic transesterification with ethanol 853
References 857
Further reading 859

Chapter 32: Scale-up of microalgae-based processes 861
Niels-Henrik Norsker
32.1 Basic microalgal metabolism 861
32.1.1 Autotrophic production 863

32.1.2 Heterotrophic production 864
32.1.3 Mixotrophic production 864
32.2 Physical processes in microalgal reactors 866
32.2.1 Mixing 866
32.2.2 Mass transfer in photobioreactors 867
32.2.3 Shear stress in photobioreactors 869
32.2.4 Light in photobioreactors 870
32.3 Scalability of cultivation systems 874
32.3.1 Tubular photobioreactors 874
32.3.2 Flat panels 875
32.3.3 Closely spaced flat panel photobioreactor 876
32.3.4 Raceway ponds 876
32.3.5 Cascade flumes 876
32.4 Scale and productivity 877
32.4.1 Large diameter tubular reactor 877
32.4.2 Small diameter tubular reactor 877
32.4.3 Medium diameter tubular reactor 878
32.4.4 Flat panel 878
32.4.5 Tube length and diameter in tubular reactors 878
References 880

Index 885

Contributors

Slim Abdelkafi Unité de Biotechnologie des Algues, Biological Engineering Department, National School of Engineers of Sfax, University of Sfax, Sfax, Tunisia

Caleb Acquah School of Nutrition Sciences, University of Ottawa, Ottawa, ON, Canada

Cíntia Jesus Almeida LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

Eleane de Almeida Cezare-Gomes Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Helena Melo Amaro Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal

André Rolim Baby Pharmacy Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Amarjeet Bassi Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON, Canada

Zheting Bi Zoetis, Lincoln, NE, United States

Nicolas Bridiau Université de La Rochelle, UMR CNRS 7266, LIENSs, Equipe Approches Moléculaires Environnement-Santé, Département de Biotechnologies, La Rochelle, France

Rosa Olivia Cañizares-Villanueva Laboratorio de Biotecnología de Microalgas, Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de Mexico, Mexico

João Carlos Monteiro de Carvalho Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Yusuf Chisti School of Engineering, Massey University, Palmerston North, New Zealand

Michael Kobina Danquah Chemical Engineering Department, University of Tennessee, Chattanooga, TN, United States

Debabrata Das Advance Technology Development Center; Department of Biotechnology, Indian Institute of Technology, Kharagpur, India

Cédric Delattre Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Mariany Costa Deprá Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Rosangela Rodrigues Dias Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Pascal Dubessay Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Hicham El Alaoui Université Clermont Auvergne, CNRS, LMGE, Clermont-Ferrand, France

Ana Filipa Esteves LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

Adrián Estrada Processes and Technology Department and Doctoral/Master Program in Natural Sciences and Engineering, Metropolitan Autonomous University Campus Cuajimalpa, Mexico City, Mexico

Mariane Bittencourt Fagundes Department of Food Science and Technology, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Imen Fendri Unité de Biotechnologie des Algues, Biological Engineering Department, National School of Engineers of Sfax, University of Sfax, Sfax, Tunisia

Francisco Gabriel Acién Fernández Department of Chemical Engineering, University of Almería, Almeria, Spain

José María Fernández-Sevilla Department of Chemical Engineering, University of Almería, Almeria, Spain

Alice Ferreira LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefinery Unit, Lisbon, Portugal

Luis Alberto Gallo-García Post Graduate Program in Food Engineering, Federal University of Paraná, Curitiba, PR, Brazil

Manuel Gomez-Ochoa Process Design and Biomass Utilization Research Group (IDAB), University of Cartagena, Chemical Engineering Program, Cartagena, Colombia

Ana Luísa Gonçalves LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

Jose Gonzalez-Diaz Process Design and Biomass Utilization Research Group (IDAB), University of Cartagena, Chemical Engineering Program, Cartagena, Colombia

Luisa Gouveia LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefinery Unit, Lisbon, Portugal

Emilio Molina Grima Department of Chemical Engineering, University of Almería, Almeria, Spain

Ana Catarina Guedes Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal

Hafiy Halim Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, China

Aidil Abdul Hamid School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia, Bangi, Malaysia

Brian He Department of Biological Engineering, University of Idaho, Moscow, ID, United States

Yulin Hu Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON, Canada

Benan İnan Yıldız Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul, Turkey

Eduardo Jacob-Lopes Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Jaison Jeevanandam Department of Chemical Engineering, Curtin University, Miri, Sarawak, Malaysia

Saurabh Milind Joshi Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, India

Vyacheslav Kafarov Center for the Sustainable Development of Industry and Energy, Industrial University of Santander, School of Chemical Engineering, Bucaramanga, Colombia

Shankar Balajirao Kausley Physical Sciences Research Area, TCS Research, TRDDC, Tata Consultancy Services, Pune, India

Anıl Tevfik Koçer Yıldız Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul, Turkey

Martin Koller Office of Research Management and Service, c/o Institute of Chemistry, University of Graz; ARENA - Association for Resource Efficient and Sustainable Technologies, Graz, Austria

Arun Kumar Department of Environmental Science, Guru Nanak Khalsa College, Yamuna Nagar, Haryana, India

Sergio de Oliveira Lourenço Department of Marine Biology, Fluminense Federal University, Niterói, RJ, Brazil

Mariana Manzoni Maroneze Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Alfredo de Jesús Martínez-Roldán CONACYT-TecNM/IT Durango, Departamento de Ingenierías Química y Bioquímica; Maestría en Sistemas Ambientales, TecNM/IT Durango, Durango, Mexico

Thierry Maugard Université de La Rochelle, UMR CNRS 7266, LIENSs, Equipe Approches Moléculaires Environnement-Santé, Département de Biotechnologies, La Rochelle, France

Jose C. Merchuk Ben-Gurion University, Beer-Sheva, Israel

Philippe Michaud Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Marcia Morales Processes and Technology Department and Doctoral/Master Program in Natural Sciences and Engineering, Metropolitan Autonomous University Campus Cuajimalpa, Mexico City, Mexico

Ana Lucía Morocho-Jácome Pharmacy Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Bernardo Llamas Moya Department of Chemical Engineering, University of Almería, Almeria, Spain

Yusuf Nazir Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, China

Niels-Henrik Norsker Bioprocess Engineering, Wageningen University, Wageningen, The Netherlands

Karina A. Ojeda Process Design and Biomass Utilization Research Group (IDAB), University of Cartagena, Chemical Engineering Program, Cartagena, Colombia

Didem Özçimen Yıldız Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul, Turkey

Tugba Özer Yıldız Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul, Turkey

Sunil Pabbi Centre for Conservation and Utilisation of Blue-Green Algae (CCUBGA), Division of Microbiology, ICAR—Indian Agricultural Research Institute, New Delhi, India

Fernando Pagels Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos; Department of Biology, Faculty of Sciences, University of Porto (FCUP), Porto, Portugal

Luana Cristina Paludo Post Graduate Program in Food Engineering, Federal University of Paraná, Curitiba, PR, Brazil

Sharadwata Pan School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany

Aniruddha Bhalchandra Pandit Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, India

Rajshree Amrut Patil Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, India

Bianca Peron-Schlosser Post Graduate Program in Food Engineering, Federal University of Paraná, Curitiba, PR, Brazil

Guillaume Pierre Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Pricila Nass Pinheiro Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

José Carlos Pires LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

Pranesha Prabhakaran Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, China

Anil Prakash Department of Microbiology, Barkatullah University, Bhopal, India

Nastasia Prybylski Université Clermont Auvergne, CNRS, LMGE; Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Maria Isabel Queiroz School of Chemistry and Food, Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil

Lucas Reijnders IBED, University of Amsterdam, Amsterdam, The Netherlands

Bárbara Rincón Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain

Catarina Rosado CBIOS—Universidade Lusófona's Research Center for Biosciences and Health Technologies, Lisbon, Portugal

Patricia Ruiz-Ruiz Processes and Technology Department and Doctoral/Master Program in Natural Sciences and Engineering, Metropolitan Autonomous University Campus Cuajimalpa, Mexico City, Mexico

Dinesh Kumar Saini Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India

Daniel Salvaterra Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos; Department of Biology, Faculty of Sciences, University of Porto (FCUP), Porto, Portugal

Eduardo Sánchez-Tuirán Process Design and Biomass Utilization Research Group (IDAB), University of Cartagena, Chemical Engineering Program, Cartagena, Colombia

Rafael Sauce Pharmacy Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Ihana Aguiar Severo Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Pratyoosh Shukla Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India

Harshita Singh Advance Technology Development Center, Indian Institute of Technology, Kharagpur, India

Jay Shankar Singh Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

Yuanda Song Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, China

Michele Rigon Spier Post Graduate Program in Food Engineering, Federal University of Paraná, Curitiba, PR, Brazil

Sean M. Tibbetts Aquatic and Crop Resource Development Research Centre, National Research Council of Canada, Halifax, NS, Canada

Claire Toucheteau Université de La Rochelle, UMR CNRS 7266, LIENSs, Equipe Approches Moléculaires Environnement-Santé, Département de Biotechnologies, La Rochelle, France

Chibuike Udenigwe School of Nutrition Sciences, University of Ottawa, Ottawa, ON, Canada

Maria Valéria Robles Velasco Pharmacy Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil

Raquel Guidetti Vendruscolo Department of Food Science and Technology, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Juliana Guerra Vieira School of Chemistry and Food, Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil

Karem Rodrigues Vieira Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Mauro Vigani Countryside and Community Research Institute, University of Gloucestershire, Cheltenham, United Kingdom

Roger Wagner Department of Food Science and Technology, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Cristina Maria Zanette Department of Food Engineering, Midwestern State University (UNICENTRO), Guarapuava, Brazil

Leila Queiroz Zepka Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

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Foreword by Michael A. Borowitzka

Microalgae are an extremely diverse group of (mainly) photosynthetic organisms which are divided into two domains/superkingdoms (Bacteria and Eukaryota). The Eukaryota include the "supergroups" Archaeplastida (Glaucophyta, Rhodophyceae, Chloroplastida), Chromalveolata (Cryptophyceae, Haptophyta, Stramenopiles, Alveolata), Rhizaria (Chlorachniophyta), and Excavata (Euglenozoa). Microalgae can be found in almost all habitats on Earth including oceans, rivers, lakes, salt lakes, and soil, and as symbionts in various invertebrates. This evolutionary diversity is further reflected in the wide variety of compounds produced by microalgae, which make these organisms attractive for bioprospecting as new sources of biomolecules and for their application in various processes.

The applications of microalgae as sources of specific products and their uses in processes such as wastewater treatment have expanded significantly in recent decades. Considering that the first successful culture of microalgae was only some 130 years ago (Beiyerinck, 1890; Miquel, 1892), which followed from the earlier studies of Cohn (1850) with *Haematococcus pluvialis* and Famintzin (1871) with *Chlorococcum infusorium* and *Desmococcus olivaceus*, immense progress has been made.

The early work on algal mass culture and applications is well-summarized by John Burlew (Burlew, 1953) in a book which is still very relevant and rewarding to read. It brings together almost all of the work done around the world at that time, including the first large-scale algae culture trials in the USA, Germany, Japan, and Israel.

The continued rapid growth of the field and the beginnings of commercial production of microalgae can be followed in the books edited by Shelef and Soeder (1980), Venkataraman and Becker (1985), Richmond (1986), Borowitzka and Borowitzka (1988), and Becker (1994). Commercial production of microalgae began in the 1960s in Japan and Taiwan with the culture of *Chlorella* for use as a health food and nutritional supplement. Commercial *Spirulina*[a] production started in the early 1970s at Lake Texcoco near Mexico City, although only natural populations of *Spirulina* in the lake were harvested (Durand-Chastel, 1980). This plant ceased

[a] The commercially produced species *Spirulina platensis* and *Spirulina maxima* were transferred to the genus *Arthrospira* by Komárek and Anagnostidis (2005) and more recently to the new genus *Limnospira* (Nowicka-Krawczyk et al., 2019), but continue to be sold under the name "Spirulina."

operation in 1995. Large-scale open raceway pond cultivation of "Spirulina" started in California and in Hawaii in the early 1980s, followed by Thailand in the late 1980s. The industrial production of high-value compounds from microalgae began in the early to mid-1980s with the industrialization of the halophilic green alga *Dunaliella salina* as a source of natural ß-carotene in Israel, the USA, and Australia (Borowitzka, 2013a). Astaxanthin production from the green freshwater alga *Haematococcus pluvialis* began in the 1990s in Hawaii (Lorenz and Cysewski, 2000), followed by the commercial production of eicosapentaenoic acid by heterotrophic culture of the traustrochytrid *Crypthecodinium cohnii* (Barclay et al., 1994).

The potential of microalgae as a source of energy has long been recognized (Harder and von Witsch, 1942; Milner, 1951), and in 1980 the US Department of Energy began the "Aquatic Species Program" (ASP) which aimed to develop algae as sources of oils for liquid fuels able to compete with fossil fuels. The history and key findings of this program are summarized in detail by Sheehan et al. (1998). Interest in algae as a source of renewable energy waned in the 1990s with the fall in oil prices until the early part of this century, when rising oil prices combined with an awareness of global warming resulted in a massive "rediscovery" of the potential of microalgae as a source of renewable energy.

In the 1990s the Japanese Ministry of International Trade and Industry (MITI) through the New Energy and Industrial Technology Development Organization (NEDO) launched an innovative research program including projects at the Research Institute of Innovative Technology for the Earth (RITE) to develop effective and clean methods for the biological fixation of CO_2 based on photosynthesis (Michiki, 1995). Unlike the US Aquatic Species Program, there is no convenient summary of the research of the Japanese RITE program; however, a brief overview of the findings can be found in Borowitzka (2013b).

The resurgence in interest in microalgae as sources of "clean" and renewable energy at the beginning of this century resulted in an almost exponential increase in research and development in microalgal biotechnology. However, the realization that microalgal renewable fuels (biodiesel, ethanol, hydrogen, etc.), although technically feasible, were not economically competitive with fossil fuels or other renewable energy sources such as solar and wind has resulted in a refocusing of microalgal research and development into areas such as nutraceuticals, functional foods, cosmaceuticals, bioactives and new high-value products (e.g., fucoxanthin, bioplastics, phytosterols) (Borowitzka, 2013c), as well as their application in wastewater treatment and agriculture (plant growth promoters, fertilizer, feed additives) (Mulbry et al., 2007; Kotrbáček et al., 2015; Coppens et al., 2016). Furthermore, advances in molecular biology, bioreactor design, and downstream processing are leading to new applications and products from microalgae. The last 20 years or so have seen the establishment of a large number of producers of microalgae (mainly small-scale), especially in China and Europe, but the main species cultured are still "*Spirulina*," "*Haematococcus*," and *Chlorella*.

Among the tens of thousands of microalgal species (eukaryotic algae and cyanobacteria) that are estimated to exist (Guiry, 2012) only a very small number have been commercialized so far. Some of the more recently commercialized species are *Phaeodactylum tricornutum* (as a source of fucoxanthin), *Euglena gracilis* (mainly as a source of paramylon), *Porphyridium cruentum* (mainly as a source of polysaccharides and phycoerythrin), and *Nannochloropsis* (mainly as a source of long-chain polyunsaturated fatty acids). Microalgae are also an essential feed component in aquaculture, especially for mollusks, and the larval stages of crustaceans and fish. The production of the algae is mainly on-site at the hatcheries and the main algae grown are species of *Isochrysis*/*Tisochrysis*, *Tetraselmis*, *Nannochloropsis*, *Pavlova*, *Chaetoceros*, *Skeletonema*, *Nitzschia*, and *Navicula*.

It is clear that microalgae represent a still largely underutilized resource and the extensive research efforts in algal culture, processing, and products being carried out worldwide will lead to new products and applications in the future. Changes in consumer preferences for natural and healthy products are also expanding the markets for algal products. The chapters of this book present up-to-date reviews and assessments of key aspects of microalgal processes and products, showing the significant progress that has been made in the field of microalgal biotechnology and the challenges facing the commercialization of microalgae.

Michael A. Borowitzka
Algae R&D Center, Murdoch University, Murdoch, WA, Australia

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PART 1

Fundamentals

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CHAPTER 1

Microalgae biotechnology: A brief introduction

Yusuf Chisti

School of Engineering, Massey University, Palmerston North, New Zealand

Chapter outline

1.1 Introduction 3
1.2 A brief history of everything 4
1.3 Algae as human food 7
1.3.1 Microalgae and seafood 10
1.4 Nitrogen for sustainable agriculture 13
1.5 Potential biofuels and other organic chemicals 15
1.6 Carbon sequestration by microalgae 15
1.7 Algae-based treatment of wastewaters 17
1.8 Harmful algae 18
1.9 Commercial production and processing of microalgal biomass 19
1.10 Concluding remarks 20
References 20

1.1 Introduction

This chapter provides an overview of microalgal biotechnology. Emergence of microalgae, their critical role in the environment, and their societal impact are discussed with reference to the major topics covered in this book.

Microalgae and cyanobacteria are distinct groups of photosynthesizing microorganisms that are often discussed together. This practice will be followed here. Microalgae are complex eukaryotes compared to the more primitive cyanobacteria, which are prokaryotes. Estimates of the number of algal species range from 45,000 to more than 100,000 (Chisti, 2018). Species occur in diverse niches, including saline, estuarine, and freshwaters; in deserts and ice sheets; on soil and rocks; and on tree barks. Algae are generally perceived as a nuisance (Chapman, 2013; Chisti, 2018): unsightly in swimming pools; a problem in eutrophicated lakes, rivers, and coastal waters; and a cause of economic loss due to periodic

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poisonings of shellfish and other aquatic wildlife by harmful algal blooms. This notwithstanding, algae are essential to the survival of life on Earth and, historically, the reason behind our existence (Chisti, 2018). For example, nearly half of all the oxygen entering the atmosphere is produced by oceanic photosynthesis ascribed almost exclusively to the diverse algae. All life in the oceans and the supply of much of our seafood depend on microalgae and cyanobacteria. A group of large algae, the seaweeds or macroalgae, has been used by humans for at least the last 4700 years as food, medicine, cosmetics, and fertilizers (Chisti, 2018), although commercial production of both macroalgae and microalgae is relatively recent (Chisti, 2018). On land, plants descended from algae provide all our food and fodder (Chisti, 2018). In short, all life can be ultimately traced to algae and their predecessors (Chisti, 2018).

Microalgae and cyanobacteria contribute to civilization in numerous ways as discussed in the various chapters of this book and other literature (Andersen, 2005; Becker, 1994; Borowitzka and Borowitzka, 1988; Borowitzka and Moheimani, 2013; Bux and Chisti, 2016; Chisti, 2018; Cresswell et al., 1989; Kim, 2011, 2015; Levine and Fleurence, 2018; Pandey et al., 2014, 2019; Pires, 2017; Posten and Walter, 2012a,b; Richmond, 2004; Sharma et al., 2014). While their economic potential is substantial, they have a much bigger ecological role in assuring continuity of life on Earth (Chisti, 2018).

Relatively few microalgae are commercially grown, generally in open ponds, often in the raceway type of open ponds shown schematically in Fig. 1.1 (Chisti, 2012, 2016; Sompech et al., 2012; Terry and Raymond, 1985) and further discussed in other parts of this book (Chapter 6). To a lesser extent, cultures are grown in fully closed photobioreactors (Acién Fernández et al., 2013; Carvalho et al., 2006; Merchuk et al., 2007; Molina Grima et al., 1999; Sánchez Mirón et al., 1999; Tredici, 1999), which are discussed further in Chapter 5 and Chapter 6. Cultivation in photobioreactors is expensive and, therefore, is used for relatively small production volumes of high-value algae. Better control of contamination and the culture conditions in closed photobioreactors allows a greater range of microalgae to be grown in these compared to open ponds. The economics of production of algal biomass have been discussed extensively in the literature (Acién et al., 2012; Amer et al., 2011; Molina Grima et al., 2003). Chapter 30 in this book provides further insights.

1.2 A brief history of everything

The Big Bang created the universe around 13.8 billion years ago. Earth formed around 4.5 billion years ago (Fig. 1.2). The early atmosphere was the result of the gases being released from the Earth through volcanic activity. The atmosphere was much richer in carbon dioxide than it is today and had barely any free oxygen (Chisti, 2018). The first life-forms emerged

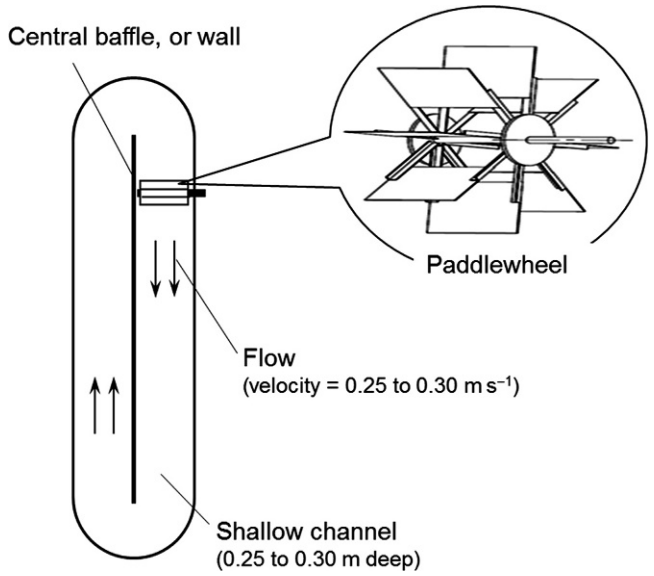

Fig. 1.1
A paddlewheel-mixed open raceway pond viewed directly from above (Chisti, 2016, 2019).

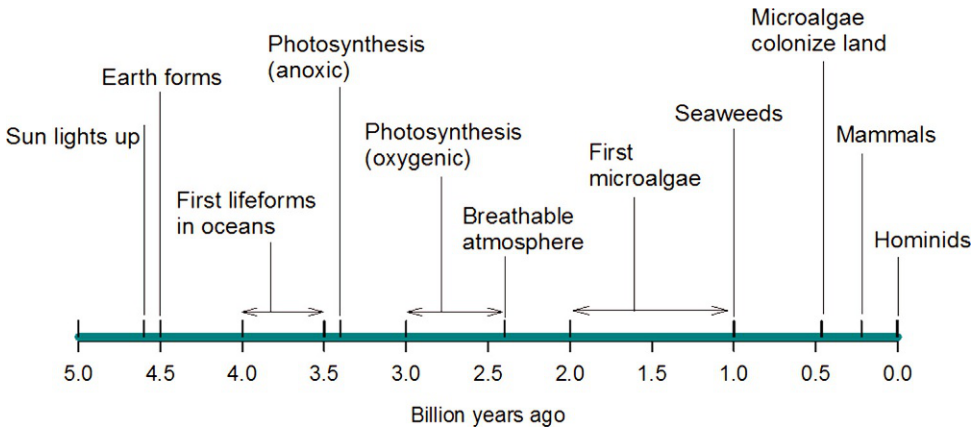

Fig. 1.2
A history of the Earth, life, photosynthesis, and algae.

around 3.5–4 billion years ago (Fig. 1.2). Photosynthesis had evolved by 3.4 billion years ago, but it was anoxic and did not release oxygen (Fig. 1.2). Oxygen generating photosynthesis came into being prior to 2.4 billion years ago (Fig. 1.2), likely as early as 3 billion years ago (Kaufman, 2014). Around 2.4 billion years ago, the Earth acquired a breathable atmosphere as a consequence of the oxygen released by microbial photosynthesis (Chisti, 2018). Oxygen

evolving photosynthesis, or oxygenic photosynthesis, uses sunlight, carbon dioxide, and water to produce organic carbohydrates and oxygen. The carbohydrates, together with other inorganic nutrients, are used to make all the structural chemicals that form the bodies of microalgae, cyanobacteria, and higher plants (Chisti, 2018).

Today's cyanobacteria and microalgae are the immediate descendants of the early photosynthesizing microbes (Chisti, 2018). Eukaryotic microalgae evolved between 1 and 2 billion years ago (Fig. 1.2) (Chisti, 2018). Seaweeds evolved later, possibly around 1 billion years ago (Fig. 1.2). Based on fossil evidence, at least four types of microalgae moved from water to land around 465 million years ago (Fig. 1.2) (Chisti, 2018). Three of these early migrants came from freshwater and only one came from saltwater (Chisti, 2018). The descendants of the saltwater alga that came ashore thrive today as the trentepohiales, a group of 60 terrestrial algal species that colonize rocks and tree bark (Chisti, 2018). Of the algae that colonized land, only the freshwater charophytes progressed to higher plants (Chisti, 2018; Delwiche and Cooper, 2015; Gerrienne et al., 2016; Harholt et al., 2016; McCourt et al., 2004). A single common lineage for all the multicelled land plants is supported by genetic evidence (Graham, 1996). Descendants of the other freshwater algae that originally came ashore exist today mostly as subaerial algae, i.e., the algae that generally grow exposed to air (Chisti, 2018).

An outcome of oxygenic photosynthesis was the oxygen-rich atmosphere that exists today. This allowed the oxygen-breathing life-forms to evolve, and so arose mammals (Chisti, 2018). Mammals, the group of warm-blooded animals to which humans belong, came into being some 220 million years ago but hominids (the group that includes all modern and extinct Great Apes) did not emerge until around 7 million years ago (Chisti, 2018). Humans have been around for only the last 200,000 years (Chisti, 2018).

Algae are believed to have influenced the evolution of humans from our hominid ancestors (Chisti, 2018). Humans are distinguished from the other hominids by the size and capacity of their brains (Chisti, 2018). Brain is mostly fat (~60% fat), and around 20% of the brain fatty acids are comprised of the omega-3 fatty acid docosahexaenoic acid (DHA) and the omega-6 fatty acid arachidonic acid (AA) (Chisti, 2018). A diet rich in DHA and AA is the easiest way to obtain these fatty acids (Chisti, 2018). Hominid diets rich in aquatic foods containing plenty of microalgae-derived DHA and AA are believed to have been a factor in the evolution of the human brain (Bradbury, 2011; Chisti, 2018; Crawford et al., 1999; Cunnane and Stewart, 2010; Cunnane and Crawford, 2014; Joordens et al., 2009). The DHA in aquatic animals originates in microalgae and is bioaccumulated through the food chain (Chisti, 2018). AA has other potential sources (e.g., oil seeds, vegetable oils), but aquatic animals were likely to have been the most readily accessible source for our hominid ancestors (Chisti, 2018). (DHA and AA can be made in the body from dietary intake of the essential fatty acids linoleic acid and α-linolenic acid found in oil seeds and vegetable oils.)

1.3 Algae as human food

Macroalgae, or seaweeds, have been used as food by humans for thousands of years. Seaweeds are grown commercially in coastal waters in more than 30 countries for food and polysaccharides (e.g., agars, carrageenans, alginates) (Fig. 1.3) (Chisti, 2018). The latter are used mainly as food thickeners and texture modifiers (Wikfors and Ohno, 2001).

Although microalgae are commercially used to produce food additives (e.g., pigments and colorants; Fig. 1.4) and nutraceuticals (e.g., β-carotene, or provitamin A) (Chisti, 2018), they are not commonly used directly as food to any significant extent. The green microalga *Chlorella* is added to some foods, but cannot be used as human bulk food because of its poor digestibility (Wikfors and Ohno, 2001). Many cosmetic products based on extracts of both seaweeds and microalgae have been commercialized (Fig. 1.5).

Other than the seaweeds, the cyanobacterium *Arthrospira* (*Spirulina*) has a long history as a successful niche food. *Arthrospira* has been used as food by indigenous peoples of Asia,

Fig. 1.3
Seaweed polysaccharides commonly used in food and other applications.

Fig. 1.4
Some algal pigments used commercially. The powders shown contain a certain amount of the pigment of interest in excipient solids such as algal biomass or/and starch.

Fig. 1.5
Examples of algae-based cosmetic products.

Africa, and the Americas for more than 1000 years (Chisti, 2018). The Aztecs and other peoples of central Mexico harvested *Arthrospira*, known to them as *tecuitlatl*, or *tequitlatal*, from local lakes for food. Fine nets were used to gather the biomass until around the 16th century. The consumption continued for nearly 100 years after the Spanish conquest (Chisti, 2018), but has virtually disappeared as lagoons and lakes of the region were filled for development.

In Chad in West Africa, *Arthrospira* grows naturally in brackish alkaline water pools that form along the sandy northeastern shores of Lake Chad (Batello et al., 2004; Chisti, 2018). Water scooped from pools is drained through cloth to concentrate the biomass slurry. In traditional processing, the thickened slurry is poured onto a circular patch of sand, which soaks up the water (Chisti, 2018). After about 20 min, the thin moist cake stuck on sand is cut into squares, peeled off, and further dried (Chisti, 2018). This thin sun-dried *Arthrospira* cake, or *dihé*, is sold in local markets (Batello et al., 2004). *Dihé* is made year-round by women of the indigenous Kanembu tribe (Chisti, 2018), and is consumed mostly as a sauce. The dry cake is crumbled or ground, mixed with water, and cooked. Other ingredients may be added. The sauce is eaten with a stiff dough made of cooked ground sorghum or millet (Chisti, 2018). More than 250 metric tons of dry *Arthrospira* are consumed annually as *dihé* (Batello et al., 2004). Globally, estimated commercial production of *Arthrospira* biomass is around 2000 dry tons (Chisti, 2018). Production in the Kanem region of Chad therefore represents more than 10% of the global production (Chisti, 2018). Modern commercial production of

Fig. 1.6

Some commercial food products containing *Arthrospira* and/or *Chlorella*: *Spirulina*-powder (A); flatbread wraps containing *Spirulina* (B); *Spirulina* suspension for use in food and drinks (C); *Spirulina* and fruit smoothie (D); yogurt containing *Chlorella* and *Spirulina* (E); *Spirulina* chips (F).

Arthrospira and its nutritional aspects are well-documented in the literature (Gershwin and Belay, 2008). Large amounts of *Arthrospira* are produced in the United States and China (Chisti, 2018). Simple production schemes are being implemented in less developed regions to supply local needs. Some food products made from *Arthrospira* are shown in Fig. 1.6. *Arthrospira* is a potential source of colorants such as C-phycocyanin and extracellular biopolymers (Dejsungkranont et al., 2017a,b). Some microalgal oils (Chapter 17) and proteins (Chapters 16 and 20) may have value as foods. Not all microalgae are edible, and some are toxic (Garcia Camacho et al., 2007; Gallardo-Rodríguez et al., 2012).

The terrestrial colonial cyanobacterium *Nostoc* (*Nostoc commune*, or *Nostoc commune* var. *flagelliforme*, or *Nostoc flagelliforme*) (Fig. 1.7) has a checkered history of human consumption as food, particularly in China. *Nostoc*, or "hair vegetable" (*fat choy* in Cantonese; *fa cai* in Mandarin) (Simoons, 1991; Zhou, 2017), has been consumed in China for more than 1000 years (Gao, 1998; Li and Gu, 2018) in medicinal remedies and specialty foods for festive occasions (Simoons, 1991). Consumption also occurs in some other Asian countries in salads and soups. *Nostoc* is claimed to be a health food with medicinal benefits (Li and Gu, 2018), although the evidence for this is questionable (Zhou, 2017). The biomass for consumption appears to have been harvested entirely from the wild, and no commercial cultivation appears to have been developed. In China, *Nostoc* was wild-harvested by being scraped off the soils of arid and

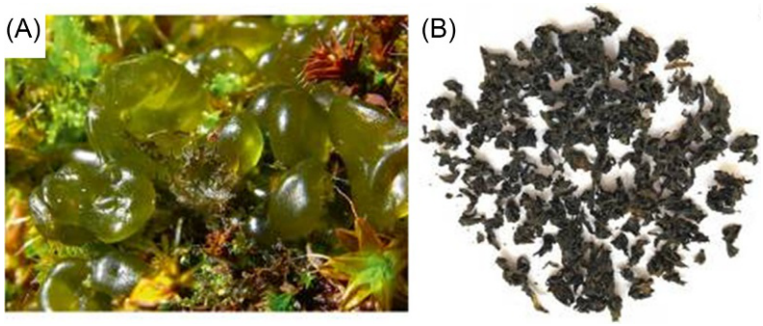

Fig. 1.7
The colonial cyanobacterium *Nostoc commune* in nature (A) and dried (B). *Photographs from Li and Guo (2018). Use permitted by a Creative Commons Attribution License.*

semiarid steppes of the northwest and Inner Mongolia. Indiscriminate harvest led to so much widespread soil erosion and desertification that harvesting had to be banned (Roney et al., 2009). Many of the products now being marketed as *fat choy* are counterfeits consisting of dyed starch noodles (Roney et al., 2009). In addition to *Nostoc*, some other colonial cyanobacteria are also consumed in China and Taiwan (Simoons, 1991).

1.3.1 Microalgae and seafood

As primary producers in most aquatic environments, microalgae and cyanobacteria are the source of all wild-caught seafood (Chisti, 2018). Since 1961, the global consumption of fish has increased more than 2.2-fold to around 20 kg per person per annum in 2018 (Chisti, 2018). Consumption of other seafood (including freshwater species) has also risen. Wild catch of fish is unlikely to rise further, as global fisheries are already overexploited (Chisti, 2018).

Fish is a major source of protein in some regions. Globally, nearly 75% of the fish catch is used for human consumption; the rest is made into fishmeal and oil for feeding farmed fish and other animals (Chisti, 2018). Aquaculture is the farmed production of seafood. Although fish have been farmed for more than 3500 years (Chisti, 2018), modern-day large-scale aquaculture is relatively recent. With the wild-caught seafood production reaching its limits, aquaculture already supplies nearly 50% of the seafood consumed (Chisti, 2018), and is set to overshadow the wild-caught supply. Production of farmed fish now exceeds global beef production (Chisti, 2018).

Aquaculture is an efficient way of producing food, as fish typically convert more of the feed into body mass compared to terrestrial animals (Chisti, 2018). The feed conversion ratio (FCR), or the kilogram of feed (dry basis) required by an animal to gain 1 kg of live mass, is relatively low for cold-blooded species. For example, around 1.2 kg of feed is required to produce each kg of salmon whereas each kg of pork and chicken require 5.9 kg and 1.9 kg of feed, respectively (Chisti, 2018). Table 1.1 provides some typical FCR values for farmed species.

Table 1.1: Feed conversion ratios of some farmed animals.

Animal species	Feed conversion ratio
Atlantic salmon	1.2
Rainbow trout	1.0–1.2
Tilapia	1.5
Farmed catfish	1.8
Tropical shrimps	1.8
Farmed fish (based only on wild harvested fish equivalent fed)	0.3
Broiler chicken	1.9
Pork	5.9
Sheep	4–6
Beef	6.8

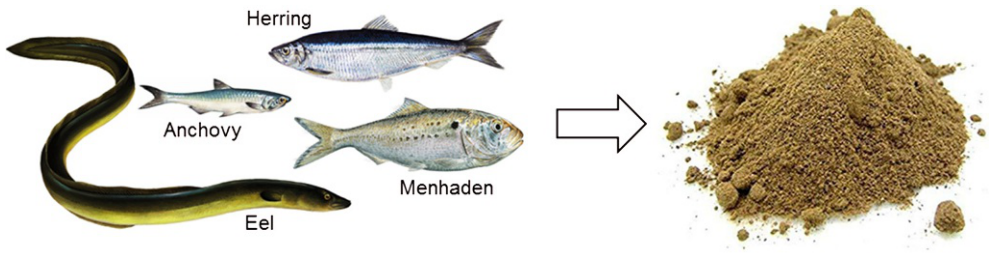

Fig. 1.8
Some fish used to make fishmeal.

The problem is that aquaculture depends substantially on the marine wild catch as the feed required needs to have fishmeal and oil (Chisti, 2018). Production of 1 kg live weight of aquacultured fish typically requires 0.3 kg fresh weight of wild-caught fish (Table 1.1), in addition to other feed components. Nearly 75% of the fishmeal comes from marine fish specifically caught for producing it (Fig. 1.8) and the bycatch of trawlers (Chisti, 2018; Jackson, 2012). Fish species with a limited market (e.g., sardines, anchovy, menhaden, sand eels) are caught for fishmeal and oil (Chisti, 2018). A smaller portion (~25%) of the fishmeal and oil are obtained from by-products of fish processing for human consumption (Chisti, 2018; Jackson, 2012). In 2018, the global production of fishmeal was around 4.727 million metric tons. Production of the top 25 producing countries is shown in Fig. 1.9. The combined average production of fish oil by the top 10 producing countries was 679,000 million metric tons per annum during the 2012–2015 period (Seafish, 2018). Data for annual global production of fish oil for the period of 2007–16 suggest a stagnating, or declining, production, possibly linked to a limited availability of the wild-caught fish (Fig. 1.10).

Wild fish catch cannot be used to sustainably increase the supply of fishmeal and oil for aquaculture. In this context, a bigger future role is envisaged for microalgae, as algal oil and meal, supplemented with other plant and animal protein, have the potential to displace, or

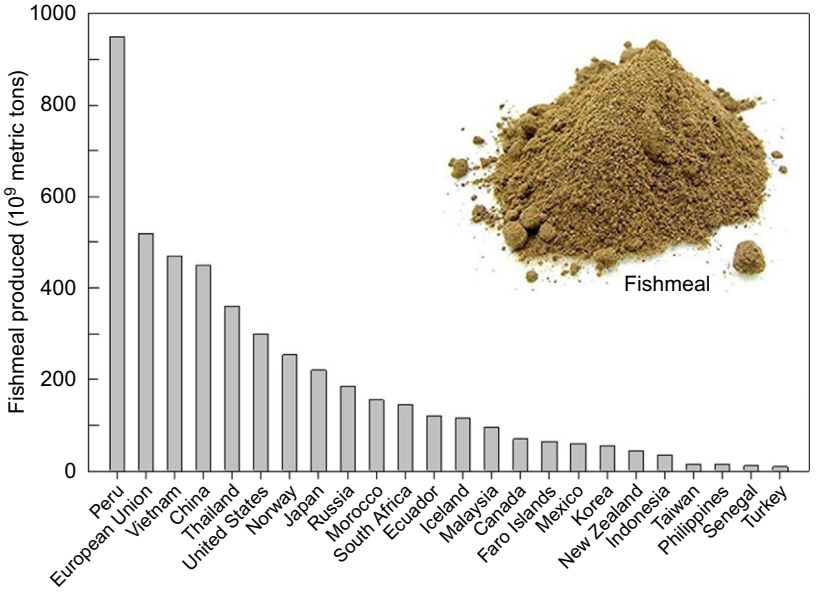

Fig. 1.9
Fishmeal production in 2018, by the top 25 producing countries. *Source: IndexMundi (www.indexmundi.com).*

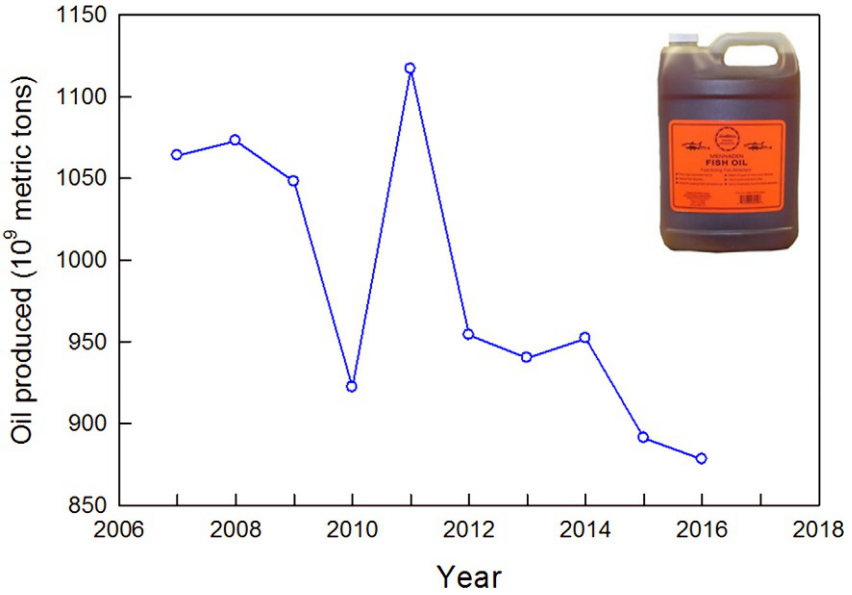

Fig. 1.10
Global annual fish oil production for the period 2007–16. *Based on data from Seafish (2018).*

greatly reduce, the dependence on fishmeal and oil in aquaculture. Crops such as soybean already provide some of the nutrients used in aquaculture feeds, but they cannot totally replace fishmeal and oil (Chisti, 2018). Plants are deficient in some of the essential nutrients provided by fishmeal and oil.

For example, the amino acids lysine, methionine, threonine, and tryptophan may be insufficient in plant proteins (Henry, 2012). Many microalgae contain high levels of all essential amino acids (Henry, 2012). Other essential nutrients that microalgae can provide, but are missing in land plants, include the nonprotein sulfonic acid taurine, which is required by many carnivorous fish (Henry, 2012). Furthermore, microalgae produce all the important long-chain polyunsaturated fatty acids (PUFA) found in fish oil (Chisti, 2018). These fatty acids are essential to the health of marine animals (Chisti, 2018). Some marine species (e.g., lobsters, crabs, shrimps, salmon, red snapper, red mullet) require microalgae-sourced bioaccumulated pigments such as astaxanthin, which can be obtained via cultured microalgae (Chisti, 2018). In principle, processed microalgal biomass can replace fishmeal and oil as aquaculture feeds (Sarker et al., 2016) as long as it is produced affordably and in sufficient quantity (Chisti, 2018).

Although the use of microalgae as aquaculture feeds is well-established, feed algae are expensive because of a low productivity of photoautotrophic culture (Chisti, 2018). This low productivity is not because of any inherent limitation in algal biology, but because of an inability to provide sufficient light to self-shaded algal cultures (Chisti, 2018). Microalgae grown heterotrophically on organic carbon obtained from higher plants have a much greater (e.g., 10-fold greater) biomass productivity relative to photoautotrophically grown cultures. Therefore, heterotrophic growth may be more likely to satisfy the potential demand for PUFA-rich algae feed for aquaculture, to replace fish oils (Chisti, 2018).

An expanded use of algal biomass as aquaculture feed may have a competitor, as oils rich in PUFA such as EPA and DHA can now be produced in genetically modified (GM) terrestrial crops (Usher et al., 2015). In principle, therefore, there is a strong future possibility of replacing fishmeal and oil completely with a combination of plant and microbial proteins and crop-derived oils in aquaculture feeds (Chisti, 2018). Of course, there is also the potential of using aquaculture feed microalgae that have been genetically enhanced to overproduce the desired PUFAs (Chisti, 2018).

1.4 Nitrogen for sustainable agriculture

Crops cannot be grown without assimilable nitrogen. Nitrogenous fertilizers are used to provide the necessary nitrogen (Chisti, 2018). Feeding the existing population would be impossible without the chemically produced nitrogen fertilizers (Chisti, 2018) which are made by the highly energy intensive Haber-Bosch process (Chisti, 2018). The world demand for nitrogen fertilizers is continuing to grow.

Biological fixation of the atmospheric nitrogen to fertilizer is possible in principle using cyanobacteria that have the natural capacity for this (Chisti, 2018; Zehr, 2011). Around 43% of global nitrogen is fixed through biological processes, and the rest is anthropogenic (Chisti, 2018). Some of the natural nitrogen fixation occurs in the oceans and other aquatic environments (Chisti, 2018). According to one estimate, 140×10^6 metric tons N (Fowler et al., 2013), or roughly 69% of the global natural nitrogen fixation, is linked to oceans (Chisti, 2018). Most of this is due to cyanobacteria belonging to the genus *Trichodesmium* (Chisti, 2018).

Freshwater cyanobacteria contribute to the fertility of agricultural land (Chisti, 2018). For example, the floating fern *Azolla* (duckweed) has for centuries been known to improve fertility in rice farming in Asia (Chisti, 2018). *Azolla* harbors the symbiotic cyanobacterium *Anabaena azollae* in its leaf cavities (Wagner, 1997), and this bacterium fixes all the nitrogen that *Azolla* and the bacterium require (Chisti, 2018). Natural decomposition of the *Azolla* biomass releases nitrogen to fertilize the rice crop (Chisti, 2018). *Azolla* may be grown with rice, or separately (Fig. 1.11) for incorporating into the soil before rice is planted (Bocchi and Malgioglio, 2010). Use of *Azolla* reduces the need for artificial N-fertilizers in paddy fields (Chisti, 2018). Plants grown in association with nitrogen-fixing microbes are also a potential source of fodder. Plant–cyanobacteria associations are further discussed by others (Santi et al., 2013).

Free-living cyanobacteria of the genus *Anabaena* also fix nitrogen and may be used as soil inoculants, or bio-fertilizers. Biological fixation of nitrogen by cyanobacteria and other microorganisms will certainly contribute to sustainable farming practices in the future (Chisti, 2018). Aspects of microalgal bio-fertilizers are covered further in Chapter 18.

Fig. 1.11

Azzolla for animal feed being grown in a concrete tank at Kerala Agricultural University, Vellanikkara, India (www.kau.in/photo-album/azolla). *Licensed under a Creative Commons International License.*

1.5 Potential biofuels and other organic chemicals

Under suitable conditions, photosynthetically grown microalgae can be made to produce diverse organic chemicals, including feedstocks for making many kinds of biofuels (Chisti, 2018). The energy embodied in these compounds is of course the solar energy captured via photosynthesis (Chisti, 2018). Starch is produced directly from glucose derived from photosynthesis, and algae can accumulate large quantities of this (Chisti, 2018). Starch can be easily hydrolyzed to glucose for fermentation to fuels such as bioethanol (Karimi and Chisti, 2017) and biobutanol. Like the vegetable oils, the triglyceride oils accumulated by algae can be easily converted to biodiesel by transesterification with methanol (Chisti, 2007). Algal biomass may be anaerobically digested to generate biogas (Montingelli et al., 2015; Valijanian et al., 2018), a mixture of combustible methane and carbon dioxide. Alternatively, the biomass may be fermented to provide energy-rich hydrogen (Chisti, 2018). Hydrogen can also be produced using live algae directly (Dubini and Gonzalez-Ballester, 2016). Algal biomass may be used to produce liquid and gaseous fuels via processes such as pyrolysis, hydrothermal treatment, and gasification (Chisti, 2013, 2019; Pandey et al., 2014, 2019). All this is technically feasible, but so many questions of economics and environmental sustainability of processes remain that production of biofuels from algae, whether native or metabolically engineered, is unlikely in the short term (Chisti, 2013, 2019). Potential algal fuels discussed in this book include biodiesel (Chapter 13), bioethanol (Chapter 14), biogas (Chapter 12), and biohydrogen (Chapter 15). Extensive other literature is available on these topics (Borowitzka and Moheimani, 2013; Chisti, 2019; Pandey et al., 2014, 2019; Bux and Chisti, 2016).

Just as a petroleum refinery can produce a great many industrial chemicals from the fossil feedstocks of crude petroleum and coal, a biorefinery (Chapter 29) can do the same using algal biomass, or other biomass. Although commercial biorefinery operations exist, the feedstock they use is mainly lignocellulosic biomass, not algal biomass (Chisti, 2018). Algal biomass may be used in principle, but it is too expensive for this purpose (Chisti, 2018). Some other algal products and potential products include: structural (Chapter 16) and bioactive proteins (Chapter 20 and Chapter 26); small organics (Chapter 25) and sterols (Chapter 22); pigments and colorants (Chapter 19); polysaccharides (Chapter 21) and bioplastics (Chapter 23); and ultraviolet light screening agents (Chapter 24). Diverse other products may be made (Andersen, 2005; Becker, 1994; Borowitzka and Borowitzka, 1988; Borowitzka and Moheimani, 2013; Bux and Chisti, 2016; Chisti, 2018; Cresswell et al., 1989; Kim, 2011, 2015; Levine and Fleurence, 2018; Pandey et al., 2014, 2019; Pires, 2017; Posten and Walter, 2012a,b; Richmond, 2004; Sharma et al., 2014).

1.6 Carbon sequestration by microalgae

According to a widely held view, prior to emergence of the oxygen evolving photosynthesis, the Earth's atmosphere had no free oxygen and was rich in gases such as methane and carbon

monoxide. In addition, the rocks in the Earth's crust were in a reduced state. Once oxygenic photosynthesis emerged, the oxygen it produced oxidized methane and carbon monoxide to carbon dioxide and resulted in oxidation of the rocks. Once these oxygen sinks were saturated, the dioxygen (O_2) began to accumulate in the atmosphere, leading to the Great Oxygenation Event (GOE), or Oxygen Catastrophe (Chisti, 2018). Photosynthesis by algae, cyanobacteria, and plants gradually removed the carbon dioxide from circulation to lower its concentration in the atmosphere (Chisti, 2018).

Around 50% of the dry biomass of algae and plants is carbon (Chisti, 2018). Almost all of this is the carbon captured from the atmospheric carbon dioxide via photosynthesis (Chisti, 2018). Most of this captured carbon was released back into the atmosphere through decay of dead plants and algae biomass (Chisti, 2018). In some cases, the dead biomass of woody plants was deposited in anoxic environments such as marshlands, and did not fully decompose (Chisti, 2018). This partly decomposed matter accumulated as peat and over a geologic timescale, under suitable conditions, some of it was converted to coal and natural gas (Chisti, 2018). Petroleum was made by a similar process from deposits of dead microalgae and cyanobacteria (Chisti, 2018). Formation of coal and petroleum over millions of years gradually sequestered carbon (Chisti, 2018).

Of course, the carbon continues to be fixed. The net global primary production, which is the amount of carbon fixed annually by photosynthesis, exceeds 100 billion tons annually (Chisti, 2018). Around half of this is fixed in the oceans mainly by algae and cyanobacteria (Chisti, 2018). In most cases, this is not a permanent removal of carbon, as much of it is released via decay of dead algal and cyanobacterial biomass (Chisti, 2018).

Beginning mainly around the 1750s, human activity started an accelerated and continuing release of the carbon sequestered over millions of years (Chisti, 2018). For example, in 2016 alone, nearly 5.6×10^9 metric tons of carbon were released in the environment only from burning of coal (Chisti, 2018). Release of carbon sequestered over eons has been driving the rapid economic expansion of the last 220 years (Chisti, 2018). The rapid release of the fossilized carbon (e.g., coal, oil shale, petroleum) is reflected in the increasing concentration of carbon dioxide in the atmosphere since the late 1700s, the start of the Industrial Revolution (Chisti, 2018).

Since the 1750s, the atmospheric concentration of carbon dioxide has increased by more than 40% (Chisti, 2018). Around 30%–40% of the carbon dioxide released by human activity is absorbed in the oceans, where it causes acidification (Chisti, 2018). Deforestation is another contributing factor to carbon dioxide buildup in the environment, as carbon stored in plants and soils is released through deforestation (Chisti, 2018).

Formation of peat, coal, and petroleum is not the only mechanism of carbon sequestration by algae (Chisti, 2018). Microorganisms including certain algae sequester carbon also by

forming inorganic deposits. For example, the white cliffs of Dover, England, were formed in this way (Chisti, 2018). These cliffs are comprised of chalk (calcium carbonate) (Chisti, 2018). Much of the chalk rock and limestone deposits were formed by sedimentation of the calcium carbonate skeletal remains of coccolithophore microalgae (Chisti, 2018). Calcite rock formation has not ceased, and coccolithophores continue to be the major calcite producers in the oceans (Chisti, 2018). Coccolithophores are estimated to dump nearly 1.4 billion metric tons of calcite a year on the ocean floors (Chisti, 2018). This process continuously removes carbon dioxide from the atmosphere, and calcite deposits are an important carbon sink (Chisti, 2018). Carbon capture by microalgae is discussed further in Chapter 8. Notwithstanding the claims in the literature, no economically viable algal technology exists to genuinely sequester (i.e., remove for periods of hundreds of years, or more) any substantial quantity of carbon dioxide from the atmosphere above and beyond what already occurs naturally.

Microalgae influence the environment in other ways. For example, microalgae release dimethyl sulfide (DMS) into the atmosphere (Bankoff, 1999). This is the cause of the distinctive smell of seaside (Chisti, 2018). Reactions in the atmosphere result in the formation of natural sulfate aerosols from DMS (Bankoff, 1999). These aerosols function as nuclei for condensation of water vapor into clouds (Chisti, 2018). Aerosols and clouds in turn increase the reflectivity, or albedo, of the atmosphere to reduce overheating by sunlight (Chisti, 2018). Annual DMS release from oceans is estimated to be around 74,556 million metric tons (Chisti, 2018). This apparently has a substantial impact on cloud formation and reduced absorption of sunlight by the Earth (Chisti, 2018).

1.7 Algae-based treatment of wastewaters

Biological treatment of wastewater using microorganisms other than microalgae is of course well-established and highly effective (Chisti, 2018). The conventional activated sludge aerobic microbial treatment method does not remove phosphorus (P) from the wastewater (Chisti, 2018), but chemical precipitation and biological treatment options have been developed for effective removal. Unless these secondary treatment options are used, the effluent discharged to natural waters such as lakes, rivers, and coastal waters adds enough N and P nutrients to result in eutrophication. The conventional biological wastewater treatment requires an average energy input of $0.5\,\mathrm{kWh\,m^{-3}}$, costing € $0.20\,\mathrm{m^{-3}}$ of water treated (Acién et al., 2016). The cost of energy represents nearly 50% of the total treatment cost (Acién et al., 2016).

Algae can be used to treat wastewater (Chisti, 2018). In principle, the N and P nutrients can be readily removed from the wastewater by growing microalgae. Such algae-based nutrient removal processes are being developed (Christenson and Sims, 2011; Park and Craggs, 2011; Rawat et al., 2011; Delgadillo-Mirquez et al., 2016; Umamaheswari and Shanthakumar, 2016) to improve the quality of the wastewater prior to discharge, or reuse (Chisti, 2018). Such a process will inevitably generate algal biomass, and therefore may be integrated with the

production of certain bulk products (Christenson and Sims, 2011; Rawat et al., 2011) such as biomass feed for animals (Chisti, 2018).

The "high-rate algal pond" (HARP) technology for treating wastewater by algae has been around since the late 1950s (Craggs et al., 2014), but is viable only for use in small communities (<6000 individuals). The HARP process uses a natural consortium of algae and bacteria for the treatment. HARPs effectively reduce both the organic load in the treated water and the levels of residual N and P (Craggs et al., 2014), although further polishing of the effluent may be required to reduce N and P to low levels. The maximum organic loading rate of a HARP is around 100–150 kg BOD_5 ha^{-1} d^{-1} (BOD_5 = biochemical oxygen demand based on a 5-day test, a measure of the biodegradable organic matter in the wastewater), depending on the climatic conditions (Craggs et al., 2014).

In principle, N removal rates of around 3.5 g N m^{-2} d^{-1} may be attainable if an algal biomass productivity of 50 g m^{-2} d^{-1} is achieved (Acién et al., 2016). In practice, depending on the season and the predominant algae species, the removal rates in a HRAP are unlikely to exceed 20 mg N L^{-1} d^{-1} and 0.4 mg P L^{-1} d^{-1}, based on data presented by Acién et al. (2016). The power consumption of HARP is relatively low (<1 W m^{-2}; Acién et al., 2016). Notwithstanding proven efficacy, the HARP treatment is not widely used, mainly because of the large land area it requires and the long residence times needed (3–10 days, depending on location and season). As noted above, HARP systems are generally practicable only in small communities of fewer than 6000 individuals.

Algae biomass carries a negative net surface charge (Chatsungnoen and Chisti, 2019) and is therefore highly effective in adsorptive removal of cations of heavy metals (e.g., Cd^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}) from industrial wastewaters. Adsorptive removal of a toxic heavy metal occurs only until the adsorption sites on the biomass are saturated. A suitable and safe method is then needed to dispose the metals-laden used biomass. Microalgae-based options for treating wastewater are discussed further in Chapter 7 and other literature (Abdel-Raouf et al., 2012; Umamaheswari and Shanthakumar, 2016).

1.8 Harmful algae

Some microalgae and cyanobacteria are harmful to humans and a source of adverse economic impact (Bankoff, 1999; Chisti, 2018). Harmful algal blooms, or red tides, are one example (Chisti, 2018; Garcia Camacho et al., 2007; Gallardo-Rodríguez et al., 2012). Such blooms have plagued civilization since ancient times (Chisti, 2018). The Bible refers to an apparent red tide event (Bankoff, 1999): Moses lifted his staff and struck the water in the Nile "and all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river; and there was blood throughout all the land of Egypt" (Exodus 7: 20–21).

Most oceanic algal blooms are harmless, but some cause significant disruption (Chisti, 2018). Harmful algal blooms are mostly caused by toxic dinoflagellate microalgae (Bankoff, 1999; Garcia Camacho et al., 2007) and affect aquatic life. Humans are affected usually by consumption of contaminated seafood (Chisti, 2018). Red tides as well as green blooms kill fish, birds, and sea animals; they cause human disease and death, and economic chaos (Chisti, 2018; Garcia Camacho et al., 2007). Algal blooms are entirely natural, but have become more frequent in some regions, possibly because of nutrient discharges linked to human activity and inadvertent transport of algal cysts in ballast waters of ships (Bankoff, 1999; Chisti, 2018). The harmful algae are of course not entirely harmful, as they are potential sources of medicinal products (Chisti, 2018; Garcia Camacho et al., 2007; Sainis et al., 2010; Shimizu, 2000).

1.9 Commercial production and processing of microalgal biomass

In addition to the microalgae biotechnology aspects mentioned in earlier parts of this chapter, numerous other aspects must be considered in developing microalgae-based production processes. Some of the fundamentals that need attention include the algal physiology and metabolism (Chapter 2) that influence production of a metabolite. The maintenance, preservation, and propagation of the algal strain require attention (Chapter 3), as the loss of a high-producer strain may jeopardize the viability of the entire commercial operation. New molecules and products may be designed for production in microalgae through synthetic biology approaches, as discussed in Chapter 4. Once a large quantity of the biomass has been produced in either open ponds (Chapter 6; Chisti, 2012; Sompech et al., 2012; Terry and Raymond, 1985) or photobioreactors (Chapter 5; Chapter 6; Acién Fernández et al., 2013; Carvalho et al., 2006; Merchuk et al., 2007; Molina Grima et al., 1999; Sánchez Mirón et al., 1999; Tredici, 1999), it must be harvested, or separated from the water (Chapter 9; Chapter 10; Barros et al., 2015; Chatsungnoen and Chisti, 2019; Molina Grima et al., 2003; Pires, 2017; Vandamme et al., 2013). If the product is not the algal biomass per se, but an intracellular metabolite, it must be extracted somehow (Chapter 11), either directly from the cells, or after the cells have been mechanically broken or lysed by other means (Chisti and Moo-Young, 1986).

Development of a commercial operation requires attention to scale up and careful integration of the diverse processing operations (Chapter 27) for an optimal outcome. Process intensification options (Chapter 28; Chisti, 2003; Chisti and Moo-Young, 1996) need to be assessed to achieve a cost-effective production process. Ethical considerations and regulatory pressures dictate that the product life cycle is evaluated (Chapter 31) to minimize environmental impact. Furthermore, considerations of cost and environmental impact require processes that are maximally energy-efficient (Chapter 32).

1.10 Concluding remarks

Sustainability of life on Earth is closely linked with microalgae and cyanobacteria. These microorganisms and their immediate descendants modified the primitive Earth to make human existence possible. Production of all wild-caught seafood depends on algae. Algae are commercially used to make diverse products and may someday provide future fuels and other useful chemicals. These and other aspects are discussed in greater detail in other chapters in this book.

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CHAPTER 2

Morphophysiological, structural, and metabolic aspects of microalgae

Maria Isabel Queiroz[a], **Juliana Guerra Vieira**[a], **Mariana Manzoni Maroneze**[b]

[a]*School of Chemistry and Food, Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil*
[b]*Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil*

Chapter outline

2.1 Introduction 25
2.2 Morphological aspects of microalgae 26
2.3 Cell ultrastructure of microalgae 27
2.3.1 Prokaryotic cells 29
2.3.2 Eukaryotic cells 29
2.4 General considerations on microalgae 30
2.4.1 Prokaryotic microalgae (cyanobacteria) 31
2.4.2 Eukaryotic microalgae 33
2.5 Metabolic aspects of microalgae 36
2.5.1 Photosynthetic metabolism 36
2.6 Concluding remarks 44
References 44

2.1 Introduction

Microalgae are the most abundant microorganisms in aquatic environments, including about 72,500 species consistently cataloged, which represent 70% of all species of algae known globally (Guiry and Guiry, 2019). Microalgae are photosynthetic microorganisms with a high growth rate, which can convert solar energy into chemical energy by fixing carbon dioxide. This terminology includes a variety of prokaryotic and eukaryotic organisms, autotrophs, and many capable of developing heterotrophically (Queiroz et al., 2013; Santos et al., 2017). By analogy, Entwhistle and Huisman (1998), in a very simple manner as reported by Borowitzka (2018), defined microalgae as being those microscopic organisms studied by phycologists.

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Microalgae have attracted worldwide attention due their important role in the ecological environment and their biotechnological applications. These microorganisms are fundamental in the global carbon cycle, where, through photosynthesis, they assimilate approximately 50% of Earth's CO_2, generating O_2 and organic matter (Falkowski and Raven, 2007). They still play an important role in the nitrogen cycle, where cyanobacteria, especially the heterocystous ones, are considered the greatest fixers of N_2 (Borowitzka, 2018). In biotechnological terms, these microorganisms can be used in bioremediation processes (wastewater treatment and greenhouse gases mitigation) and to produce biofuels (biodiesel, bioethanol, and biohydrogen), high-value compounds (polyunsaturated fatty acids, carotenoids, and phycobiliproteins), foods, and feeds (Garrido-Cardenas et al., 2018).

Microalgae-based processes and products have several advantages over conventional biological sources, including high photosynthetic efficiency, the possibility of using nonarable land, and the ability to recycle nutrients of wastewater and flue gases (Maroneze and Queiroz, 2018). On the other hand, the consolidation of these processes and products on an industrial scale is still at an early stage and presents several bottlenecks that need to be overcome (Gifuni et al., 2018; Pereira et al., 2018).

In view of the above, it is of fundamental importance to understand the basic aspects of these microorganisms, mainly in terms of morphology, structure, and metabolism. Only with knowledge of these aspects will it be possible to move toward optimized and commercially viable processes and products based on microalgae. Thus, the purpose of this chapter is to elucidate the fundamentals of microalgae biology, mainly in terms of morphology, ultrastructure, and metabolism.

2.2 Morphological aspects of microalgae

The morphological, physiological, and structural characterization of microalgae make these organisms beings of extraordinary adaptive capacity, capable of surviving in diverse environments. The term morphology is intended to describe the shape, size, and growth of an organism. Microalgae are not only highly adaptable in physiological terms, but also highly diversified in shape and size, conferring large morphological variations, presenting a wide size range (0.5–200 μm). This morphological variation happens not only among genera and species, but also among different growth phases of the same species (Barsanti and Gualtieri, 2014).

The morphological conformation of the microalgae is called a thallus, independent of being unicellular or pluricellular, and may present as unicellular, colonial, and multicellular stalks. Single-celled stalks are present in all algae and may be aflagellate or flagellated as in the *Dunaliela salina* microalgae belonging to the Chlorophyta division, which is unicellular

biflagellate. In the divisions Cyanophyta and Dinophyta, flagellate organisms can also be found. The aflagellate unicellular forms may be present alone or form colonies, such as the Cyanophyta *Aphanothece microscopica* Nägeli and the Rhodophyta of the genus *Porphyridium* (Guiry, 2012).

Colonial forms are created by an aggregation of physically joined cells by mucilage. There are two types: amorphous, where there is no organization of cells in the colonies (Cyanophyta, Chlorophyta, Rhodophyta), and coenobium. Coenobium is a stalk in which the cells are arranged in an organized way with a defined number of cells, and it can be found in microalgae of the Chlorophyta division as the genus *Scenedesmus*. An example of this conformation is the colon-forming *Scenedesmus quadriculata* species, which may contain 2, 4, 8, 16, and rarely 32 cells, arranged linearly within the umbilicus, ranging in size from 8.2 to 9.6 μm in length and 2.4 to 8.2 μm wide (Bicudo and Menezes, 2006).

The stalks are characteristic of multicellular organisms formed by two or more cells, including filamentous structures that may form filamentous branched and unbranched stalks. The unbranched ones are formed by successive cell divisions, always in the same plane, and may occur in Cyanophytas, Chlorophytas, and Dinophytas. Branched stalks are characterized by a change in the plane of the cell divisions, and may consist of a single series of cells (uniseriate) or two or more series (pluriseriate) (Andersen, 2013).

Filamentous forms can be found in individuals belonging to the Chlorophyta and Cyanophyta divisions, such as the *Spirulina* filamentous microalgae, which consist of blue-green filaments with 1–12 μm cylindrical cell diameters, as described by Richmond (1988).

As a general rule, cyanobacteria are individuals with small cell diameters ranging from typical bacterial cell sizes (0.5–1.0 μm) to specimens that may have diameters of up to 30 μm. This indicates that eukaryotic microalgae may reach sizes much greater than those presented by prokaryotic microalgae. The reason why Cyanophytes do not reach the size of the other divisions of eukaryotic algae can be justified by a biological rule, which emphasizes that the metabolic rate is inversely related to the size of the organism. Considering that the metabolic rate determines the rate of growth, this relationship implies that the larger an organism, the more slowly it grows (Queiroz et al., 2017a,b).

2.3 Cell ultrastructure of microalgae

The microalgal cell ultrastructure is quite diverse, especially when compared to those found in animal and plant cells. This is a result of 3.5 billion years of evolutionary change, the broad phylogenetic diversity of these organisms, and their ability to adapt to environmental changes (Andersen, 2013). The main characteristics of the major microalgae groups are summarized in Table 2.1.

Table 2.1: Main characteristics of the major microalgae groups.

Phylum	Chlorophylls	Main carotenoids	Phycobilins	Thylacoid arrangement	Main storage product	Cell wall	Representative genera
Cyanobacteria	*a*, *d*, *f*	β-Carotene Myxoxanthin Zeaxanthin	Allophycocyanin c-Phycoerythrin c-Phycocyanin	Single with phycobilisomes on surface	Cyanophycean starch	Peptidoglycan-polysaccharide complex	*Anabaena, Aphanothece, Arthrospira, Nostoc, Oscillatoria, Phormidium*
Chlorophyta	*a*, *b*	α-, β-, γ-Carotene Astaxanthin Lutein Neoxanthin Violaxanthin	n.d.	Single or partially appressed	Starch	Cellulose and noncellulose polysaccharide walls	*Chlamydomonas, Chlorella, Dunaliella, Haematococcus, Scenedesmus*
Euglenophyta	*a*, *b*	β-Carotene Diadinoxanthin Echinenone	n.d.	Single or in groups of three	Paramylon	Do not have cell wall; that cell is surrounded spiral strips, termed the "pellicle"	*Astasia, Euglena, Phacus, Strombomonas*
Haptophyta	*a*, c_1, c_2	β-Carotene Diadinoxanthin Fucoxanthin Zeaxanthin	n.d.	Groups of three	Chrysolaminaran Paramylon-like storage carbohydrate	Cell covered with plate-like cellulose scales, absent or composed of calcium carbonate	*Chrysochromulina, Emiliania, Pleurochrysis, Pavlova, Rebecca, Diacronema*
Dinophyceae	*a*, c_1, c_2	α-, β-Carotene	n.d.	Groups of three	Starch, (1,4;1, 6-α-glucan)	Cell surrounded by a pellicle, usually with cellulose plates	*Alexandrium, Amphidinium, Ceratium, Gymnodinium, Karenina, Symbiodinium*
Eustigmatophyceae	*a*	β-Carotene Vaucheriaxanthin Violaxanthin Zeaxanthin	n.d.	Groups of three	Chrysolaminaran (β-1,3-glucan)	Polysaccharide walls	*Eustigmatos, Nannochloropsis, Microchloropsis*
Rhodophyta	*a*, *d*	β-Carotene Fucoxanthin Zeaxanthin	Allophycocyanin r-Phycoerythrin r-Phycocyanin	Single	Florideans starch	Firm inner layer of cellulose and a mucilaginous outer layer	*Cyanidium, Porphyridium*

n.d., not detected.

2.3.1 Prokaryotic cells

The cell of cyanobacteria is relatively simple, with a prokaryotic structure, measuring from 2 to 5 μm in diameter (Fay, 1983). Prokaryotic cells do not have organelles; however, it is possible to distinguish between two main regions of the cytoplasm: the peripheral and the central nucleoplasmic regions.

The central nucleoplasmic region (the centroplasm) hosts the cellular DNA. The DNA fibrils are arranged in a complex helical and folded conformation and are distributed homogeneously in this region. Ribosomes in cyanobacteria cells are distributed throughout the cytoplasm and mostly concentrated in the centroplasm. All prokaryotes have 70S (Svedberg units) ribosomes, which are dissociated into large 50S and small 30S subunits (Fay, 1983; Demoulin et al., 2019).

The photosynthetic apparatus of prokaryotic microalgae resides in the intracellular membranes (thylakoid). In most species, the thylakoids are oriented parallel to the cell membrane, in the peripheral region, also called the chromatoplasm. This is the case in many unicellular and filamentous cyanobacteria, like *Phormidium*, *Anabaena*, and *Nostoc* (Smith, 1982). On the other hand, in some microorganisms the thylakoids are arranged perpendicular to the longitudinal cell wall, as found in *Spirulina* and *Oscillatoria*. Thylakoids arranged radially were observed in *Phormidium* and in some species of *Oscillatoria*. The major components of these membranes are proteins (like cytochromes, platocyanin, and ferredoxin, which are the components of the electron transport chain), lipids, chlorophyll *a*, and carotenoids. In addition, cyanobacteria have a valuable complement of the photosynthetic pigments, the phycobiliproteins, located in the phycobilisomes. The phycobilisomes are arranged near to the thylakoid membranes, in parallel rows on their external surface (Fay, 1992).

The cell is enclosed by a membrane, the plasmalemma, which is fortified by a multilayered cell wall. Outside the wall, the cyanobacteria may still be surrounded by a gelatinous layer of exopolysaccharides. In some species, this may form a kind of capsule, which can delimit cell growth and even dictate the cell form (Soule and Garcia Pichel, 2019).

2.3.2 Eukaryotic cells

The ultrastructure of eukaryotic cells is more complex than prokaryotic ones due to the development of specialized compartments called organelles. The organized structure of eukaryotes is a result of small changes throughout its evolutionary history, covering about 1.5–2.0 billion years. The term organelle means “small organ,” and each one performs specialized cellular functions. These organelles comprise the nucleus, mitochondria, chloroplasts, endoplasmic reticulum, Golgi apparatus, and lysosomes (López-García et al., 2017).

Typically, the most prominent and largest organelle in a eukaryotic cell is the nucleus, which has a diameter of approximately 5 μm. The nucleus houses the genetic information of the cell through the DNA molecules. The nucleus is where, in addition to storing genetic material, DNA replication, transcription, and RNA processing also occur. In this organelle, the process of gene expression (interpreting) begins, but it ends in the cytoplasm (Andersen, 2013).

Apart from the nucleus, the chloroplast and mitochondrion are two other organelles that carry out particularly important roles in these cells. Chloroplasts are organelles where photosynthesis occurs. In eukaryotic cells, the thylakoid membranes are inserted in the chloroplasts, and it is in these membranous sacs that the photosynthetic light-harvesting pigments are found. Mitochondria are often referred to as "energy factories" because they are responsible for the production of ATP (adenosine triphosphate), the universal energy source of all biological systems. ATP is generated in cellular respiration, a process that breaks down energic molecules such as glucose and other nutrients, also releasing carbon dioxide and water (Wayne, 2019).

Chloroplasts and mitochondria also contain their own DNA. However, this DNA is not like nuclear DNA; typically these are circular chromosomes, similar to those of prokaryotic organisms. From this similarity of mitochondrial and chloroplast DNA to prokaryotic DNA, scientists have developed the hypothesis of endosymbiosis, which states that these organelles are likely to have evolved from engulfed prokaryotes. In short, this evolution was the result of a long and successful symbiotic relation of prokaryotic cells inside eukaryotic host cells (Lane, 2017; López-García et al., 2017).

Eukaryotic cells of microalgae also present some single-membrane-bound organelles, including the endoplasmic reticulum (ER), Golgi apparatus, and lysosomes. The ER is a complex membranous system distributed throughout the cytoplasm, which performs many essential functions in the cell, among which are involvement in the synthesis and processing of proteins, lipid synthesis and transfer, detoxification of compounds, and compartmentalization of the nucleus (Wayne, 2019). The Golgi apparatus—also commonly called the Golgi complex—consists of a series of disk-like membranes arranged in a stack. The Golgi apparatus is responsible for transporting, modifying, and packaging molecules received from the ER in small vesicles, which are marked to be delivered to the proper destination. In addition, this organelle also synthesizes carbohydrates, such as simple sugars or polysaccharides, and sometimes bonds these compounds to other molecules (Ito et al., 2014). The lysosome is a specialized vacuole that digests intracellular components such as old organelles, cells, or cellular components through its great variety of hydrolytic enzymes (Muffly, 2007).

2.4 General considerations on microalgae

As already addressed, the term microalgae is routinely used to designate a polyphyletic group of both prokaryotic organisms, in which cell organelles are not delimited by membranes, and eukaryotic organisms, in which they are (Borowitzka, 2018). Historically, the main phyla of

microalgae were classified based on their pigment composition, membrane thylakoids and chloroplasts organization, chemistry and arrangement of the cell wall, and ultrastructure. Currently, there are considered to be 16 classes of these microorganisms; however, there are still several divergences on the phylogenetic classification of these groups. While these issues are still being resolved, we will discuss here aspects of the major groups: Cyanobacteria, Chlorophyta, Euglenophyta, Haptophyta, Dinophyceae, Eustigmatophyceae, and Rhodophyta (Barkia et al., 2019).

2.4.1 Prokaryotic microalgae (cyanobacteria)

The cyanobacteria, or blue-green algae, were the first organisms that used oxygen photosynthesis as metabolism; consequently, they are the oldest group of algae (Andersen, 2013). It is commonly agreed that, in evolutionary terms, cyanobacteria are ancestral to the eukaryotic algae. Blue-greens have fundamentally transformed the geochemistry of Earth, since there is sufficient evidence to state that these organisms were responsible for the oxygenation of Earth, about 2 billion years ago. At the present time, these microorganisms, as the major oxygen producers on Earth, continue to affect life on this planet. In addition, the nitrogen and carbon-fixing capacity of some genera of cyanobacteria makes them one of the most important contributors to nitrogen fixation and carbon fixation chains worldwide (Soule and Garcia Pichel, 2019).

Structurally, they are nonmotile Gram-negative bacteria, with their own characteristic in relation to other bacteria, because they have chlorophyll *a* and have enzymatic complexes capable of light as a reducing element, producing the driving force for transporting electrons, called photosystems I (PS I) and photosystems II (PS II), which makes them the only prokaryotic organisms that perform oxygenic photosynthesis. The cyanobacteria composition of pigments is a remarkable difference of all others groups. Most cyanobacteria have chlorophyll *a*, phycocyanin, allophicocyanin, and phytoerythrine as light-collecting molecules, which give them a blue-green color (Borowitzka, 2018; Andersen, 2013; Viskari and Colyer, 2003). Some genera (e.g., *Acaryochloris*, *Prochloron*, *Prochlorococcus*) also contain in their pigmentary package other types of chlorophylls *b* and *d*, as well as divinyl derivatives of chlorophylls *a* and *b*.

Still in relation to the pigments, cyanobacteria have been pointed out as a promising source of carotenoids. Among them, β-carotene appears to be universally present in all cyanobacteria, while the presence and abundance of different xanthophylls (echinenone, zeaxanthin, oscillaxanthin, myxoxanthophyll) varies according to species (Huang et al., 2017). The reserve polysaccharide is typically cyanophycean starch, a predominantly α-1,4-linked polyglucan (Andersen, 2013).

Blue-greens can occupy the largest number of different environments across the world, from aquatic to terrestrial, being generally the dominant organisms of the planktons of tropical, temperate, and frigid lakes and oceans (Fay, 1983). Cyanobacteria can also be found in

extremely hostile and most peculiar habitats, due to the amazing character of adaptability that these microorganisms have acquired over their long evolutionary history. For example, the cyanobacteria *Tolypothrix* and *Anabaena* were found growing on volcanic ash after the Krakatoa volcano eruption (Gaysina et al., 2019). Genera of cyanobacteria tolerant to high temperatures are also reported, such as *Mastigocladus* and *Synechococcus*, which can grow at temperatures above 70°C (Borowitzka, 2018).

Cyanobacteria constitute one of the largest subgroups of Gram-negative prokaryotes, covering about 150 genera and more than 8000 species (Guiry and Guiry, 2019). Genera such as *Arthrospira* and *Nosto*c are representatives of the Cyanophyta group that have been extensively studied (Borowitzka, 2018).

The denomination *Arthrospira* is often also referred to as *Spirulina*, a name previously given to this commercially exploited species. *Arthrospira* is a distinct genus consisting of more than 30 different species, including *A. platensis*, *A. maxima*, and *A. fusiformis*. *S. platensis* is well-known not only for its use as a nutritional supplement, but also as a source of blue pigments used in cosmetics and food, in particular as a source of vitamin B12 and protein (Parmar et al., 2011; Komárek, 2015). In addition, many authors have reported that the use of *Spirulina* as a diet supplement has health benefits—for instance, in preventing hypercholesterolemia, hyperglycerolemia, certain inflammatory diseases, allergies, cancer, and viral infections. The beneficial effects of *Spirulina* on cardiovascular disease, based on its hypolipidemic, antioxidant, and antiinflammatory activities, are well-known (Parmar et al., 2011; Keeling 2013; Haase et al., 2012).

The genus *Nostoc* has been highlighted for its metabolic ability to fix atmospheric nitrogen and can be used in soil rehabilitation and as a natural nitrogen fertilizer in rice fields (Ripka et al., 1979). It is distributed in aquatic and terrestrial environments, and presents filament silks that may contain heterocysts and akinetes. These akinetes are characteristic of the *Nostocaceae* and of a few genera belonging to other families (Ripka et al., 1979). This microalga forms large micro- and macroscopic colonies, which consist of uniseriate trichomes embedded in a soft, inner colonial gel matrix; the outer colony surface has a leathery consistency (Andersen, 2013). It is highly resistant to temperature, and many species can resist desiccation for several years. Biotechnologically, some species are of great interest for the function of the compounds that they can accumulate, as is the case of the *Nostoc muscorum* species, which has demonstrated the ability to produce polyhydroxybutyrate, a precursor of natural plastic (Ripka et al., 1979; Halperin et al., 1974).

Despite the few cataloged species being commercially explored, a range of species of cyanobacteria has been studied under different points of view, with very promising results from a biotechnology point of view. An example is the *Aphanothece microscopica Nageli*, a cyanobacterium that forms amorphous macroscopic colonies with abundant mucilage; it is

firm, rigid, of dark bluish-green coloration, elliptical to cylindrical adult cells, measuring 9–9.5 μm × 4.2 μm, about 2.1 times longer than wide. This microalga has been studied in different aspects regarding the treatment of effluents and reuse of industrial wastes in order to use biomass as a source of protein, carbohydrates, fatty acids, and bioactive compounds (Queiroz et al., 2013; Streit et al., 2015, 2017; Masayuki and Shigeki, 2015; Rossi et al., 2017).

2.4.2 Eukaryotic microalgae

2.4.2.1 The green algae (Chlorophyta/Charophyta)

Chlorophyta, or green microalgae, is one of the most profoundly studied microalgae groups, and is described as the most abundant. The members of this phylum are mainly found in freshwater; only 10% of them are common inhabitants of marine environments (Lewin and Whiters, 1975). The morphology of the stalks of these microalgae is presented as unicellular, forming filamentous colonies. The cell wall consists of cellulose and noncellulose polysaccharide. It presents chlorophyll *a* and *b*, and has starch as its reserve material (Leliaert, 2019).

Green microalgae are considered the most promising group for biotechnological applications. A classic example is *Chlorella*, which has achieved scientific fame as a consequence of its use in fundamental studies on photosynthesis and also due to being the first commercially exploited microalga (Borowitzka, 2018). Initially, these microorganisms were proposed as a protein-rich source for human and animal nutrition. In recent years, several researchers have demonstrated several other applications for *Chlorella* in different industrial areas, such as food, pharmaceuticals, cosmetics, and fuel. *Chlorella* cells are spherical or ellipsoidal, with diameters ranging from 2 to 15 μm. Currently, 44 species of this genus have been described. Among them, *Chlorella vulgaris* is a highly cultivated species with high biomass production, being recognized as an important source of protein, fatty acids, and natural pigments (Safi et al., 2014).

In addition to chlorophyll *a* and *b* as a characteristic of Chlorophyta, these microalgae contain the carotenoids β- and γ-carotene, and several xantophylls as accessory pigments, which can be accumulated and commercially exploited, as is the case already presented for the microalgae *Dunaliella salina* and *Haematococcus pluvialis*, responsible for the commercial production of β-carotene and astaxanthin, respectively. *Dunaliella salina* occurs in hypersaline environments, and is a unicellular green microalga, with two flagella, with elliptical, ovoid, spherical, or fusiform cells. *Hematococcus pluvialis* in nature is found in shallow temporary water bodies, such as depressions in rock; these microalgae have a

gelatinous extracellular matrix. Both microalgae can be cultivated in autotrophic, heterotrophic, and mixotrophic conditions (Cezare-Gomes et al., 2019).

2.4.2.2 Euglenophyta

The members of this division are unicellular free-living motile cells, found in freshwater. About two-thirds of these species are exclusively heterotrophic because they do not have chloroplasts. The autotrophic cells have pigmentation similar to green microalgae, since they have chlorophylls *a* and *b*, β- and γ-carotenes, and xanthophylls. The energy storage product is paramylon, a β-1,3-linked glucan polysaccharide, which is arranged outside the chloroplasts. The most representative genus of Euglenophyta is *Euglena*, which has about 337 cataloged species, but only 164 have been taxonomically accepted. *Euglena* has been identified as a promising source of biotechnologically exploitable metabolites such as lipids, carotenoids, tocopherol, and paramylon (Barsanti and Gualtieri, 2014; Kottuparambil et al., 2019).

2.4.2.3 Haptophyta

The haptophytes present about 500 species, which are divided into two classes: the Coccolitophyceae and Pavlovophyceae. They are mostly unicellular motile, palmelloid, or coccoid, but some are colonial and filamentous. Photosynthetic pigments comprise chlorophyll *a*, c_1, and c_2, besides the carotenoids, β-carotene, fucoxanthin, and other xanthophylls, that give the golden yellowish-brown appearance of these algae (Andersen, 2013). These are found mainly in marine environments, and in smaller numbers in freshwater and terrestrial habitats. Haptophytes microalgae are a representative part of phytoplankton in seas around the world, blooming seasonally at polar, equatorial, and subtropical latitudes. These species can cause changes in the regional climate because they produce volatile dimethyl sulfide, which can increase cloud cover through the production of cloud condensation nuclei. In addition, species like *Chrysochromulina polylepis* and *Prymnesium parvulum* can be toxic to fish and cause death by asphyxiation (Zapata et al., 2004).

2.4.2.4 Dinoflagellates (Dinophyceae)

The dinoflagellates include a single class, Dinophyceae, with 150 genera and about 2000 species (Guiry and Guiry, 2019). They are unicellular, biflagellate organisms, found in all ecosystems, but in greater abundance in the sea and brackish water. Only about 50% of the dinoflagellates are photosynthetic; the other species occur as symbionts with reef-building corals, and are therefore essential in the formation of coral reef systems. In terms of photosynthetic pigments, the chloroplasts contain chlorophylls *a* and c_2, and several accessory carotenoids, of which peridinin is the most important, since it is responsible for the usual golden color (Borowitzka, 2018).

Dinoflagellates are perhaps well-known to the public because they are responsible for harmful algae blooms, caused by changes in environmental conditions and increased nutrients.

These algae blooms, also known as red tides, are a serious public health problem, since they lead to the deaths of fish and other marine animals, as well as various types of human illness caused by their toxins (diarrhetic shellfish poisoning, neurotoxic shellfish poisoning, paralytic shellfish poisoning, and ciguatera) (Carty and Parrow, 2015). In addition to the safety point of view of marine products, the biotoxins produced by such organisms have also been identified as having potential for use in biomedical, toxicological, and pharmacological applications due to their bioactivity (Gallardo-Rodríguez et al., 2012).

2.4.2.5 The diatoms (Bacillariophyceae)

The members of the class Bacillariophyceae are unicellular or colonial organisms, presenting as a reserve material the polysaccharide chrysolaminarin and lipids. The wall is constituted by two valves, denominated frustules, which overlap, contained in silicon oxide. Bacillariophyceae can be found in freshwater and marine environments. They are highly represented in marine phytoplankton; however, the vast majority of species are benthic. Regarding the photosynthetic pigments of diatoms, these are characterized by presenting chlorophyll *a* and *c* containing fucoxanthin and other xanthophylls, such as neofucoxanthin and diadinoxanthin (Huang et al., 2017).

One of the most widely studied diatoms is the species *Phaeodactylum tricornutum*. This microalga is recognized as an important source of lipids, with appreciable content in eicosapentaenoic acid as well as carotenoids, in particular fucoxanthin, which can be used as an antioxidant and as pigments in aquaculture for feed to be used in cultivation of economically important species, such as oysters (Bowler and Falciatore, 2019).

2.4.2.6 Eustigmatophyceae

Eustigmatophytes are a distinct group of ochrophyte algae. In their vegetative state, Eustigmatophyceae cells are spherical, ovoid, polyhedral, or irregularly shaped coccoid unicells. The coccoid cells generally appear as solitary or, less frequently, in pairs, or as colonies (Eliáš et al., 2017). They have only chlorophyll *a* and violaxanthin is the dominant xanthophyll. Eustigmatophytes are found primarily in freshwater and terrestrial habitats, with the exception of the genus *Nannochloropsis*, which is exclusive marine. Eustigmatophyceae is considered a small class, containing only eight genera and about 30 species. Among these, species of the genus *Nannochloropsis* have become a focus of commercial and scientific interest due to their potential for production of biofuels and other bioproducts, especially long-chain polyunsaturated fatty acids and pigments (Ott and Oldham-Ott, 2003).

2.4.2.7 The red algae (Rhodophyta)

Considering photosynthesis as the common point of all microalgae, that cyanobacteria are the first organisms to perform oxygenated photosynthesis, and, judging by the pigment packet, the cell walls of reserve material of the members that make up the Rhodophyta groups, it is

assumed that these organisms are the first link between prokaryotic and eukaryotic algae. Rhodophyta, or red algae, are characterized by presenting chlorophyll *a* and phycobiliproteins as pigment accessories, which can often mask the green color of chlorophyll *a*. In addition, it contains the Florideans starch (polysaccharide similar to glycogen), which works as a reserve carbohydrate, similar to what occurs in cyanobacteria (Borowitzka, 2018).

Red algae are a morphologically diverse group. Most of them inhabit marine environments. To date, about 7000 species have been described, with the genus *Porphyridium* being the most studied so far (Guiry, 2012). These microalgae have spherical and avoid cells, measuring 5–16 μm in diameter; they have no flagella, and can form loose colonies in a mucilaginous matrix. The species of this genus have drawn attention due to their potential for exploration as a source of PUFAs, as arachidonic acid and EPA, sulfated polysaccharides, pigments (phycobiliprotein and phycoerythrin), and also as a source of protein (Borowitzka, 2018).

2.5 Metabolic aspects of microalgae

Microalgae are extremely versatile in relation to their ways of obtaining energy. However, the main energy model is naturally photosynthesis, in which light is the source of energy for the synthesis of cellular organic material, but many of these microorganisms are able to use different energy metabolisms such as respiration and nitrogen fixation (Masojídk et al., 2013; Přibyl et al., 2016; Gallon, 1992).

2.5.1 Photosynthetic metabolism

The photosynthetic process can be defined as the synthesis of organic compounds by fixing carbon dioxide from the absorption of luminous energy by the pigmentary system of the organism. It is a unique process of sunlight energy conversion, where inorganic compounds and light energy are converted in organic matter by photoautotroph organisms. In these organisms, carbonic acid and bicarbonates are transported through the plasma membrane and then accumulated in the cell as a reservoir (Masojídk et al., 2013).

Photosynthetic light reactions in higher plants and eukaryotic algae take place in the internal membranes of the chloroplasts—the thylakoids—where the photosynthetic pigments (chlorophylls, carotenoids, and phycobilins) and the enzymes necessary for the use of light and fixation of carbon dioxide are found. The pigments are located in highly organized structures, called photosystem I (FSI), or reaction center (P700), and photosystem II (FSII), or reaction center (P680), which are interconnected through a series of electron carriers. The photosystems are enzymatic complexes capable of using light as a reducing element, producing the driving force for the transport of electrons. In prokaryotic microalgae, these

structures are found in thylakoids. When light is absorbed, a series of oxy-reduction reactions are initiated (Smith, 1982).

2.5.1.1 Photosynthetic pigments

2.5.1.1.1 Chlorophyll

Chlorophylls are the essential pigments of photosynthesis. They are flat, polyhedral structures, belonging to the class of porphyrins. They are formed by four pyrrole rings intertwined by methyl bridges, with magnesium as the central atom. In the fourth pyrrole ring is a molecule of propionic acid esterified by a long chain acyclic alcohol, in general the phytol, which confers a hydrophobic character of chlorophyll (Queiroz Zepka et al., 2019). Four types of chlorophyll, *a, b, c* (c_1, c_2), and *d* are recognized, whose maximum wavelengths of absorption when solved in methanol are 665, 652, 630, 696, and 707 nm, respectively. Chlorophyll *b* differs from chlorophyll *a* because it presents a residue of aldehyde (–CHO) in the place of the methyl group (–CH3) contained in the porphyrin ring that surrounds the magnesium ion of the molecule. In addition, chlorophyll *b* is the most abundant pigment in superior plants and green algae (Chen and Blankeship, 2010; Chen et al., 2012). Chlorophyll *c* differs from chlorophyll *a* because it does not present the phytol in its chain, while chlorophyll *d* of the structure, similarly to chlorophyll *a*, presents in the first ring of the molecule the aldehyde group replacing the methyl group. With regard to chlorophyll *f*, its molecule structurally resembles chlorophyll *b*, very much presenting, however, the replacement of the methyl radical in the first pyrrole ring (Queiroz et al., 2017a,b).

Among these chlorophylls, chlorophyll *a* stands out for its role as the main photochemically active element of the light energy receptor in the photosynthetic process, converting it into chemical energy. The other types of chlorophylls and the other pigments work as accessories. The light energy absorbed by them is transmitted from molecule to molecule until it reaches a specialized chlorophyll *a* molecule, which is identified by the maximum absorption peak (700 nm), which is above the maximum absorption peak of chlorophyll *a* (580–670 nm). This chemically active chlorophyll molecule is called 700 (P700); it is associated with other chlorophyll molecules and other pigments and constitutes the FSI. When the FSI is illuminated, a small reduction occurs in the absorption at 700 nm, resulting from the photooxidation of the reaction center of this system.

A similar process occurs with FSII (P680). Thus, the pigments absorb light of various wavelengths, transferring the energy to chlorophyll *a*, triggering the release of electrons to the transport chain. Chlorophylls *a*, *b*, and *c* were identified more than 100 years ago. In 1996, chlorophyll *d* was for the first time identified in the cyanobacterium *Acaryochloris marina*, which manages to extend the absorption limit at a wavelength 40 nm longer than chlorophyll *a* (Miyashita et al., 2003; Murakami, 2004). More recently, in 2010, chlorophyll *f* was discovered in the cyanobacterium *Halomicronema hongdechloris*, which has the property of

absorbing luminous energy at a longer wavelength than any other molecule chlorophylls (Chen et al., 2012). Thus, the fundamental importance of chlorophylls *d* and *f* in relation to the other chlorophyll molecules is to allow the light absorption spectrum to be extended, enabling the photosynthetic process to be carried out in the infrared region. This increment increases the number of available photons by 19% (Queiroz Zepka et al., 2019).

2.5.1.1.2 Carotenoids

Carotenoids, as well as chlorophyll *a*, are present in all microalgae. Structurally, they consist of a terpene formed of 40 carbon atoms, with absorption of luminous energy in the range of 400–550 nm, presenting yellow, orange, and red coloration (Rodriguez-Amaya, 2015; Maroneze et al., 2019). They are classified into two major groups: xanthophylls, which contain oxygen, and carotenes, which are pure hydrocarbons without oxygen. Carotenoids do not play an essential role in the photosynthetic process in microalgae; their absorption spectrum is complementary to that of chlorophyll *a* (Rodrigues et al., 2014). These pigments stand out for their antioxidant activity, protecting cells from oxidative damage caused by free radicals and reactive species, in addition to protecting systems from oxidative damage, which can lead to enzymatic inactivation and cell death (Rodrigues et al., 2015). The characteristic of long, extensive systems of conjugated double bonds (chromophore group) is responsible for the color and for the antioxidant activity. This property is associated with several biological activities of this compound that act in the prevention of noncommunicable diseases, such as cancer and degenerative diseases (Jacob-Lopes et al., 2019).

Carotenoids are synthetized in the chloroplasts, and they accumulate when the organisms are subjected to a stress condition. Several species of microalgae show the ability to synthesize specific carotenoids, which makes the uniqueness of the microalgae carotenoid profiles ideal for chemotaxonomic identification. For example, Cyanophyta are the only ones that present myxoxanthophyll, echinenone, and neoxanthin, while Chlorophyta are rich in fucoxanthin, violaxanthin, and lutein (Patias et al., 2017; Huang et al., 2017; Maroneze et al., 2019).

2.5.1.1.3 Phycobiliproteins

Phycobiliproteins are intensely colored macromolecules which include three groups of algae: Cyanophyta, Rhodophyta, and Cryptophyta. Phycobiliproteins are water-soluble molecules composed of proteins with linear tetrapyrrole chromophore groups called phycobilins, united by covalence, by triesters bonded to cystine residue (Santiago-Santos et al., 2004; Přibyl et al., 2016). These accessory pigments absorb light in a range between 470 and 670 nm, which makes them responsible for around 50% of the light capture required in the photosynthetic process (Mishra et al., 2012).

The phycobiliprotein, in addition to increasing the spectrum of light uptakes such as chlorophylls and carotenoids, acts as a reserve of nitrogen. In stressful situations, the protein fraction of the pigment is degraded, releasing essential metabolic requirements. However, when

nitrogen availability is resumed, these molecules can be resynthesized (Bermejo, 2015). The phycobiliproteins can be divided into three categories according to their energy absorption properties. Those of high energy (540–570 nm) are called phycoerythrin (red); those of intermediate energy (610–620 nm) are phycocyanin (blue); and those of low energy are allophycocyanin (blue) (Přibyl et al., 2016).

Phycoerythrin are recognized as the main pigments in the process of capturing light from the red region to the green of the visible light spectrum. As a function of its absorption spectrum, phycoerythrin can be divided into three classes: β-Phycoerythrin (545–565 nm), R-Phycoerythrin (499–565 nm) and C-phycoerythrin (565 nm). β-Phycoerythrin has been highlighted since it is particularly useful due to its fluorescence properties. Phycocyanin has been described as the main phycobilin that constitutes the pigments that characterize the cyanobacteria. Considering the characteristic variation among species, phycocyanin, along with allophycocyanin, represents the highest fraction of total biomass pigments (Cunnane and Crawford, 2014; Santiago-Santos et al., 2004; Streit et al., 2017).

The importance in the industrial environment of phycobiliproteins is intrinsically linked to dyes and bioactive properties of this class of pigments, highlighted, as with the other pigments of microalgae, in the food, chemical, and pharmaceutical industries (Queiroz et al., 2013; Zhang et al., 2017). These compounds represent one of the main commercial sources of pigments, having as the main producer of phycocyanin the cyanobacteria *Arthrospira platensis* and the representative of the group of Rhodophyta *Porphyridium cruentus* (Přibyl et al., 2016).

2.5.1.2 Photosynthesis

As already noted, an ancestor of the current cyanobacteria was the first organism that developed photosynthetic apparatus capable of using water as a reducer and therefore performing photosynthesis with release of oxygen, such as the superior plants and eukaryotic algae (Maroneze et al., 2016). However, these organisms and many species of eukaryotic microalgae, even preferably using the photosynthetic metabolism, are able to grow in the dark using organic carbon sources. The type and quantity of inorganic or organic carbon to be used depend on the environmental conditions and the microalgae species (Maroneze and Queiroz, 2018).

Oxygen photosynthesis is classically divided into two phases: the light phase, in which the light energy is used for synthesis of ATP and NADPH, and the dark phase, in which these compounds are used for the fixation of carbon dioxide. The fixing of carbonic gas is not directly dependent on the light and the process, so it is called reaction in the dark (Wijffels et al., 2013).

In the first stage or "light-dependent reactions," the energy absorbed by the FSI is transferred to the reaction center of the photosystem, P700, which, when excited, loses electrons by becoming a reducer. These electrons are carried by an ordered array of compounds to the NADP, the ultimate acceptor, which is reduced to NADPH. A similar process occurs in FSII, which loses electrons through P680. The electrons emitted by the P680 are received by a

chain of electron acceptors whose final acceptor is P700. In this way, the P700 electrons are replaced at the expense of P680, which, when it loses electrons, becomes a strong oxidizer. In short, the result of the light action in FSII is the oxidation of P680 and the reduction of P700. The reduction of P700 occurs through electrons from the photolysis of water responsible for the release of oxygen in the oxygenic photosynthesis characteristic of higher plants, algae, and microalgae, both eukaryotic and prokaryotic. Thus, the function of the FSI is to generate a reductant capable of forming NADPH and the function of the FSII is to generate an oxidant capable of cleaving the water (Masojídk et al., 2013; El-Khouly et al., 2017).

The connection between PSI and PSII is performed by a sequence of electron carriers with increasing oxy-reduction potentials, leading to the interaction of photosystems, transfer of electrons from water photolysis to NAD, and concomitantly to the generation of a gradient of protons for the synthesis of ATP (Foyer, 2018).

In the second phase of the photosynthetic process, called the light-independent reactions phase, the reduction of carbon dioxide from ATP and NADPH generated in the light phase occurs through a set of reactions that integrate the Calvin-Benson-Bassham cycle. This stage is often mistakenly called the dark phase; however, these reactions do not occur in the dark, since they occur simultaneously with the light reactions, although the light is not directly involved (Fay, 1983).

In the Calvin-Benson-Bassham cycle, CO_2 is assimilated, leading to the synthesis of an organic molecule, catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This cycle involves a total of 13 reactions in sequence, beginning with the step where CO_2 is added to the 5-carbon sugar ribulose bisphosphate (Ribulose-bis-P) to form two phosphoglycerate (P-PG) molecules catalyzed by the RuBisCO. The 3-PG is then phosphorylated by ATP, resulting in 1,3-diphosphoglycerate, which is reduced to glyceraldehyde 3-phosphate, whose reaction is catalyzed by the enzyme glyceraldehyde 3-phosphate dehydrogenase specific for NADPH. The sequence leads to the production of a series of phosphate sugars, while the Ribulose-bis-P is regenerated for a further sequence of CO_2 fixation (Fay, 1983; Masojídk et al., 2013; El-Khouly et al., 2017).

2.5.1.3 Respiration

In the autotrophic metabolism, the carbon source originates in the internal process of photosynthesis. In the heterotrophic process, the carbon is captured in the external medium. The main sources of energy in heterotrophic growth are carbohydrates such as glucose, acetate, glycerol, and wastewater, and, to a lesser extent, organic acids (Queiroz et al., 2013; Francisco et al., 2015; Santos et al., 2018). Given that glucose is the substrate most used in microalgal heterotrophic cultures, it can be defined as a set of oxy-reduction reactions through which an organic molecule is degraded to simple molecules. When the final acceptor of hydrogen produced results in an organic substance, the process is defined as fermentation. When substances with lower energy content such as CO_2 and water are formed, it is called

respiration. Thus, in respiration, the organic nutrients are oxidized mainly to carbon dioxide, while de-molecular oxygen is reduced to water. Respiration results from a complete degradation of the substrate with release of electrons and protons, where the final acceptor is the molecular oxygen (Perez-Garcia et al., 2011).

In microalgae, the initial metabolism of glucose (glycolysis) can occur by two metabolic pathways: by glycolysis or the Embden-Meyerhof pathway (EMP) and the pentose phosphate pathway (PPP), both of which are aerobic. The initial route most used by heterotrophic microalgae (under darkness) is the PPP, originated by the hexose monophosphate pathway, while the EMP is the preferred route for breaking glucose under light conditions. Among the final products of the PPP are fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate, which are intermediates of glycolysis, produced from glucose 6-phosphate (G6P). In this way, the PPP acts as a preparation to generate ATP for the glycolytic route, from the citric acid cycle (TCA). This route is strictly aerobic and provides pentose required for synthesis of nucleic acids and prosthetic groups containing nucleotides, as well as the elements necessary for the synthesis of aromatic amino acids and vitamins. The pentose route does not provide energy directly, but $NADPH_2$ may constitute a source of ATP if electrons are reported to oxygen through the respiratory chain. $NADPH_2$ can also be used for lipid metabolism (Ramos-Martinez, 2017).

Although the enzymes of the glycolytic pathway are identified in several eukaryotic microorganisms, and especially in the Cyanophyta, their activities are extremely low; in these organisms, a respiratory metabolism may cause growth in the dark. The energy yield in the dark of these microalgae is dependent on the route of the pentose, where the G-6-P is oxidized and decarboxylated in two stages: the ribulose-5-phosphate catalases the enzymes G-6-phosphate dehydrogenase and gluconate dehydrogenase, respectively. Both enzymes are present in high concentrations in Cyanophyta. These organisms differ from other prokaryotes because they present low rates of endogenous respiration and limited ability to use organic substances as a carbon source in the dark; these microalgae do not have complete Krebs cycle or tricarboxylic acid cycle (TCA). Some enzymes of the TCA cycles, such as α-oxoglutarate dehydrogenase, succinyl-COA-synthetase, and succinic dehydrogenase, are in low concentrations or absent in these microalgae, which prevents the intermediate flow of the TCA cycle and thus the use of substrates such as acetate, pyruvate, or carboxylic acids for efficient energy production and growth in the dark. Even with these enzymatic deficiencies, these microalgae are able to synthesize amino acids and lipids. In addition, enzymes of the glyoxylate route are also in low concentrations in Cyanophytas. Thus, these routes allow a limited carbon flux of isocyanate to succinate on the synthesis of compounds such as chlorophyll and phycobilins (Fay, 1983; Shmetterer, 1994).

In respiration, the opposite of photosynthesis occurs, where oxygen is consumed with the parallel production of carbon dioxide, with organic substrates directed to growth and cell divisions. The respiratory metabolism in microalgae has two main functions: to serve as the

exclusive source of energy for maintenance and biosynthesis, and to provide the carbon building blocks for biosynthesis (Jacob-Lopes et al., 2010).

The application of this metabolic model makes it possible to overcome the major limitation of photosynthetic microalgal cultures, which is the dependency on light, which significantly complicates the process. Additionally, in heterotrophic cultures it is possible to integrate wastewater treatment processes with the production of metabolites of interest, establishing a biorefinery concept (Francisco et al., 2015).

2.5.1.4 Nitrogen fixation

Microalgae are also able to metabolize different forms of nitrogen to support cell growth and maintenance. Various nitrogenous compounds can be used—for example, organic forms such as urea and amino acids have access to the interior of the cells through active transport. However, ammonia is the metabolically preferred form, since its absorption and assimilation are faster and require less energy (Diez and Karolina, 2015).

Regarding the use of molecular nitrogen, cyanobacteria are the major regulators of the global nitrogen cycle. These microorganisms use nitrogenase to reduce N_2 gas into biologically available ammonium (Mishra et al., 2019). Biological nitrogen fixation is performed only by some free-living prokaryotes, such as cyanobacteria or associated with other organisms. Cyanophytas are the largest group of prokaryotic organisms capable of fixing nitrogen, with some filamentous species representative of the *Ocillatoria, Nostoc*, and *Anabaena* genera, and some diatoms, such as those from the *Rhyzosolenia* and *Hemiculus* genera, which are known for their ability to use molecular nitrogen as the only source of nitrogen (Rossi et al., 2017).

Biological nitrogen fixation can be defined as the reduction of the molecule nitrogen to ammonium ions, through the catalysis of a complex enzymatic system, the nitrogenase (Saha et al., 2003). The enzymatic system nitrogenase is basically constituted by two proteins: the dinitrogenase reductase, a molybdenum iron protein that binds N_2, and the nitrogenase reductase, a smaller iron protein that acts as an electron donor to the first protein (Berges and Mulholland, 2008). In this way, the proteins present activity not alone, but in symbiosis (Diez and Karolina, 2015).

The enzymatic complex nitrogenase is highly sensitive to oxygen and can only function under anaerobic conditions; direct exposure of these enzymes to free O_2 results in irreversible inactivation of its catalytic capacity. This sensitivity to oxygen, coupled with the fact that all known N_2-fixing organisms are prokaryotes, suggests that the ability to fix molecular nitrogen originally evolved during the early anoxygenic period of Earth's history (Mishra et al., 2019).

Considering that today, Cyanophyta perform oxygenic photosynthesis, the N_2 fixation process becomes incompatible with its photosynthetic nature. In this way, cyanobacteria developed two strategies to segregate and use both metabolic processes. These strategies are a biological

circadian clock to segregate the two processes temporally, and multicellularity and cellular differentiation to separate them spatially. Species that use the first strategy are, for example, *Cyanothece* sp. a unicellular strain that performs photosynthesis during the day and fixes nitrogen at night, while *Trichodesmium erythraeum* is a nonheterocystous filamentous cyanobacterium that fixes nitrogen during the day due to its morphological and physiological differentiation, which separates photosynthetic and nonphotosynthetic regions. To protect the enzymatic system, other species develop highly specialized cells to provide the nitrogen fixation in a filament. The heterocysts allow nitrogen fixation in the environment in the presence of oxygen (Kumar et al., 2010).

Heterocysts are developed in some species, usually filamentous, when under limited conditions of nitrogenous sources, especially NO_3^- e NH_3/NH_4^+, which results from the metamorphosis of vegetative cells. It is chemically constructed from a polysaccharide stratum followed by a glycolipid layer, making it difficult to exchange gas between the cells and the external environment, guaranteeing the anaerobic conditions necessary to ensure nitrogenase activity (Fay, 1992; Markow et al., 2012).

In morphological terms, heterocysts present a more rounded form and are larger than vegetative cells, being distributed along the filament. There is a small area of contact between vegetative cells and heterocysts, in a way that prevents the entry of O_2, but allows the transport of sugars from the vegetative cell to the heterocyst, since the heterocystous Cyanophytes do not present the FSII and, therefore, do not perform photosynthesis. The source of carbon used is then obtained through sugars provided by the vegetative cells. Although heterocystous microalgae lack FSII, they maintain the FSI to supply the energy needed for nitrogen fixation (Fay, 1983; Magnuson and Cardona, 2016; Mishra et al., 2019).

In the process of nitrogen assimilation, ammonia is incorporated into the carbonated chain through intermediate components (oxoglutarate) of the Krebs cycle. As already discussed, heterocysts are formed under conditions that limit other nitrogenous forms; as a result, there is a scarcity of substrate to react with oxoglutarate, so it accumulates inside the cell. The increase of the oxoglutarate levels triggers the maturation of the heterocyst; consequently nitrogenase is produced and the cell ceases to produce the ribulose 1,5-diphosphate carboxylase, inactivating the FSII, thus ceasing to fix the carbon, but continues depending on the phosphorylation bound to the FSI for ATP production. There is an increase in respiratory rate and subsequent consumption of O_2, which makes the intracellular environment ideal for the activation of nitrogenase (Magnuson and Cardona, 2016).

In this context, the pentose route plays an important role in the N_2 fixation process, providing the necessary energy and reducing power to develop the process. The ammonia produced is then incorporated into amino acids, especially glutamine, which will be transported to the vegetative cells. The fixation of the molecular nitrogen has been studied from a biotechnological point of view, aiming its use for fertilizer production (Kumar et al., 2010; Markow et al., 2012).

2.6 Concluding remarks

The discussion of microalgae's morphophysiological, structural, and metabolic aspects in this text leads us once again to consider that the migration of the beneficial effects of microalgae from the laboratory to the application in favor of humanity has been an extremely slow process, and as always, a conclusion is that many problems remain to be solved. However, in the last decades this process has occurred slightly faster around the world with the growing interest in technologies for the production of natural products that present bioactive effects and are based on renewable resources. As a result, it is critical that researchers and companies around the world focus increasingly on developing strategies to improve the performance of processes and consequently improve viability. In order to do so, it is vital for these individuals to have knowledge about the biological foundations of the microorganisms in question.

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CHAPTER 3

Microalgae culture collections, strain maintenance, and propagation

Sergio de Oliveira Lourenço

Department of Marine Biology, Fluminense Federal University, Niterói, RJ, Brazil

Chapter outline

3.1 Living biological collections 49
3.2 The operation of microalgae culture collections 50
3.2.1 Basic laboratory infrastructure 52
3.2.2 Identification of cultures 61
3.2.3 Propagation of cultures 63
3.2.4 Conditions of keeping microalgae in culture collections 66
3.2.5 Strain quality control and routine problems 68
3.2.6 Cryopreservation of microalgae in culture collections 70
3.2.7 Microalgae as models of organisms in genomics, proteomics, and GMO studies—The role of culture collections 77
3.3 Some microalgae culture collections across the world 78
3.4 Concluding remarks 83
References 83

3.1 Living biological collections

Biological collections constitute a fundamental heritage of information and knowledge about fauna, flora, and microbiota. They consist of units specialized in keeping specimens of organisms, or part of them, for scientific studies and various applications. They are essential for building the knowledge base on biodiversity, as they act as safe depositaries of biological materials, contributing significantly to the acquisition of taxonomic, physiological, genetic, morphological, chemical, and ontogenetic information, among others. Biological collections may harbor living or nonliving collections. In the case of living collections, they are called culture collections and their uses and functions extend even further as they can act as centers of conservation of genetic resources and supply of biological materials for biotechnological, educational, and educational uses, for example.

Among the culture collections, those dedicated to microorganisms (bacteria and fungi) are the most common in the world. The oldest known micro-culture collection is the Kral collection,

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established in 1890 in Prague (Czech Republic) to provide pure cultures of pathogenic bacteria for comparative studies and species identification. Throughout the 20th century, several living biological collections were created in Europe, the USA, and Japan, in response to the growing demand for microorganisms for research and commercially valuable applications. This has also led to a growing diversification of the activities performed by biological collections, arising from advances in industrial microbiology (1960s), biotechnology (1980s), and genetic and genomic engineering (1990s onwards).

Culture collections can be classified into two basic categories: service collections and work collections. The former are endowed with large collections of strains, professional curation, and fully computerized systems for obtaining miscellaneous information (e.g., growing conditions, chemical composition information, derived products). Service collections are heavily funded by government agencies because of their strategic role in the development of scientific research and economically important applications involving the distributed strains. Service collections have enormous national importance and, in most cases, their performances reach international demands. Work collections are simpler, have smaller collections, usually do not have a recognized professional curator inside and outside the institution, and typically lack robust documentation, collection management, and specialized and efficient delivery service. Still, work collections have local, regional, and sometimes national importance, fostering various forms of research development and applications of living organisms. Even though there is universal recognition of the strategic importance of biological collections, only in the last 40 years has there been an effective consolidation of these entities around the world.

Ideally, all living biological collections should be affiliated with the World Federation for Culture Collections (WFCC; available at http://www.wfcc.info). Founded in 1963, the WFCC is an entity that collects living biological collections of all natures in the world. About 770 culture collections from 76 countries are currently registered at the WFCC International Data Centre, with varying degrees of organization and activities (research, services, comprehensive collections, professional curation, etc.). Only about 5% of WFCC-linked collections can be classified as service collections. The WFCC holds periodic events (e.g., the 15th International Culture Collections Conference, held in Chile in November 2019), publishes documents and studies, organizes thematic meetings, and, through organized discussions among its members, establishes actions and defines standard procedures for the operation of biological collections, such as storing strains, distributing control, security procedures, organizing collections, etc. In addition, the WFCC offers a scientific advisory service to help organize malfunctioning collections.

3.2 The operation of microalgae culture collections

Isolating a microalga can be a long and laborious process. However, once isolated, in principle a microalga can be kept in culture without a predetermined time limit. However, the maintenance of cultivated species is not always of interest to researchers who have isolated them, who may

not have the resources or time to do so. An alternative to address this problem is the establishment of microalgae culture collections. These are laboratories specially prepared to receive and keep microalgae in culture indefinitely, but always presumably in the long term. Labs that have the physical space and basic resources can function as collections of microalgae in cultivation. However, laboratories that maintain few strains are generally not called collections, and this term is assigned to laboratories that house at least a few dozen strains and effectively function as repositories of living algal material. However, there is no strict concept in this respect and eventually laboratories with small numbers of strains are also called microalgae culture collections as long as they have a continuous (not occasional) strain maintenance activity.

Culture collections of microalgae are organized mainly according to the existing strains, and secondarily by the microalgae species. This treatment results from counting each culture derived from an isolation event as a unit, regardless of the number of species involved. Thus, very specialized microalgae collections are recognized, with a very small number of species, but with many strains in cultivation. A good example is the Chlamydomonas Resource Center at the University of Minnesota, which has thousands of strains (from all over the world) of one genus, most of which are *Chlamydomonas reinhardtii*. Lorenz et al. (2005) classify microalgae collections into three basic categories:

1. Diverse collections dedicated to research and educational purposes
2. Collections with well-defined delimitations of species or strains for research or practical study; and
3. Collections of genetically well-defined and stable strains (often formed by a single species or a few species) for molecular studies, development of biotechnological applications, etc.

Whatever the focus of the microalgae collection, it faces certain universal characteristics regarding the maintenance of living cells in cultivation. One is the need to carry out aseptic propagation of cultures, usually as batch cultures inoculated with aliquots of preexisting cultures. A second feature concerns the space occupied by tens to thousands of flasks and other cultivation objects. The use of various liquid and/or solid culture media is a necessity to satisfy the different species or strains kept in the laboratory. Some problems are also universal to virtually all culture collections and are inherent in the artificiality of the laboratory environment. Examples of these problems are differences in the quality and quantity of light radiation, nutrient availability, movement of culture flasks, etc. These factors may cause certain strains to exhibit morphological characteristics different from those typically seen in nature (e.g., reduced size of diatom frustules, pigmentation changes, loss or reduction of cellular projections). The possibility of accidental mixing of strains (especially distinct strains of the same species) or misidentification of culture flasks is a constant concern in the routine of culture collections.

3.2.1 Basic laboratory infrastructure

Cultivation environment means the specific place where microalgae cultivation is carried out, whatever its size. Ideally, in a laboratory, microalgae cultivation can be performed in climate-controlled rooms (Fig. 3.1) or incubator chambers (Fig. 3.2). An air-conditioned cultivation room is a large environment that allows people to enter (these rooms are called "walk ins"), the installation of cultivation benches and shelves, and the use of larger experimental flasks (Fig. 3.3). Even cabinets can be installed to house experimental flasks inside climate-controlled cultivation rooms. Incubator chambers are appliances with similar physical characteristics to refrigerators that can only hold smaller culture flasks. Climatized cultivation rooms and incubators should be sufficiently thermally insulated to maintain a stable temperature within a certain tolerable range, depending on the needs of the activity. The environment must be reserved for circulation and access to be controlled, restricted to authorized persons, reducing

Fig. 3.1
Image of one of the cultivation rooms of the Thailand Institute of Scientific and Technological Research (TISTR). The room is climatized and has vertical steel shelves where small vials (test tubes and Erlenmeyer flasks) are kept with the TISTR Culture Collection. *Photo taken by the author.*

Fig. 3.2
Panoramic view of an incubator chamber of the Department of Marine Biology of the Fluminense Federal University, Brazil, where microalgae can be grown in small flasks. *Photo taken by the author.*

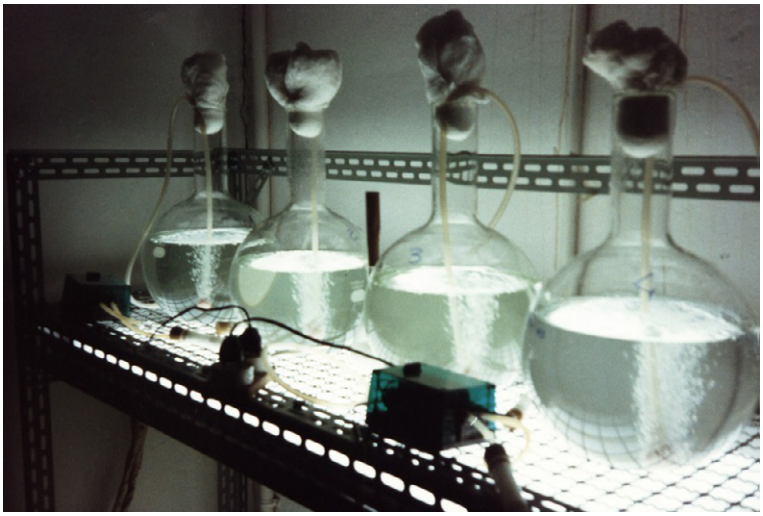

Fig. 3.3
Image of an experiment with 6.0 L flasks cultivated with the chlorophyte Tetraselmis gracilis, carried out on a horizontal bench in a climate room of the Marine Biology Department of the Fluminense Federal University, Brazil. Illumination is provided by 40 W lamps, radiating the experimental vials from the flat bottom through steel grating. *Photo taken by the author.*

the accumulation of dirt and heat exchanges with adjacent spaces, especially in the case of climatic cultivation rooms. Using an incubator makes it easier to control the environment but restricts activities to small-volume jar cultivation.

Climatized rooms and incubators are important, but they are not obligatory items for microalgae cultivation in the laboratory. Although many species are sensitive, there are several microalgae tolerant to less stable environmental conditions, allowing them to be grown even in a makeshift environment. Thus, in the impossibility of setting up an air-conditioned cultivation room or purchasing an incubator, it is possible to grow microalgae in a laboratory space provided there is an efficient cooling or air circulation system and adequate lighting. However, such conditions rarely exist in laboratories, which are affected by either high temperatures in the tropics or remarkable fluctuations of temperatures in temperate climates, for instance.

In the case of growing rooms, the vials can be placed on open shelves (Fig. 3.3) to allow for greater air and cooling circulation and to facilitate cleaning. Cultivation flasks are arranged on the shelf racks and can be lit from side, ceiling, or bottom-up in the case of clear glass shelves (Fig. 3.4) or leaky metal shelves, such as those found in household refrigerators (Fig. 3.5). Hollow shelves allow for better air circulation between the culture flasks. If the rack is metallic and therefore capable of receiving a large load, a larger number of culture flasks can be used, allowing for better use of available space. It may also be interesting to have horizontal metal

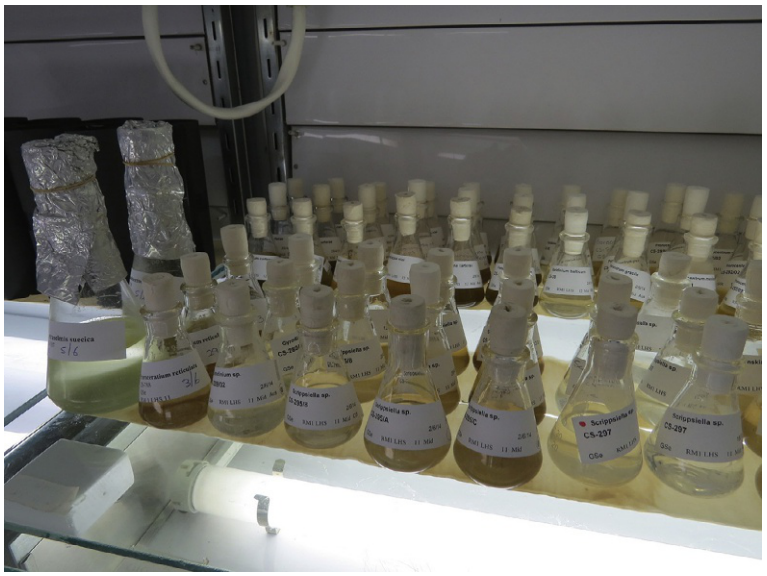

Fig. 3.4
Microalgae growing facilities at the Australian National Algae Culture Collection, Hobart, Australia. Note the shelves of clear glass and the lighting, which allows bottles to radiate from the bottom up and vice versa. *Photo taken by the author.*

Fig. 3.5
Microalgae culture vials inside an incubator of the Thailand Institute of Scientific and Technological Research, under controlled temperature. *Photo taken by the author.*

rods for hanging test tubes near a light source, for example (Fig. 3.6). This feature allows for great space utilization while preventing test tubes from casting shadows on one another. The use of incubators restricts the placement of culture flasks directly on the shelves or on small test tube racks on the shelves.

Temperature is one of the factors that most affect the metabolic rate of organisms. The room temperature should be chosen according to the needs of the species present and the purpose of the cultures. For example, tropical species may be grown at temperatures between 20°C and 25° C, species from temperate environments can be grown at lower temperatures, between 10°C and 20°C, while species originating from polar environments are typically grown at temperatures up to 5°C. These are, however, dangerous generalizations, as tropical species may require higher or lower temperatures than those indicated above to achieve optimal growth.

Fig. 3.6
Arrangement of horizontal rods to hang test tubes around a light source in Microalgae Collection at the University of Göttingen (SAG, Sammlung von Algenkulturen der Universität Göttingen), Germany. *Photo taken by the author.*

The same may be true for certain species from temperate environments. In fact, the decision about temperature stems from knowing the needs of each species. If many species must be kept at the same time in the same growing environment, a tolerable temperature for all species can be chosen (e.g., 20°C), even if the chosen temperature does not favor optimal growth of all species present. If there is space and resources, as well as many microalgae in cultivation, it is appropriate to divide the species into culture environments with temperatures appropriate to each group: a room or incubator at 15°C, another at 20°C, and a third at 25°C, for example.

Constant temperatures, whatever they may be, are desirable for algae cultivation as they provide more stability in experiments and even in routine cultivation operations. As a result, there is a tendency for greater reproducibility and predictability of species responses. The thermal stability of a climate room can be achieved by installing robust cooling systems, similar to those used in cold rooms, which maintain an approximately constant temperature, with small

variations, often below 0.5°C. Alternatively, common air conditioners, which are cheaper than the abovementioned cooling systems, may be used as long as they provide adequate temperature control, which means small temperature variations around the desired value. The use of incubator chambers involves simpler solutions for the control of temperature variations, since the appliances are equipped with internal heat distribution systems, according to domestic refrigerators. Manufacturers can even prepare incubators according to customer specifications regarding operating temperature range (e.g., 10–40°C), lamp arrangement, shelf position, etc.

Temperature control is an important point for microalgae cultivation, and in the case of air-conditioned rooms, special care should be taken to choose a cooling system suitable for the size of the cultivation environment. For example, a 10 m^2 indoor air-conditioned room with a ceiling height of 3 m can easily be kept at 20°C if a 12,000 BTU/h air conditioner is installed. However, two air conditioners must be installed, although only one of them is switched on at a time. This allows the alternation of use between the two devices, resulting in less wear and tear and longer service life. In addition, any technical malfunctions in the operation of one of the refrigerating appliances can be remedied while the second air conditioner continues to operate. Finally, it is important to stress that an air-conditioned room must be well insulated thermally in order to optimize the operation of the cooling system. Whole air-conditioned container rooms are available on the market which can be purchased directly from the manufacturers. These are laboratories with basic electricity and water installations, which are thermally insulated by using low-conduction materials such as fiberglass on their walls. While practical, these portable laboratories are relatively expensive and have installation restrictions, requiring a flat area and sufficient space to be carried by trailers and maneuvered by large machines to the desired position. On the other hand, if the air-conditioned room is built in the laboratory itself, one can simply use 10–15 mm Styrofoam sheets below the plaster, which results in good thermal insulation results.

Thermometers need to be distributed in different positions within climate-controlled cultivation rooms to ascertain existing temperatures. Depending on the size of the room, the efficiency of the cooling system, the arrangement of shelves and worktops, and the characteristics of the furniture used (e.g., height, shape of the shelves), significant differences in temperature measurements may occur in distinct parts of the environment. Cultivating shelves and benches may exhibit higher temperatures during the photoperiod than the center of the room, for example. Digital thermometers are often installed in very visible parts of the room, but these may not correspond to those where temperatures eventually reach higher or lower values. Certainly, the temperatures that matter most within a growing room are those recorded with the growing flasks. As a criterion for temperature investigation, priority should be given to measurements next to the experimental flasks. With these measures, it becomes possible to make decisions about the best way to organize the cultivation room and obtain the desired temperatures.

Lighting is one of the cornerstones of microalgae cultivation and thus requires special attention to the success of the activities. The best results with microalgae cultures are achieved with artificial fluorescent lighting. Thus, each shelf or bench where cultures are placed should be illuminated by fluorescent lamps, providing more energy to the experimental flasks than just the diffused ambient light. Diffused light is often insufficient to provide adequate microalgae growth, except for species with affinities for low light environments such as several cyanobacteria (Jeffrey et al., 1997). In the market there are several types of fluorescent lamps, and those used most commonly for cultivation of microalgae are cold light, warm white light, or daylight. In many situations, different types of lamps can be used in combination. However, it is undoubtedly more appropriate to choose a single lamp type and use it in an experiment without combining it with other lamp types, as some microalgae species may exhibit different responses to existing lamp types. In addition, the selection of a lamp type is also important in case of possible repetition of experiments, since the lack of control on this aspect can add artificial variability to the results. Lamps of the same type and wattage (e.g., 40 W daylight light) may differ in their spectral behavior if produced by different manufacturers. This is one more aspect to consider when preparing a bench or shelf for experimental cultures.

Daylight lamps best simulate the wavelength range (350–700 nm) required for photosynthesis; however, they can cause warming of cultures, especially if the lamps are too close to the experimental vials. This problem can be overcome if the room cooling system is very efficient or, alternatively, if fans are installed next to the lamps, promoting their cooling. Cold lamps do not generate heat because the wavelengths of the red region are not generated and emitted. These lamps are widely used in building installations and indoor environments in general because, since they generate less heat, they cause lower expenses with cooling systems. However, in microalgae cultures, cold light bulbs can generate unsatisfactory growth responses, as many species are strongly affected by the absence of red band wavelengths. Thus, the use of this type of lamp can be disastrous for some species. Incandescent light or direct sunlight often causes culture problems due to the heat they release and should be avoided. It is possible, however, to use sunlight to stimulate microalgae growth as long as it is not excessive. There are laboratories in which culture flasks are arranged near windows to receive natural light, but avoiding direct sunlight on the flasks (Fig. 3.7). The optimal condition for phytoplankton growth will depend on the light intensity, duration, and wavelength to which algal cells are exposed.

Generally 40 W or 20 W lamps are used, which correspond to the wattages most commonly found in the market. Forty watt lamps should preferably be used because they provide a better fit between useful space and power. If, however, small surface cultivation facilities are made, it may be more convenient to use 20 W lamps as they are smaller. Two or three lamps per shelf should be installed to maintain small-volume flasks in a culture collection.

Fig. 3.7
Arrangement of culture flasks near a window for exposure to natural light. Note that the incidence of direct sunlight is avoided. Göttingen University Microalgae Collection (SAG, Sammlung von Algenkulturen der Universität Göttingen), Germany. *Photo taken by the author.*

The electrical circuit that controls the illumination of the culture flasks must have a timer attached, which allows the photoperiod adjustments to be made that are usually required. Although it is possible to cultivate microalgae in continuous light, whether for research, maintenance of live microalgae in the laboratory, or commercial production, in the laboratory, the use of some photoperiod regime is more common. It is particularly common to use 12 h of light: 12 h of darkness for laboratory culture maintenance purposes and, for example, 16 h of light: 8 h of darkness for experimental cultures, depending on the purpose of the activity. The adoption of long photoperiods (e.g., 18 h of light: 6 h of dark) or continuous light is more common in commercial cultures, where exposure of microalgae to longer light tends to stimulate further development of them. Although valid, this statement is often only partially true. Some microalgae grow only slightly more in continuous light than in a 12-h photoperiod (e.g., 10%–20% more), as one must also consider that certain biological rhythms change little, even when there is a strong stimulating agent. Ocean species, for example, generally do not

tolerate cultivation under continuous light. Often there is a biochemical or genetic control of biological processes, which thus become relatively little influenced by external factors such as longer light cycle duration. Thus, the verified responses are not necessarily linear, especially in more sensitive species.

For experimental microalgae cultivation, a photonic flux density of 60–350 $\mu E\,m^{-2}\,s^{-1}$ may be used, while for strain maintenance in test tubes in a culture collection, 20 $\mu E\,m^{-2}\,s^{-1}$ is sufficient to provide good growth. It is important to keep in mind that the efficiency of fluorescent lamps decreases over time, reducing the amount of photosynthetically active radiation to microalgae. Thus it becomes necessary to replace the lamps periodically. It is convenient to measure the light radiation incident on the cultures frequently by means of a quanta meter.

It is virtually impossible to establish universal adequate light intensities for standard microalgae cultivation. This stems from the fact that the luminous intensity actually relevant for determining the condition of sufficiency, deficiency, or light saturation is that which occurs within the culture flasks. However, the measurements performed and expressed in most studies refer to those that affect the external surface of the culture flasks. This tendency to measure light radiation on the outer surface of the vials is due to the fact that measurements are easier to perform and also because most light radiation sensors cannot be immersed in water. Thus, this picture makes the researchers "get used" to the relative expression of irradiance values. The mere expression of an experiment performed with 70 $\mu E\,m^{-2}\,s^{-1}$ irradiation does not mean that there is sufficiency or lack of light. If the experiment is being performed with 15 mL volume test tubes, the irradiance is very likely to be high and may cause photoinhibition. On the other hand, if the experimental flasks contain 5.0 L of cultivation, the most likely situation would be light limitation. In culture collections, the photonic flux density is typically low and this is suitable, since fast growth of the cultures is not the purpose of the activity.

Finally, it is recommended that climate-controlled cultivation rooms and incubators be prepared with white internal walls, as this makes the environment very clear and makes better use of the available light radiation, as well as facilitating the identification of spots of dirt. Ideally, even the floor should be light in color. The existence of many dark surfaces and objects within the cultivation environment implies a greater absorption of light radiation by other bodies, in addition to making dirt spots more difficult to locate.

The use of transfer chambers is very important in culture collections. The main purpose of transfer chambers is to create a space with a minimum of dust and likelihood of contamination. These units can be assembled in the laboratory itself or can be purchased ready-made from specialized companies. Transfer chambers are the places where cultures are handled, involving opening vials and inoculating fresh culture media with algae materials. Fundamentally, the transfer chambers are small apparatuses enclosed by metallic or formic walls and arranged just above the researcher's waist height to facilitate the manipulation of the vials. They should

contain a smooth (ideally steel) surface for ease of cleaning, on which the vials are arranged and handled. If the surface is punctured, less dirt tends to accumulate. One or more ultraviolet lamps should be installed inside the transfer chamber to radiate the environment at least for a few minutes before use, which causes destruction of microorganisms. Whatever the environment used for culture transfers, before use the work unit surface should be thoroughly cleaned with 70% ethyl alcohol, which will help to eliminate possible microbial contaminants. Iodized alcohol solutions can also be used for cleaning the surfaces of the culture handling area. Air circulation in the environment should be reduced during culture handling; the air conditioner or fan should be turned off if they are present in the room. The preexisting culture vials themselves (containing live cells) and the new culture medium vials should also be irradiated with UV before opening, as they always have microorganisms on their lids and outer surfaces. Opening the culture flasks and inoculating the culture media should be done within inches of the sterile area produced around the flame of a Bunsen burner, an internationally recognized antimicrobial procedure for nearly 150 years since the classical experiments of Louis Pasteur.

One such transfer chamber is the laminar flow chamber, which circulates free air of microorganisms in the internal work area. In a laminar flow, particles that are present in a fluid (gas or liquid) travel well-ordered and parallel paths or layers. In true laminar flow, there is no mixing or intersection of different layers of fluid. This principle means that microorganisms are not spread inside a laminar flow chamber and are led to a special high efficiency filter where they are retained. This special filter is known as the high efficiency particulate air filter, and can be placed on the ceiling (establishing a vertical air flow) or behind the inner wall of the chamber bottom (establishing a horizontal air flow). If space is available, it is very convenient to install transfer chambers in a small room apart from the common laboratory work area to make it more private. A few square meters are sufficient for this. It is also recommended to install ultraviolet lamps in the room, so that minutes before handling cultures inside the transfer chamber (where there should be another UV lamp), the presence of microorganisms in the surrounding environment will be significantly reduced. Ultimately, this reduces the possibility of microbial contamination of the culture flasks.

3.2.2 Identification of cultures

The maintenance of multiple strains in cultures necessarily implies the existence of an adequate identification system of each component of the collection. When few strains exist in a laboratory, controlling the identification of each material is easy and often this aspect is of little importance, as one simply writes the name of the species on the culture flask. In some laboratories, identification is done by an abbreviation of the species name, followed by elements that allow the strain in question to be readily identified. Thus, hypothetically, the cyanobacterium *Synechocystis pevalekii* could be identified as "SYN PEV." An isolated strain

from Guarapari waters, in Brazil, could be incorporated as SYN PEV GR1, whereas a second strain of the same species isolated from the same place could later be incorporated into the collection as SYN PEV GR2. If a new strain of the same species is isolated in another location, such as São Luís (also in Brazil), the strain could be classified as SYN PEV SL1. The date on which the culture was inoculated should be indicated on the flask and the use of a diary, in which a continuous record of routine collection maintenance activities is made, may be sufficient to keep the cultures under control.

On the other hand, in a collection of hundreds of strains, the complexity of the strain identification problem is greatly increased and it is generally not possible to work with such simplified forms of identification. Adoption of mere abbreviations may manifest itself as a poor criterion for identifying the components of a large collection, as algae with similar names may be confused (e.g., *Synechocystis* and *Synechococcus* genera). The same can be considered for codes concerning localities of origin (e.g., Guarapari and Guaratuba, both in Brazil). Thus, it is often necessary to create a numbering system for each strain incorporated in the collection. In addition, it is also generally interesting to indicate the name of the species and the culture medium used, in addition to the date of subculture. The best option for incorporating so much information without taking up too much space is to use inkjet or laser printed paper adhesive labels (Fig. 3.4). Fig. 3.8 presents the information contained in the labels used in the Elizabeth Aidar Microalgae Collection, Fluminense Federal University, Brazil, to identify the strains in the collection. The only drawback to using adhesive labels on culture flasks is their removal. Often labels will tear when being removed, and adhesive glue will usually accumulate on the flask, which requires more effort to clean the flasks externally before washing. Acetone is quite

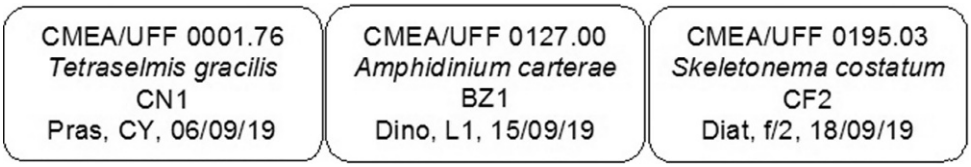

Fig. 3.8

Information contained on labels used to identify strains kept in the Elizabeth Aidar Microalgae Collection, Fluminense Federal University, Brazil. In the first line appears the name of the collection (CMEA/UFF), the sequential number of strains of CMEA/UFF (0001, 0127 and 0195), followed by the year of strain isolation (1976, 2000 and 2003), with only the two last digits. In the second line, the name of the species is indicated. The third line shows the strain record: CN1 = first strain of Cananéia (SP); BZ1 = first strain of Armação dos Buzios (RJ); CF2 = second strain of Cabo Frio (RJ). This information can be omitted when there is only a single strain of the species in the collection. The fourth line gives an abbreviation for the name of the large group to which the species belongs (Pras = prasinophyte; Dino = dinoflagellate; Diat = diatom), the culture medium used (CY = Conway–Walne medium, 1966; L1–Guillard and Hargraves, 1993); f/2–Guillard, 1975) and the date of inoculation of the vial (day/month/year).

useful for removing glue residue. It may be prudent to label collection flasks in two separate locations to minimize any accidental problems that make it difficult or impossible to read labels (e.g., seawater splashing that may scatter printed ink or accidentally dropping a label while handling culture flasks). Thus, the vial can receive the strain number in a second place, handwritten with a glass or plastic pen. Finally, it is also worthwhile keeping the vials in fixed positions within the collection; as well as facilitating the location of a strain of interest, this also helps to identify a vial whose label has been lost or damaged.

Large culture collections necessarily involve species and strains with very different growth rates, which lead to different frequencies of subculture. For example, a given fast-growing species (e.g., the eustigmatophycean *Nannochloropsis oculata*) can be propagated three times within 50 days, while a second slow-growing species (e.g., the diatom Coscinodiscus wailesii) be propagated only once in the same period. The frequency of propagation of the collection should take into account the needs of the strains, and never a purely convenient criterion for the researcher (e.g., propagation all algae once a month, in a single week—a very long time for fast-growing species and too short for slow-growing species). Thus, in a month, several subcultures of different batches of strains can be propagated and the follow-up of these routine activities requires more than a simple diary to record the procedures. Ideally, computerized files should be created for each strain's or batch of strains' propagation to allow for prompt accumulation of all information of interest. This can be done in database-specific software or, more simply, in more commonly used types of software that allow the creation of spreadsheets or tables.

3.2.3 Propagation of cultures

As discussed above, it is imperative that cultures be propagated at appropriate intervals for each strain or set of strains in the collection. The interval should allow sufficient time for the culture to reach a high density, but it should not be too long for problems of death and/or deterioration of large portions of the culture due to cell senescence. Virtually all microalgae culture collections keep cells alive through batch cultivation. Ideally, we should subculture the cultures while they still have healthy developmental conditions—for example, at the end of the exponential phase or at the beginning of the stationary phase of growth. It is noteworthy that stocks of culture collections are maintained under suboptimal conditions (see Section 3.2.4), so that growth occurs at lower rates, increasing the duration of each growth phase. Small cells tend to grow at high rates, which necessitates subculturing at short intervals (e.g., 2 weeks). On the other hand, large cells or delicate colonial species typically grow at low zrates, so that subcultures can be made over long periods (e.g., 2 months). It is important to stress that the propagation intervals should not be considered with extreme rigor. Thus, if problems occur that prevent a strain that is typically propagated every 21 days from being propagated at 28 days, this should not be a concern. A safe subculture interval should be c. one-quarter of the maximum

strain survival time under current growing conditions. This means that a week beyond the optimum subculture time is unlike to result in culture loss.

Culture propagation should follow the classical procedures, such as the need to sterilize vials, pipettes, Nicrom handles (for subculturing solid media), caps, Pastettes (disposable polyethylene pipettes), culture media, etc. All materials cited should preferably be handled within an aseptic chamber (e.g., laminar flow chamber) or at least close to the flame of a Bunsen burner. All vials (with preexisting cultures or new culture medium) should be opened for the shortest possible time to minimize the possibility of microbial contamination. Glass pipettes should have their wide end filled with nonabsorbent cotton to reduce the potential for microbial contamination. At the wide end of glass pipettes, rubber or plastic teats can be connected to aspirate some culture volumes at subculture, if needed. By being squeezed to release the liquid from the pipette, any microorganisms present inside the teat can be transferred along with the inoculum to a fresh culture medium, a possibility that would be reduced with the use of cotton at the wide end of the pipette. The same protection occurs when pipetting is eventually performed using mouth aspiration (although this procedure has already been abolished in most laboratories). Regardless of the risk of transferring microorganisms to the culture, there is also the possibility of accidental ingestion of culture aliquots and aspiration of gases emanating from them. Even though relatively few microalgae species contain toxins harmful to humans, it is assumed that the simplest procedure to be adopted is to abolish pipetting by mouth.

In the case of propagating cultures from a collection, before starting to subculture, it is essential to check carefully all the vials to be handled to avoid any errors (e.g., subculture from the wrong vials). In addition, it is much more rational for the vials that are to be inoculated to be previously identified, and this procedure is highly recommended. In a collection of microalgae in cultivation, each unit is kept in small volume vials such as test tubes and reduced capacity Erlenmeyer flasks (e.g., 50–125 mL), which often suggests the possibility of propagating many flasks at a time, to save time or make the culture renewal procedure more practical. However, it is absolutely inconvenient to propagate many vials at once, as several undesirable implications occur. Propagating a few vials at a time: allows greater care for each one, with less chance of microbial contamination; minimizes the occurrence of mechanical errors derived from sequence manipulation; allows more free space within the aseptic chamber for accommodating and handling the vials and cultivations and the proper disposal of used materials; and makes the activity less exhaustive and subject to a lower probability of errors.

Propagation of liquid cultures consists of transferring small aliquots from a preexisting culture to the fresh culture medium vial. If subculture is prepared within a laminar flow chamber, all the vials should be irradiated with UV light for at least a few minutes before handling (following the instructions of each apparatus). In addition, the mouths of all tubes should be buckled into the flame of a Bunsen burner if work is done outside a laminar flow chamber (or even if done within a laminar flow chamber if deemed appropriate by lab staff). Coccoid cell cultures

(e.g., the chlorophyte Chlorella minutissima) should be shaken briefly to promote good cell distribution in the liquid before being pipetted to inoculate the new vial. The use of vortex tube stirrers is appropriate but not essential. Monadal species (e.g., the cryptomonad *Rhodomonas salina*) should also be agitated, since even with flagella they can accumulate at the bottom of the flask if kept undisturbed in the growing environment. Delicate colonial species (e.g., the primnesiophyte Phaeocystis globosa) should be agitated rapidly and without vigor to avoid damage to cells or colony arrangement. Benthic habit cells can be scraped from glass pipette vials, glass rods, or Nicrom handles before being shaken and collected for inoculation by pipetting (e.g., filamentous cyanobacterium *Phormidium ectocarpii*, which forms biofilms firmly attached to the bottom of cultivation bottles).

It is also important to stress the need to maintain simultaneously a few culture flasks of each strain. This is essential to make routine culture propagation work safer, as problems resulting from potential accidents (e.g., breaking a cultured flask) and contamination (from one algae to another or even the growth of bacteria and fungi), which are natural in a microalgae collection, can be easily bypassed by using additional culture flasks. The minimum number of vials for each strain is two, but undoubtedly this is a small and dangerous number to work with in a collection. Maintaining three vials for each strain is more convenient. In this case, these are usually three generations in which the older culture (colloquially called the "grandmother culture"—A) is used to inoculate the intermediate culture ("mother culture"—B), which in turn is used to inoculate the youngest culture ("daughter culture"—C). When the "daughter culture" reaches a high cell density, it will be used to inoculate a new culture, D; this will then become the "mother culture" and culture A, which at this time is likely to be quite old and damaged, can be discarded to make way for the new incorporated culture (culture D). Importantly, disposal of old cultures should take place only after certification that new vials have been effectively inoculated. In the SAG collection, Germany, only one set of culture flasks is used for each strain, but there are always at least five flasks of different ages for each collection component (Lorenz et al., 2005). Regardless of whether they are distinct strains of strain propagation, it is highly prudent that at least one of the vials is kept in a separate location from the others. For example, if there are four vials of each strain, three are kept in a growing room and the fourth vial is kept in an incubator chamber. This minimizes potential data derived from problems that may occur in growing environments, such as a refrigeration system malfunction; if all the vials are together in the same place, the risk of loss of every one is high.

An even more interesting situation is the maintenance of independent cultivation series. For example, each strain may be kept in the collection in medium (50 mL capacity) and small (15 mL capacity) test tubes. In this case, cultures kept in large test tubes would serve to inoculate other large test tubes, while small tube cultivations would always be subcultured to other small tubes. This procedure is adopted at the Elizabeth Aidar Microalgae Collection, Fluminense Federal University, and further reduces the potential problems of contamination of cultures in stock, as contamination accidents often occur, but are only noticed a long time after

their occurrence. Remember that regular microscopic examination of cultures is not always possible and very similar-looking microalgae strains (culture color and even cell shape) can sometimes be picked up without the problem being noticed. One situation that illustrates this problem well is the accidental contamination of a Tetraselmis gracilis culture by Tetraselmis suecica, two extremely similar species. The existence of independent series of culture propagation would allow the immediate solution of the problem, since there is minimal possibility that the same contamination accident happened in the second series of flasks. Independent series of culture flasks should not necessarily be of different capacities, but this option is often appropriate because certain species may exhibit differences in growth rates in different containers. Growth is usually faster in larger vials, presumably due to the larger space available (reducing the effect of factors such as shading by neighboring vials) and the possible greater movement of the experimental vials.

It is highly recommended that microalgae from a collection also be kept in solid medium, unless of course species are unable to grow out of a liquid medium. Microalgae are typically propagated in a solid medium at longer intervals (usually after a few months of cultivation), providing less manipulation and less chance of contamination and other accidents. The maintenance of microalgae in a solid medium does not eliminate the need to keep cultures in a liquid medium.

Finally, it may be quite interesting that certain strains of special importance are shared by some independent collections. For example, strains from a total of 438 isolated by the German researcher Ernest G. Pringsheim (1881–1970) are present in four of the world's largest collections of freshwater microalgae (UTEX, USA; SAG, Germany; CCAP, United Kingdom; CCALA, Czech Republic). Nine strains are shared by the four collections, three of them share 84 strains, and two of them have 121 strains in common (Day et al., 2004). This virtually guarantees the propagation of these strains of historical importance indefinitely. However, duplication of strain maintenance efforts by large collections tends to be restricted to special components only, as space must be made available for isolates of local, regional, or national importance. It tends to be more convenient for a given large collection to share some strains with various small collections or laboratories in the country itself. Thus, it acts as a safe depository for strains of interest to researchers, educational and research institutions, and even national companies, reinforcing its role in coordinating microalgae cultivation work.

3.2.4 Conditions of keeping microalgae in culture collections

In a microalgae collection, strains can be kept under different growing conditions, depending on the intended use. In most cases, stocks are not kept under conditions such that they can be used immediately in an experiment, for example. Strains are almost always kept under suboptimal growth conditions, as this prevents algae from growing too quickly and requires intensive work of subculture and culture manipulation. The adoption of suboptimal cultivation conditions

involves factors such as temperature and light, manifested as the use of a nonideal but nondeleterious temperature (e.g., 20°C for a hypothetical strain whose optimum would be 23° C; 15°C, for example, could be a deleterious temperature) and/or a weak irradiance (e.g., $20\,\mu E\,m^{-2}\,s^{-1}$). Nonideal culture media are rarely used to maintain microalgae under suboptimal conditions, as this can lead to very unsatisfactory growth conditions, since this may result in cell damage or long recovery times when strains are transferred to another culture medium. Cultures are recommended to be propagated to optimal growing conditions only when they are about to be used. Suboptimal conditions should lead to slower growth, but should not promote algae stress, otherwise there is a risk of loss of strains or even difficulty propagating to larger volumes. Most algae collections keep their culture stocks in test tubes of varying sizes, mainly due to space savings. Most algae exhibit better growth responses if grown in larger vials, such as 125 mL capacity Erlenmeyer flasks. If the growth of a strain is too slow, the culture can be propagated to a larger flask and subjected to optimal growth conditions. Once optimal growth is restored, cultivation can be reincorporated into the collection under standard cultivation conditions.

Some large collections of algae in cultivation also adopt keeping freshly prepared cultures in optimal condition for 4–15 days (depending on the growth rate of the strain) before arranging the vials in the collection under suboptimal growing conditions. The short period under more stimulating growth conditions provides faster adaptation to the culture medium. On the other hand, it also allows examination of the culture for possible contamination before its actual incorporation into the collection. During this short observation period after subculturing, the culture can be kept under higher temperatures or irradiance, for example. This procedure is not essential, of course, and can be waived, especially if the collection has limited staff support and limited space.

In the case of cultivation in a liquid medium, it is advisable for the stock storage vials to be shaken periodically. The agitation of the culture flasks contributes to keep the cultures healthier, as some living cells that possibly decant to the bottom are resuspended and interact more with the culture medium. Agitation tends to be especially suitable for flagellate-free cells and should be avoided (or only mildly) only in the case of more sensitive strains such as naked (not armored), fragile dinoflagellates or delicate colonial forms of various algal groups.

Except for very slow-growing strains, algae generally adapt quickly to new (and better) conditions, in a range that almost always corresponds to 7–10 days, with at least one propagation in the period. Eventually a longer time frame may be required for microalgae to reach full acclimatization conditions to desired conditions (e.g., 20 days), with more propagations in the period, but this assessment depends on the researcher's experience and the algae's own behavior. Due to the need to acclimate strains over a variable time, microalgae collections tend to take a 4-week time frame to meet strain requests made by external users.

This procedure allows adequate time to perform the propagation necessary to deliver a material under full use conditions. It is therefore necessary to plan the receipt of strains several weeks in advance, otherwise there is a risk of receiving cultures under inadequate growing conditions. This need, however, is not always understood by users who request strains of microalgae from collections, especially when it comes to requests from companies that cultivate microalgae. Often, the public is unaware of the conditions for maintaining strains in microalgae collections (necessarily suboptimal) and do not understand the need for a few weeks to deliver cultures. Due to this condition, certain microalgae collections keep strains requested more often under more favorable growing conditions (e.g., higher light intensity and optimal temperature) and also in larger culture flasks. Some large collections, such as the Provasoli-Guillard National Center for Marine Algae and Microbiota (USA), even offer the so-called "aquaculture kit," which consists of a set of species typically used by US aquaculture companies (e.g., *Nannochloropsis oculata*, *Chaetoceros calcitrans*, Isochrysis galbana, etc.). The set is marketed as a package. Of course, such procedures are only feasible when the collection has sufficient support staff, adequate physical space, and a high demand for strains.

Transferring a stock condition culture from a collection for use in experiments or delivering to external applicants is relatively simple. Fundamentally, the cells are simply inoculated in a fresh culture medium and the new culture kept under optimal growth conditions for a variable time, depending on the strain. Additional subcultures are made after a few days by which the cultivated volumes can be increased. If strains in the collection are grown under suboptimal (but not stressful or strongly limiting) conditions, adapting the material after changing to new conditions is quick and easy. The propagations reported here refer to cultures in liquid medium. If the strain is cultivated in a solid medium, the time required to reach a suitable condition for experiments is substantially longer but fully achievable.

New strains incorporated into collections after recent isolation should ideally undergo a set of growth tests to establish optimum conditions of temperature, culture medium, salinity, and other abiotic factors relevant to their maintenance in the collection. These results are fundamental for establishing the ideal conditions for algae growth, providing important subsidies for the design of experiments. This type of basic study can and should be guided by information already available concerning strains or similar species, so that the researcher can proceed to the survey with some useful indications. However, this procedure is laborious and may be impracticable if the collection does not have enough staff. It is worth remembering that sets of experiments like these are simple, but have sufficient scientific value to constitute lower impact publications, or at least to be included in a strain catalog, for example.

3.2.5 Strain quality control and routine problems

It is recommended that periodically all strains in a collection be examined for contamination (by bacteria, fungi, or other microalgae species). The standard procedure is to prepare slides for examination of cell appearance by light microscopy. Ideally, a slide should be prepared about a

week after inoculation of the culture. This simple procedure can be difficult if the collection is made up of many strains, taking a long time to perform on a routine basis.

In addition to slide preparation, cultures should be periodically examined for coloration, as the different species/strains have characteristic colorations in culture. After a routine period in the laboratory, with culture media suitable for each algae, those responsible for maintaining the collection tend to recognize the typical aspect of healthy cultivation of each microalgae (shades of green, brown, red, etc.). Thus, the occurrence of a 10-day-preparation *Synechocystis pevalekii* culture test tube with a yellowish-green or brown coloration may mean contamination with chlorophyte algae or diatoms, for example. On the other hand, very pale cultures, after a long time since inoculation (e.g., 2 weeks), may result from the use of an inoculum with many cells in a poor physiological state or the accidental use of a culture medium not suitable for growth (in a culture collection, various culture media tend to be used). Another indicator of inadequate culture status is the accumulation of many cells at the bottom of the test tube, which may indicate inadequate growth conditions or, if the culture is still young, the use of an inoculum with many dead cells. Possible contamination by bacteria or fungi can also be checked for by examining the coloration of the cultures. The presence of bacteria makes the cultures whitish and eventually even bacterial pellets (aggregations visible to the naked eye) can be seen. Filamentous fungi produce mycelia usually visible to the naked eye, which look like cotton flakes.

In a collection, it is possible to keep many strains (or all of them) axenic, but this is a task that requires permanent care. Once the condition of purity is reached, microorganisms can again contaminate the culture through almost imperceptible oversights. However, many microalgae collections do not operate with axenic cultures, but simply keep the strains under low bacterial contamination. The presence of few bacteria in the cultures may not bring relevant damage to the activity, depending on its application. More than this, there are strains that grow best when bacteria are present, so axenic cultures in this case may even be inconvenient (Lorenz et al., 2005).

Important problems are related to the preparation of culture media and use of culture vessels. Examples of such problems are salt precipitation during autoclaving (which may subject the algae to nutrient limitation), pH outside the tolerance range of the species, omission of some component solution from the culture medium, inoculation of the strain into the wrong culture medium, and use of culture flasks with waste cleaning materials, among others. Problems of this nature should be avoided by carefully preparing the culture media and culture flasks used, and by adopting safe culture propagation practices, such as subculturing a few cultures at a time. Still with respect to culture media, it may be appropriate to subject cultures maintained with defined media to any mixtures with natural seawater or indefinite media. Contact with substances from another chemical environment can be stimulating to the growth of certain microalgae, especially strains that have sudden periods of slow or unsatisfactory growth. This may be a symptom of a lack of some component of the culture medium, the availability of

which is sufficient for other species, but not for certain strains to achieve better growth performance.

Other relevant factors in a microalgae collection are temperature, salinity, and lighting. Climate-controlled cultivation rooms may be set to a certain temperature, but eventually the temperatures of the shelves or countertops where the algae are deposited may differ by a few degrees Celsius. Depending on the sensitivity of the species, this may lead to poorer growth responses, which may even compromise strain maintenance in the collection. It is recommended that growing rooms have a layout that promotes homogeneous heat distribution throughout the room, and temperature measurements are taken at critical points in the room. Regarding salinity, it is recommended that culture media be checked for this variable, as well as seawater used for the possible preparation of semidefined and undefined culture media. Although the vast majority of strains from coastal environments are tolerant to some salinity variations, some of them may be more sensitive, such as oceanic species. Control of this factor consists in maintaining salinity within the known tolerance limits for each strain. Light radiation presents several types of possible problems. Excessive light can cause photoinhibition of cultured cells, especially cyanobacteria and other phycobilisome-containing algae (Lorenz et al., 2005). Thus, it is essential to avoid exposing the cultures to bright light. On the other hand, too-weak light radiation can stimulate the synthesis of more photosynthetic pigments; if the culture flasks are too close together, the likelihood that self-shading will cause algae growth problems is very high. Racks full of test tubes should be avoided in culture collections, and greater culture distribution should be sought for available space. Photoperiod duration is also a potentially critical factor. Strains from temperate and polar environments tend to be more tolerant to large variations in photoperiod duration, but often strains from tropical environments tend to exhibit poor growth responses if a very different 12-h light regime is adopted. This does not mean that tropical species cannot be grown, for example, in continuous light. The arguments presented here refer only to the likelihood of success or failure of the enterprises, whose best decision must be made by testing.

3.2.6 Cryopreservation of microalgae in culture collections

The various aspects of maintaining a microalgae culture collection, discussed above, clearly show that it is a laborious activity that consumes a large portion of researchers' time, even in collections with adequate technical support. Microalgae collections play an essential role in contributing to the assessment of algal biodiversity, whether by providing strains, being used for conducting research, or conserving representative samples of the genetic heritage of various regions, countries, lakes, seas, and oceans. Some fundamental questions arise from the continuous manipulation of metabolically active microalgae indefinitely. Do genetic, morphological, and physiological changes occur in microalgae kept in cultivation for long periods? What is the physical limit of maintaining microalgae strains in collections? The

questions exemplified address entirely different problems, but they have in algae cryopreservation an alternative answer.

Cryopreservation is the maintenance of living organisms (or part of them) under an ultra-cold temperature (typically below −130°C) so that they remain able to survive after thawing (Day and Brand, 2005). The applications of cryopreservation in the biomedical, agrarian, and environmental sciences are very wide, also involving important commercial applications. The biological mechanisms that guarantee the conservation of life under very low temperatures are not yet fully understood, but many groups around the world have been advancing in this field. Although some general principles are the same in different applications (e.g., use of very low temperatures, long-term conservation, need to protect cells against intracellular ice formation, etc.), there are many specificities regarding the use of cryopreservation with different organisms. Therefore, specific studies are needed to enable the use of cryopreservation according to the nature and peculiarities of different species. Characteristics such as the existence or not of a cell wall, the amount of macromolecules in cells, presence of flagella, etc. are influential as to the success of the enterprises. In the field of phycology, researchers dedicated to the functioning of living microalgae collections are some of the most involved with the development of cryopreservation techniques, as some important advantages derive from their use to maintain microalgae strains in a metabolically inert condition. There are also some disadvantages regarding the use of this practice in relation to traditional methods of maintaining microalgae collections treated in previous sections. Table 3.1 presents favorable and unfavorable arguments for the use of cryopreservation in microalgae collections.

The cryopreservation process occurs in controlled steps, which must be performed in rigid sequences. The most common way to obtain ultra-low temperatures is by using liquid nitrogen

Table 3.1 Advantages and disadvantages of cryopreservation over metabolically active cell culture methods.

Advantages	Disadvantages
Provides stability against changes in genetic content over time due to selective pressure and/or genetic drift	Requires an investment in specialized equipment, special consumables, and training
Protects cultures against problems arising from microbial contamination, handling errors and identification of culture flasks, as well as problems arising from failures in the culture environment	Requires duplication of cryopreservation equipment and all cultures under conservation for reliability, as errors resulting from improper thawing under uncontrolled conditions, even brief, may lead to death
Requires less storage space per culture, as typically only 1.0 or 2.0 mL of each culture is stored	Requires a regular (at least monthly) supply of liquid nitrogen or a reliable freezer for ultra-low temperature generation (−150°C or less)
Reduces long-term maintenance cost when many strains can be maintained	Usually takes 2–3 weeks to generate adequate volumes of cultures from containers under cryopreservation

After Day, J.G., Brand, J.J., 2005. Cryopreservation methods for maintaining microalgal cultures. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier/Academic Press, San Diego, pp. 165–187.

(−196°C), although freezers capable of generating temperatures of −150°C can also be used. The major concern with the use of freezers refers to power supply fluctuations that cause temperature increases, a problem that does not exist with the use of liquid nitrogen. Considering the use of liquid nitrogen, the process is much more controlled than simply throwing aliquots of cultures into cryogenic canisters (Fig. 3.9). Although some species survive direct exposure to liquid nitrogen, such as *Chlorella protothecoides* (which tolerates an incredible cooling rate of $-2000°C\ min^{-1}$), the vast majority of species lack this extraordinary capacity. Small volumes of cultures (1.0–2.0 mL) should be placed inside properly identified cryovials; cultures must be in a very healthy state, corresponding to cultures without any incidence of stress or limitation. Cryoprotective substances (or cryoprotective agents) are used to prevent excessive damage to cells during freezing and thawing processes (see discussion below). Specific culture transfer velocities or rates need to be established from room temperature to very low temperature values. The same is true for the reverse process, from removing cells from the ultra-cold environment to culture temperatures (in reality, cooling and sample heating protocols are required). These are just a few basic observations to illustrate the complexity of handling cryopreservation samples; many other aspects must be considered that are not dealt with in this work, which is not intended to address this subject in detail.

Microalgae cryopreservation involves an assessment of percent viability, understood as the proportion of cells that remain alive and regain their metabolic activity after the culture is

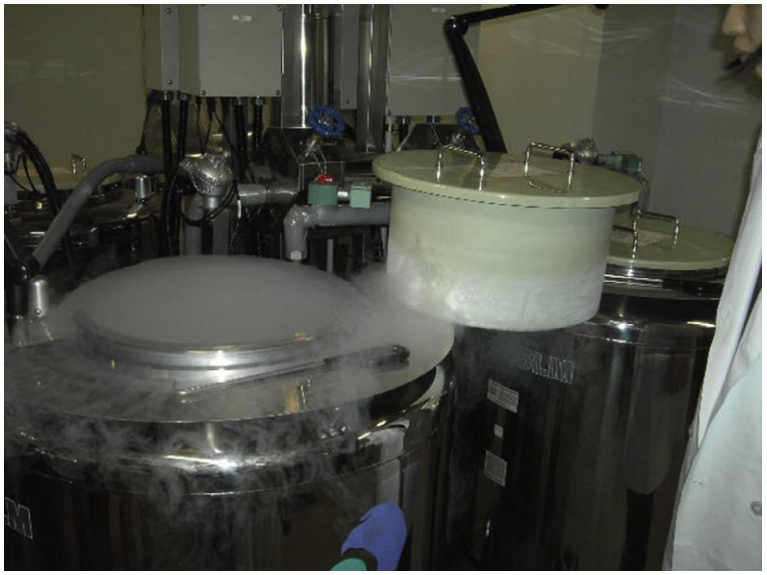

Fig. 3.9
Opening of a cryogenic cylinder of the Microbial Culture Collection at National Institute for Environmental Studies, in Tsukuba, Japan, for cryopreservation of microalgae samples. *Photo taken by the author.*

thawed. It is absolutely normal and expected that a portion of the population undergoing cryopreservation will die. Thus, it is necessary to establish values to allow an evaluation of the success of the practice. A viability of 60% is considered optimal for most species, but lower values are accepted depending on the difficulty of retrieving the different algae tested. Thus, the occurrence of only 10% of viable cells can be perfectly accepted when dealing with species with difficult cryopreservation. In addition, it is necessary to consider what the application of the culture is, because for various purposes there is no important implication derived from low viability. Information about the maximum algae conservation time is still scarce, mainly because the application of cryopreservation to microalgae is still quite recent, with less than 50 years of systematic research. However, information obtained by Day et al. (1997) suggests that microalgae are capable of maintaining a constant level of viability for at least 20 years.

Losses during the cryopreservation process derive from cell damage. Damage can occur at any stage of the process, but it is very rare during the storage of algae in liquid nitrogen (or freezer) itself, as conditions at this stage are extremely stable. It is precisely for this reason that it is assumed that specimen conservation can occur over extremely long time frames, as there are potentially no changes in the environment surrounding cells maintained under inert metabolism. Undoubtedly, the vast majority of damage occurs during freezing and thawing of samples. According to Day and Brand (2005), the main causes of cell damage are: exposure to freezing temperatures (temperatures insufficiently low to provide cryopreservation, with consequent ice formation); mechanical stress during extracellular ice formation; osmotic stress; intracellular ice formation; and free radical formation under low temperatures.

Brand and Diller (2004) describe some of the transformations undergone by the cells during the freezing period, reproduced below. As the temperature decreases, intracellular water remains in the liquid state after onset of ice formation in the extracellular medium. This ultra-cooling results from the lack of intracellular ice nucleation sites. However, the conditions for ice nucleation are eventually met. When ice formation occurs well below the equilibrium temperature, solidification can be triggered in an extremely rapid process which can be observed microscopically. Ice formation within cells is potentially harmful and must be minimized for successful cryopreservation. High concentrations of intracellular solutes and/or cryoprotective agents interfere with ice formation. Methanol (MeOH), dimethyl sulfoxide (DMSO; Me_2SO), and glycerol are the three most widely used cryoprotectants in algal procedures. These substances penetrate freely through cell membranes to balance the extracellular medium and intracellular matrix. They reduce osmotic changes during the cooling process and reduce ice formation if they are in high concentrations. Despite these beneficial effects on cryopreservation, these cryoprotective agents can be toxic to many species of algae, making their use in sensitive organisms unfeasible. Brand and Diller (2004) further report that when a seaweed culture is rapidly cooled, it reaches a temperature at which osmotic transport of water and cryoprotectant ceases before algal cells have time to become highly dehydrated, thus

favoring intracellular ice formation. An intermediate rate of cooling, such as 1°C min^{-1}, in the presence of a suitable cryoprotectant often promotes sufficient osmotic dehydration to prevent frost damage while avoiding excessive cellular dehydration. Resuscitation of cells after the cryopreservation period generally involves keeping the samples at room temperature for a few minutes, collecting the decanted material at the bottom of the cryotube and transferring the cells to a fresh culture medium. These procedures are done in the dim light and the cells inoculated in the fresh culture medium should be protected from light for several hours (e.g., over 12 h or overnight). After this time, the flasks may be exposed to light and viable cells should start growing within 1–2 days. Growth can also be evaluated on agar plates (except for species that do not grow in solid medium) by examining colony development after incubation. By counting cells immediately after the end of the cryopreservation cycle and after the short incubation period, the viability of the cells can be estimated. Controls should be employed to allow better estimates of viable cell rates.

The progress in cryopreservation of algae has been happening gradually, by testing for each strain. Eukaryotic (freshwater and seaweed) algae are cryopreserved with highly variable efficiency, but seaweeds in general are more difficult to successfully cryopreserve than freshwater and terrestrial algae. More success has been achieved with cyanobacteria and soil algae. In terms of taxonomic groups, there are also significant differences. Cryopreservation of most dinoflagellates, cryptophytes, synurophytes, and raphidophytes is difficult. Interestingly, marine diatoms have been cryopreserved more successfully than freshwater cultures. Culture collections search for specific protocols for successful cryopreservation testing strain by strain.

More and more microalgae cultured collections are expected to use cryopreservation as an alternative for maintaining their holdings. A great example of the interest of algae collections in cryopreservation was the implementation of the COBRA project: "Conservation of a Vital European Scientific and Biotechnological Resource: microAlgae and Cyanobacteria." Funded by the European Union, the COBRA project was initiated in the late 1990s and involved institutions from five countries (Germany, France, Portugal, the United Kingdom, and the Czech Republic) and important algae collections (ACOI, CCALA, CCAP, PCC and SAG—see Table 3.2 for information on acronyms), in search of the development of universal methods of cryopreservation of microalgae. This networking has promoted a major advance among the European countries involved, as well as the natural benefits of bringing together the different participating algae collections.

The search for alternatives to enable the cryopreservation of still problematic organisms should increase and may bring innovative solutions that combine unusual processes at the moment, following the trends of so-called cryobiology. An alternative that is already being tested with algae is the combination of vitrification with cryopreservation. This approach uses cryoprotectant vitrification for the conservation of aliquots of algal cultures in liquid nitrogen.

Table 3.2 Examples of microalgae culture collections operating in different countries.

Collection name, location, internet address and characteristics
– Algobank-Caen, Coleción de Cultures de Microalgues, Université de Caen-Basse, Normandie (France); https://www.unicaen.fr/algobank/accueil The collection features more than 200 strains of marine phytoplankton components, with an emphasis on flagellated organisms
– Coimbra Collection of Algae (ACOI) (Portugal); http://acoi.ci.uc.pt The collection has more than 3000 strains of freshwater and terrestrial environments from Portugal
– American Type Culture Collection (ATCC) (USA); https://www.atcc.org The largest US culture collection, ATCC involves a variety of microorganisms and viruses, including algal strains
– Canadian Center for the Culture of Microorganisms (CCCM) (Canada); http://cccm.botany.ubc.ca Located at the University of British Columbia, this was born from the fusion of three collections of microalgae and fungi. It currently contains 300 strains of marine and freshwater microalgae
– *Chlamydomonas* Resource Center at University of Minnesota (USA); https://www.chlamycollection.org The largest algal collection in the world in number of strains of the same genus, with more than 3000 strains of *Chlamydomonas*; it places an emphasis on genetic studies
– Australian National Algae Culture Collection (ANACC) (Australia); https://www.csiro.au/en/Research/Collections/ANACC Australia's reference collection for microalgae cultivation comprises 1000 marine and freshwater strains of major algal groups
– Canadian Phycological Culture Centre (CPCC) (Canada); https://uwaterloo.ca/canadian-phycological-culture-centre Formerly known as the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC) the collection now is housed at the University of Waterloo and comprises 500 microalgae strains from all major groups, with a predominance of freshwater strains
– Culture Collection for Algae and Protozoa (CCAP) (United Kingdom); https://www.ccap.ac.uk This national reference culture collection for microalgae and protozoan in the UK contains around 3000 strains of marine and freshwater microalgae
– Collection of Algae at the University of Cologne (CCAC) (Germany); http://www.ccac.uni-koeln.de The CCAC maintains approximately 5000 strains from around the world, with many marine and freshwater flagellates
– Culture Collection of Algae of Charles University of Prague (CAUP) (Czech Republic); https://botany.natur.cuni.cz/algo/caup.html This contains 256 strains of freshwater algae, mainly chlorophytes, cyanobacteria, and chrysophytes
– Culture Collection of Autotrophic Organisms (Czech Republic); http://ccala.butbn.cas.cz This collection comprises 510 strains of freshwater microalgae and cyanobacteria, as well as strains of seedless plants
– Culture Collection of Baltic Algae (CCBA) (Poland); https://ccba.ug.edu.pl/pages/en/home.php The collection is part of the University of Gdań sk and comprises Baltic phytoplankton and freshwater microalgae and macroalgae, totaling 200 strains
– Marine Biotechnology Institute Culture Collection (MBIC) (Japan); http://www.mbio.jp/mbic This collection of marine microorganisms (bacteria, fungi, and microalgae) has about 300 strains of microalgae

Continued

Table 3.2 Examples of microalgae culture collections operating in different countries—cont'd

Collection name, location, internet address and characteristics
– Microbial Culture Collection at National Institute for Environmental Studies (MCC-NIES) (Japan); https://mcc.nies.go.jp The largest Japanese collection of microalgae in culture, it has about 1400 freshwater, marine and terrestrial strains of the main groups of algae
– Pasteur Culture Collection of Cyanobacteria (PCC) (France); https://webext.pasteur.fr/cyanobacteria This is one of the Pasteur Institute's collections of microorganisms, specializing in cyanobacteria, with 750 axenic, freshwater, and marine strains
– Plymouth Marine Collection (MBA Collection) (United Kingdom); https://www.mba.ac.uk/facilities/culture-collection The collection is part of the Marine Biological Association of the UK and comprises 400 marine phytoplankton strains
– Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) (USA); https://ncma.bigelow.org/cms/index/index The US National Reference Collection for Marine Phytoplankton, featuring more than 2200 strains from around the world
– Roscoff Culture Collection (RCC) (France); http://roscoff-culture-collection.org This collection contains 5000 strains of marine microalgae, macroalgae, protozoans, bacteria, and viruses, most of which are isolates belonging to the marine picoplankton
– Sammlung von Algenkulturen der Universität Göttingen (Culture Collection of Algae at Göttingen University) (SAG) (Germany); https://www.uni-goettingen.de/de/sammlung+von+algenkulturen+%28sag%29/184982.html This contains more than 2300 strains in cultivation of all groups of algae, mostly freshwater and terrestrial environments
– Norwegian Culture Collection of Algae (NORCCA) (Norway); https://niva-cca.no Maintained and owned by the Norwegian Institute for Water Research (NIVA) and the University of Oslo (UiO), this includes 2000 algal strains from the two institutes and the former Danish algal culture collection SCCAP, which has been recently closed.
– Thailand Institute of Scientific and Technological Research Culture Collection (TISTR Culture Collection); https://www.tistr.or.th/tistr_culture The largest Thai culture collection, TISTR involves a variety of microorganisms, including 560 algal and cyanobacteria strains, mainly freshwater strains from Thailand
– University of Texas Culture Collection of Algae (UTEX) (USA); https://utex.org Leading collection of freshwater microalgae in the USA, with about 3000 strains; some strains are marine and brackish water

Vitrification has been widely used in medical cryobiology and for the preservation of animal, human, and flowering plants germplasms, but has only recently begun to be tested with algae under the COBRA project (Harding et al., 2004). Pure water and dilute aqueous solutions form a vitreous state if cooled extremely rapidly ($> -1000°C\ min^{-1}$), which can be easily achieved in the laboratory. Highly viscous aqueous solutions can be glazed with a lower cooling rate

(Day and Brand, 2005). Vitrification of the medium in which the cells are subjected can be achieved by immersing them in highly concentrated sucrose solution shortly before cryopreservation procedures are performed.

Another alternative is algal encapsulation-dehydration, adapting procedures developed a few decades ago for flowering plants. This basically consists of trapping small aliquots of microalgae cultures into calcium alginate droplets, followed by osmotic dehydration (usually by incubating for 24 h in 0.5–1.0 M sucrose solution) and desiccating the contents to a 20–30% moisture content (Day and Brand, 2005). Dehydrated drops are cooled to cryogenic temperatures, which makes the small water content highly viscous, virtually completely vitrified. Drops containing vitrified algal cells are kept under cryogenic conditions indefinitely.

3.2.7 Microalgae as models of organisms in genomics, proteomics, and GMO studies—The role of culture collections

For more than a century, microalgae have contributed significantly to increasing knowledge about complex biological processes. A prime example is the use of microalgae and cyanobacteria in the elucidation of various stages and chemical reactions of photosynthesis, from pioneering works (e.g., Warburg, 1919) to the present day. The simplicity of cells and their intense growth are strong arguments that justify their use in various research activities. This trend has not changed today when the so-called era of genomics and proteomics begins.

One of the most important steps to insert microalgae into this new context was the complete study by a multidisciplinary and interinstitutional team of the genome of Thalassiosira pseudonana, a marine diatom (Armbrust et al., 2004). T. pseudonana was one of the first organisms of no medical or agricultural importance to have its genome fully elucidated. The species was chosen because of its accentuated importance in North Atlantic waters, and belongs to a group (the diatoms) that may account for one-fifth of the planet's primary production. The study identified the genes responsible for several fundamental cellular processes, such as nitrogen metabolism, as well as genes that date back to the secondary endosymbiosis event suffered by the diatom group.

The study by Armbrust et al. (2004) was a very important milestone in this context, opening new possibilities for research with microalgae. A natural development is the development of techniques that allow a better understanding of gene expression for various metabolic characteristics (Parker and Armbrust, 2005) or the realization of genetic transformations in the species itself (Poulsen et al., 2006), for example. At present, dozens of microalgae have had their genomes fully sequenced and studied (e.g., Chlorella vulgaris in 2015; Chrysochromulina parva in 2018; *Cyanophora paradoxa* in 2012; *Emiliania huxleyi* in 2013; *Nannochloropsis gaditana* in 2014; Phaeodactylum tricornutum in 2009; Porphyridium purpureum in 2013; and *Volvox carteri* in 2010, among other microalgae), significantly expanding the basic knowledge

and possible commercial applications of microalgae. All of these strains are deposited in different collections of microalgae cultivation around the world, which serve as safe deposits of the biological diversity. Thus, culture collections play a central role in genomic and proteomic studies involving microalgae.

The use of transgenic algae in productive activities or toward the generation of substances of medical importance seems to be a matter of time (possibly a short time). The chlorophyte Chlamydomonas reinhardtii is possibly the best species for genetic transformation studies because its nuclear genome has already been studied in detail and the expression of its chloroplast genes is already known. Genes from other organisms have already been introduced into the C. reinhardtii chloroplast, and their characteristics are expressed, such as the production of luciferase (bioluminescent cellenterate genes Renilla reniformis) and the viral protein FMDV.VP1 (foot-and-mouth virus genes). In addition, transformations in C. reinhardtii mitochondrial DNA have also been successfully achieved (Walker et al., 2005). Certainly in the coming decades, microalgae genomics will be present among the most valued studies involving these beings. Knowledge of commercially important microalgae and cyanobacteria genomes and proteomes (e.g., *Arthospira platensis*) may offer more and better support for their use in more efficient ways. On the other hand, these studies allow the creation of genetically modified microalgae with higher productivity or with specific roles in the production of substances of interest. These applications reinforce the key role of culture collections of microalgae.

In recent years microalgae culture collections have also taken on a special role in the genetic authentication of microalgae. This role derives from the existence of many gene sequences in large international depositories and from the very advance of bioinformatics. Modern genomic and metabolomic tools have provided the possibility of generating and interrogating large data sets that can provide answers to previously imponderable taxonomic, evolutionary, ecological, and physiological questions (Day et al., 2017). Today's large culture collections of microalgae are highly qualified centers for combining microscopic identification of microalgae and the use of advanced DNA barcoding techniques (Brand et al., 2013). However, the curatorial tools needed to provide and maintain the relevant biological resources on which new knowledge can be built have not kept pace with this meteoric rise in scientific capacity, its associated activity, or the huge increase in published science (Day et al., 2017). This is one of the great modern challenges of the large culture collections of microorganisms.

3.3 Some microalgae culture collections across the world

Large microalgae collections are part of the reality of some countries, providing an important service to the public. Examples of such centers are the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) (East Boothbay, Maine, USA), Sammlung von Algenkulturen Göttingen Universität (SAG) (Göttingen, Germany), Culture Collection of

Algae and Protozoa (CCAP) (Oban, United Kingdom), and Australian National Algae Culture Collection (ANACC) (Hobart, Australia), among others. The aforementioned institutions function as units that centralize the national distribution of strains to users for all types of culture uses and applications. These are centers maintained with government resources, but which have the sale of strains as a source to cover (at least partially) the expenses associated with the maintenance of the collections. As an example, CCMP charges US$ 150.00 per 20 mL culture for universities/nonprofits, in addition to post office expenses. CCAP (United Kingdom) charges per 20 mL of culture £50.00 and £120.00 (GBP) for universities/nonprofit organizations and businesses, respectively, plus postage costs. SAG (Germany) charges per bottle of 20 mL of cultivation €165.00 (euros) for companies and €40.00 for researchers and educational and research institutions that qualify for subsidies, in addition to post office expenses.

Microalgae culture collections are of strategic importance, as they are fundamental to stimulate scientific research, the commercial production of microalgae and didactic activities, for example. Large collections take on an even more prominent role for their home countries and often receive support within national scientific development planning. Some historical aspects of three large collections of microalgae (SAG, NCMA, and CCAP) illustrate very well the relevant role they play.

SAG's beginnings date back to the 1920s, when the founder of the collection, Ernest G. Pringsheim, worked at Prague's German University in the Czech Republic. For more than 30 years, Pringsheim accumulated strains from the institutions in which he worked, including Cambridge University in the UK. Upon returning from exile in 1953, Pringsheim went to Göttingen University, accepting an honorary researcher position in botany. In Göttingen, he founded a large collection of microalgae gathering all their isolates and harboring new strains, isolated by their new collaborators, in addition to the great help of his wife, Olga. Currently SAG still maintains 311 original isolates from Pringsheim. The collection received great institutional support, and in 1954 Wulf Koch was appointed curator of the collection, a position he held until his retirement in 1979. During Koch's management, the collection began to develop strain distribution services for the scientific community and companies. The first strain catalog appeared in 1964, indicating the existence of 912 strains and 625 species of microalgae. Uwe G. Schössler served as SAG's curator from 1979 to 1999, increasing the collection's collection and the organization of available strains. During this period, SAG achieved greater international visibility and substantially increased the strain distribution service. With Schössler's retirement in 1999 came the third generation of researchers at SAG, which is now headed by Thomas Friedl and curated by Maike Lorenz. Currently the collection has 2141 strains, corresponding to 1228 species and 486 genera, and is one of the five largest collections of microalgae in cultivation in the world. In the period 2006–2011, the SAG received some 600 orders per year and dispatched an average of 2100 cultures per year (Friedl and Lorenz, 2012). Friedl and Lorenz were responsible for holding the first international symposium bringing

together researchers who run microalgae collections in 2002 in Göttingen, entitled "Culture Collections of Algae: Increasing Accessibility and Exploring Algal Biodiversity." Dozens of microalgae collections from around the world were represented at the event.

The Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) is the national reference collection for marine microalgae cultivation in the USA, with an annual supply of 2100 cultures for users inside and outside the United States. Its creation was recommended in 1980 by specialists assembled by action of the National Science Foundation (NSF), the leading US funding agency for scientific research. At that time there had already been a large national reference collection of freshwater microalgae cultivation in operation at the University of Texas, Austin since 1976. This culture collection previously worked at Indiana University for 20 years, coordinated by Richard C. Starr, until it was transferred to Texas. The American researchers understood that there was a need for the creation of a large national reference collection for marine microalgae cultivation, with an efficient strain distribution service. A marine microalgae collection was created in 1981 at the Woods Hole Oceanographic Institution (Massachusets), originally derived from the merger of marine microalgae collections maintained by Luigi Provasoli and Robert R.L. Guillard. The following year, the collection was transferred to the Bigelow Laboratory for Oceanic and Atmospheric Sciences (Maine), becoming a special unit of the institution from 1985. Always with financial support from NSF (as well as other government and private funders), the collection grew considerably in subsequent years, with the isolation of new strains and also the deposition of algal materials by researchers from various institutions. In 1992, the collection acquired a new name, being designated a "center" and not a "collection" of microalgae cultivation, reinforcing its tradition in research. In addition, the names of the two major researchers who provided most of the original collection were incorporated into the name of the center. In 2011, the NCMA acquired its current name. The addition of microbiota to the collection and name seemed especially fitting given Luigi Provasoli's discoveries more than 50 years ago that many algae require associations with bacteria to grow normally. Despite the name change, the NCMA identifier remained on all algal strains, ensuring continuity with current, past, and future phytoplankton research.

CCAP is part of a network of institutions specializing in the cultivation of different groups of organisms, such as fungi, bacteria, and animal viruses, all with independent headquarters and highly specialized scientific and technical staff—this is the UK National Culture Collections (available at http://www.ukncc.co.uk). The British National Microalgae Collection functioned for a few decades, divided into two headquarters, one specializing in marine microalgae (Oban, Scotland) and another in freshwater microalgae (Ambleside, England). In 2004, the two collections were merged into a single headquarters (Oban) into an entirely new building to house the large collection, built under the auspices of the UK National Culture Collections and the Scottish Association for Marine Science (http://www.sams.ac.uk). The CCAP algae collection has strains that have been isolated over many decades, and its origin is linked to the time when

Ernest G. Pringsheim lived in exile in the United Kingdom (in London and Cambridge). Pringsheim lived in the United Kingdom (London and Cambridge) in exile. Living biological collections in the UK, such as CCAP, have at least five doctors on staff, one of whom is the curator of the collection and the rest are dedicated to other aspects of work such as isolating and identifying strains, life cycle studies, physiology, and ecology of organisms. These professionals do not have didactic responsibilities; they are fully dedicated to research activities and the maintenance of collections. In addition to these, there is also a technical staff formed by professionals with college and middle level, supporting routine activities. Technicians are tasked with receiving external strain requests, preparing them and dispatching them under the supervision of one of the team's researchers. The professional model described for the British collections, in fact, is quite similar to those found in many of the large collections of microalgae in cultivation around the world.

While collections teams such as CCAP and NCMA are only researchers, other large collections, such as the University of Texas at Austin (USA), the University of Waterloo (Canada), and the University of Coimbra (Portugal), have university professors on their staff, dividing their time between research and maintenance of collections and teaching. These centers have strong institutional technical support as well as the workforce of undergraduate and graduate students.

In developed countries with a small territorial extension and a long tradition of microalgae use, a model of creation of national reference units tends to be adopted. Such units centralize and control the national distribution of strains to all users. Relative climate uniformity, small territories and the ease and speed of distribution of strains nationally have enshrined this model in European countries such as the United Kingdom (through CCAP) and Portugal (University of Coimbra). It is possible to maintain a very large portion of the national algal flora under cultivation, without the need to simulate very varied environmental conditions (temperature, photoperiod, etc.) in the laboratory. In contrast, in countries with large territorial extensions, climatic diversity, and/or large numbers of users in universities and companies, the natural option has been the creation of centers focused mainly on regional service. This is the case of the USA, which has large and medium-sized microalgae collections in some parts of the country, especially existing collections in Maine (NCMA) and Texas (University of Texas). Other countries such as Australia, Canada, and Japan also have a network of laboratories with large collections of microalgae. Interestingly, Japan falls into this category because despite having a relatively small territory, it has a huge number of users and a remarkable tradition in cultivation of aquatic organisms.

Large collections of microalgae in developed countries do not eliminate the existence of numerous smaller collections. These usually involve few strains and are generally dedicated to maintaining the algae used in local studies. Large collections assume the fundamental role of regulating public service and providing high-quality controlled source biological material for

the various possible applications. Another function of large collections is the organization of available information on the species/strains studied internationally. Therefore, it is imperative to have libraries with specialized collections and professionals designated to carry out bibliographic research on the production of knowledge with microalgae. Taxonomic updates are activities that require good-quality bibliographic material and highly qualified technical personnel.

The division of functions is very common, always having the figure of a curator, one or more assistants of the curator, a general director, and some technicians; naturally teams can be bigger. The curator is a researcher responsible for direct manipulation of the collection and is usually a doctor. He or she has the function of overseeing all routine activities of the collection, such as purification of strains, identification of materials sent to external applicants, growth experiments with different culture media, etc. One or more curator assistants are responsible for specific sectors of the collection (e.g., aquaculture algae, potentially toxic algae, cryopreservation). Some technicians, with or without higher education, join the team and work preparing culture media, propagating algae, and preparing materials that are distributed to users outside the collection. The general director is the researcher who coordinates the work developed in the culture collection and connects it with the scientific community and the general public. It is also increasingly common to have a professional to act as secretariat of the collection, receiving external requests for strains and coordinating their service, in addition to organizing all internal administrative activities. Microalgae collections are not only used for performing routine activities. Research is also conducted by the team, focusing on various topics such as taxonomy, physiology, and microalgae phylogeny, for example. Research opportunities in collections also attract temporary staff. Usually some doctor researchers without permanent ties to the collection are part of the temporary team, acting as postdoctoral fellows, for example. Their projects usually involve aspects of relevance to the development of the collection, such as genetic studies of strains, cryopreservation methods, extraction of algal substances, etc. Undergraduate and graduate students add to the collections' workforce. Finally, visiting researchers also contribute to the collections and make use of the various material resources in specific short-term studies.

Typically, a collection of 1000 strains and the preparation of 400–500 aliquots of cultures per year for the external community involves at least eight people, with three to five permanent staff members being common. Large collections can also be supported by a scientific committee, composed of some doctoral researchers who are not part of their permanent staff. The members of the scientific committee act as advisers and provide contributions to the scientific development and actions of the collection. The websites of the large collections of microalgae in cultivation often offer lists of available strains (with detailed information on each one), basic instructions on microalgae cultivation, media formulations, algae images, historical information, key bibliographic references, instructions for ordering and payment, etc. Table 3.2 presents summary information on some of the world's leading microalgae collections.

The large collections of microalgae in cultivation offer other services than simply sending live microalgae in culture medium to applicants. Diversification of services (especially in the USA) has become increasingly common, such as: purification of strains in isolation by researchers and companies; preparation of ultra-dense cultures for aquaculture or biotechnology applications; delivery of concentrated and lyophilized microalgae; extraction and concentration of nucleic acids (DNA and RNA) from species of interest to users; maintenance service of strains isolated by other researchers/institutions (without incorporating them into the collection collection); preparation and dispatch of culture media or solutions for enrichment of water used in cultures; teaching materials (seaweed cell models and educational games) and science fairs; and marketing of species sets for specific applications such as aquaculture and ecotoxicology, for example. All these specialized services are self-funded and payment terms are generally diverse, including credit cards. Short courses on microalgae cultivation or other more specific topics (e.g., culture isolation and purification, sterilization and washing of cultivation materials, aquaculture algal biomass production) are also offered by some microalgae collections and almost always paid for.

3.4 Concluding remarks

Science has moved at an extremely rapid pace since collections first started to maintain examples of microorganisms in the 19th century. Microalgae culture collections are a key component of life science research, biotechnology, and emerging global bio-based economies. Culture collections are valuable resources for the sustainable use of microalgae diversity, distribution of organisms, and its conservation, as well as the documentation and diffusion of associated data. They serve as a taxonomic reference, and provide for the long-term preservation of strains and organisms for all known uses. They distribute strains of known identity, provide information for their successful culturing, and offer diverse services to microalgal industry, research, and education communities. Most large collections maintain both actively growing and cryopreserved cultures. It is absolutely plausible and expected that the demands for specialized and reliable services from microalgae culture collections will increase and diversify more and more. The collections of today must adapt and change to meet new demands. Ideally, microalgae cultivation collections should look for ways to share their experiences and skills to meet the enormous tasks ahead.

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CHAPTER 4

Synthetic biology applied to microalgae-based processes and products

Dinesh Kumar Saini[a], **Sunil Pabbi**[b], **Anil Prakash**[c], **Pratyoosh Shukla**[a]

[a]Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India [b]Centre for Conservation and Utilisation of Blue-Green Algae (CCUBGA), Division of Microbiology, ICAR—Indian Agricultural Research Institute, New Delhi, India [c]Department of Microbiology, Barkatullah University, Bhopal, India

Chapter outline

4.1 Introduction 85
4.1.1 Aspects of synthetic biology 86
4.2 Feasibility of microalgae for synthetic biology to produce various products 88
4.2.1 Biofuels 88
4.2.2 Various other bioactive products 91
4.3 Challenges to developing microalgae as a chassis for synthetic biology 92
4.4 Future perspective 94
4.5 Conclusion 94
Acknowledgments 95
References 95

4.1 Introduction

In recent years, microalgae have gained attention globally as they have the potential to produce a wide range of compounds for industries like pharmaceutical, nutraceutical, food, cosmetic, bioenergy, and biofuel (Moreno-Garcia et al., 2017; Saini et al., 2020). These algae are related to plants as they contain both chlorophylls a and b in their photosystems for performing photosynthesis. They are ubiquitous and found in various habitats, including marine, freshwater, terrestrial, and even in harsh environmental conditions such as hot springs, deserts, etc. (Harwood and Guschina, 2009). They are rich sources for the production of metabolites such as polyunsaturated fatty acids (PUFA), carotenoids, polysaccharides, and particularly biofuels (bioethanol, biodiesel, lipids, etc.) (Nguyen et al., 2019). Microalgae are a diverse group of single-cell photosynthetic microorganisms that have been extensively studied as an

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alternative source for biofuel as many of these contain 40%–50% of triacylglycerols (TAG) and lipids by dry weight (Mata et al., 2010).

Synthetic biology involves the intersection of three fields: biological science, engineering, and chemistry. It aims to modulate or manipulate genetic circuits using various metabolic or genetic tools to achieve ultimate goals (Benner, 2003; Mukherji and Van Oudenaarden, 2009). It includes novel approaches to develop new genetic circuits and redesign metabolic and genetic activities of cells to produce specific products (Anand et al., 2017; Canton et al., 2008). The basic difference between molecular and synthetic biology is that synthetic biology uses molecular methods to create defined and predictable genetic structural design to perform complex processes. These processes include regulation of gene expression (via insertion of strong, native, or foreign promoters, enhancers, etc.), redesigning endogenous pathways to develop specific biomolecules, and many other functions. For example, recombinase, an enzyme, is used by molecular biologists to develop transgenic and knockout organisms, whereas synthetic biologists use it to build genetic circuits and repress or enhance expression of genes in the cell to achieve certain products. Moreover, advanced tools such as CRISPR/Cas9 are also used to control multilevel gene expression. Synthetic biology can be used in organisms in which reverse genetics approaches are applicable, and tools of molecular genetics like selection markers, genetic transformation, promoters' engineering, and other advanced tools such as ZFN, TALEs, and CRISPR can be applied (Jagadevan et al., 2018). Hence, in the case of microalgae, synthetic biology is limited to a few model organisms such as *Chlamydomonas reinhardtii*, *Cyanidioschyzon merolae*, *Nannochloropsis* sp., *Ostreococcus tauri*, and *Phaeodactylum tricornutum*. Synthetic biology tools need to be modified and optimized by understanding basic molecular mechanisms using various omics experiments for every individual organism. *C. reinhardtii* is a well-studied model organism in microalgae and has proven its ability to produce metabolites like fatty acid, hydrogen, and recombinant proteins. For example, in *C. reinhardtii*, DNA is inserted into its chloroplast using homologous transformation. A new expression system has been developed that accumulates 80% heterologous proteins, which are most effective in the algal nuclear expression system.

4.1.1 Aspects of synthetic biology

Synthetic biology is an emerging discipline that aims to redesign or rebuild artificial life through existing biological methods to achieve certain goals. Recently, synthetic biological research focused only on a few model organisms like *E. coli* and yeast, thereby providing and enhancing in-depth knowledge of their biological mechanism and their actual potential to produce various products. These techniques are used in metabolic engineering for the production of industrial enzymes. First, using omics experiments and system biology, different rate-limiting steps or enzymes of particular metabolic pathways can be identified (Baweja et al., 2016). After that, using different tools of synthetic biology, their expression can

be improved or regulated with respect to our goals (Kumar et al., 2017b). Microalgae are photosynthetic organisms that use solar irradiances and inorganic nutrients to grow and to form various chemicals and commodities. These organisms also absorb CO_2, which is a kind of greenhouse gas, during the process of photosynthesis. Additionally, they are related to plants but grow faster and are easy to manipulate due to their simpler genetic background. As already stated, these microalgae produce biofuel, pigments, biodiesel, lipids, and other bio-commodities, which make them attractive aspirants for renewable sources of these products (Fig. 4.1).

To increase biofuel/biodiesel and other compounds production in microalgae, the following properties need to be improved: (1) increase photosynthetic efficiency through metabolic engineering, which enhances productivity, oil production, and carbon sequestration rate in algal biomass; (2) convert carbon flux into useful products; and (3) develop committed microalgal cells, which help in lowering the large-scale production cost. For this, we can use synthetic biological tools as a potential approach to fulfill our goals. To apply any engineering approaches, proper characterization and optimization of each step have to be done for manufacturing any product from biological cells. In the case of microalgae, very few model organisms are present, as mentioned before, to date. Among them, *C. reinhardtii* green alga is a well-known model organism as it is fast-growing, haploid, and has a well-sequenced genome. In synthetic biology, the development of BioBricks is an important task. BioBricks are the part of DNA that are interchangeable and regulate genetic and metabolic functioning of the biological cell. Various BioBricks such as promoters, ribosome-binding sites, enhancers, etc. are used in synthetic biology to modulate and manipulate the genetic makeup of model organisms.

Promoters are sequences that regulate expression of a particular gene, e.g., *Hsp70A-RbcS2* chimeric promoter, alcohol-inducible promoters, nitrate-inducible promoters, light-inducible promoters (*PsaD*, *RbcS2*, and *LHCSR3* promoters), etc. To increase productivity, a strong promoter can enhance the expression of any gene that directly increases productivity (Lee et al., 2018; Kong et al., 2019). Both native and foreign promoters can be used for expression in a microalgal cell. For example, a promoter from the 5′ untranslated region (UTR) of the RuBisCO small subunit gene of *C. reinhardtii* has been used for *Chlamydomonas* transformation. Additionally, many foreign strong promoters are used in microalgae; for example, CaMV 35S and SV40 promoters taken from viruses have been used to express target genes (Wang et al., 2010). Many promoters previously used in plants to express transgenic genes are also used in microalgae as these organisms have a certain genomic homology; for example, the AlcR-$P_{alc\text{-}A}$ system has been recently used in *Chlamydomonas* (Lee et al., 2018). Negative regulation of gene regulation techniques also plays a crucial role in the regulation of gene expression at the transcription, translation, and posttranslation stages in the cell (Huang et al., 2010). There are many molecular techniques that can be used to downregulate an expression such as the use of a repressor as a negative transcription factor, which is used

in *E. coli*, using inducible systems of gene expression, degradation tags, and even the recently developed RNAi technique (Work et al., 2012). Endogenous enhancers are sequences that can enhance the rate of transcription of particular short gene clusters or certain protein sequences. Eichler-Stahlberg et al. (2009) inserted three introns from *C. reinhardtii* RBCs gene into recombinant luciferase, which resulted in a fourfold increase in erythropoietin expression level.

4.2 Feasibility of microalgae for synthetic biology to produce various products

Algal biotechnology is one of the prime targets to produce high-value metabolites. Cyanobacteria have already been proven as potential candidates for synthetic biology. They have been used to produce terpenoids, biofuels, sugars, fatty acids, and phenylpropanoids at lab-scale using synthetic biology, and the ability to use sunlight and CO_2 is undoubtedly one of the biggest advantages of these organisms (Wang et al., 2012,b; Huang et al., 2010). Along with cyanobacteria, microalgae with the ability to produce various compounds are also emerging as a potential resource. These are fast-growing eukaryotes with higher energy conversion efficiency due to their microscopic size and larger surface area, and with advance, molecular tools used via synthetic biology can enhance their ability for the production of biofuels and other metabolites (Banerjee et al., 2016). Some of the products that microalgae produce are summarized in Table 4.1, and the problems associated with their production are sorted out using synthetic biology.

4.2.1 Biofuels

Microalgae are considered to be one of the highest growth potential photoautotrophs in comparison to plants and seaweeds. They use light energy and inorganic matter for their growth and produce various compounds (Shah et al., 2018). These are attractive feedstocks for biofuel as they contain around 50%–60% of lipid content per unit dry weight (Chen et al., 2018; Jagadevan et al., 2018). Moreover, as they grow, they effectively impact on environmental issues by reducing CO_2 emissions and also alleviate problems related to water pollution, thereby providing the benefits of biomass-derived biofuels. Microalgae accumulate large amounts of triacylglycerol and starch, which are further converted into biodiesel and bioethanol, respectively (Chen et al., 2013).

Bioethanol is a well-known fuel, which is produced in Brazil and the United States of America from crop resources such as sugarcane and corn (Balat and Balat, 2009). Bioethanol is easy to develop as well as to distribute and is superior, characterized for automobiles, but its production from crops such as sugarcane, corn, and other agricultural feedstock competes with requirements for agricultural land and needs a lot of freshwater. Therefore, it is not economical

Table 4.1 Summary of the various synthetic tools used in microalgae and their outcomes.

S. no	Host organisms	Synthetic biological tools	Source	Outcomes	References
1.	*Chlamydomonas reinhardtii*	Transformation		Various human therapeutic proteins are expressed in algal chloroplast	Rasala et al. (2010)
2.	*Dunaliella salina*	RNA interference (RNAi)		Transcription level of *pds* gene was reduced up to 28% after transformation of plasmid *p*BIRNAI.Dsa containing homologous sequence of *pds* gene in comparison to control cell	Sun et al. (2008)
3.	*Nannochloropsis* sp.	Transformation		Shows efficient protocol for homologous recombination and gene knockout	Kilian et al. (2011)
4.	*Chlamydomonas reinhardtii*		*Synechoccocus* sp. PCC 7942	Shows expression of two isoforms of D1 protein; cyanobacterial low light isoforms expressed in host organism and shows 11% increase in dry mass	Gimpel et al. (2013)
5.	*Chlamydomonas reinhardtii*	RNAi		Single RNAi sequence used to silence LHC proteins which show lower expression mRNA of LHC proteins up to 26% and 68% less chlorophyll than parental strain	Mussgnug et al. (2007)
6.	*Chlamydomonas reinhardtii*	Nuclear transformation	*Chlorella zofingiensis* (*CzPSY*)	Phytoene synthase gene (*Psy*) key enzyme of carotenoids synthesis was inserted into *C. reinhardtii* showing 2.0- and 2.2-fold increase in carotenoids violaxanthin and lutein, respectively, than the transformed cell	Cordero et al. (2011)
7.	*Nannochloropsis oceanica*	Multiple gene overexpression		Constructed a genetic engineering toolkit containing bidirectional promoters and marker genes, transformed in host and shows increase in production of ω-3 long-chain polyunsaturated fatty acids (LC-PUFAs)	Poliner et al. (2018)
8.	*Nannochloropsis oceanica*	Overexpression		Constructed stress-inducible endogenous promoter (NoD12, a lipid droplet surface protein (LDSP) promoter) to alter fatty acid composition under nitrogen starvation conditions	Kaye et al. (2015)
9.	*Nannochloropsis* spp.	CRISPR/Cas9-based genome editing		Precise deletion of five base pair using CRISPR/Cas9-based genome editing in nitrate reductase translation, which makes the mutants unable to grow in NaNO medium	Wang et al. (2016)
10.	*Chlamydomonas reinhardtii*	RNA interference		Decrease in expression of *Chlamydomonas* phosphoenolpyruvate carboxylase isoform 1 (*CrPEPC1*) gene mRNA using RNAi directly increase TAG concentration up to 20%	Deng et al. (2014)

for large production because competition for agricultural land lowers the food productivity, which directly influences food security of any nation. Along with agricultural land, these crops require nutrition, pesticides, etc. for their growth (Nigam and Singh, 2011). On the other hand, microalgae can be grown on nonagricultural land, degraded wastelands, and in seawater and wastewater, and do not compete with agricultural land or its productivity.

Production of bioethanol is currently classified into three generations: (1) first-generation bioethanol (utilizing starch-based crops); (2) second generation (utilizing lignocellulosic substrate, e.g., agricultural waste of crops like rice, wheat); and (3) third generation (utilizing algal feedstock). First-generation production is based on various seasonal crops, which exploit agricultural land and their resources such as water, fertilizers, and pesticides. Production costs using these well-established technologies are very low, for example, 0.16–0.22 US$L^{-1} in Brazil, but this is not applicable globally, especially in countries in which less arable land is available (Gaurav et al., 2017; Lee and Ofori-Boateng, 2013). In the second generation, lignocellulosic material is used, which requires potential pretreatment due to the presence of high lignin content. This pretreatment is time-consuming and increases the production cost of the bioethanol in comparison to first-generation alcohol production (Naik et al., 2010). In the third generation, photosynthetic microorganisms such as microalgae and cyanobacteria are used, which have higher growth rates, low lignin content, and simpler cellular structures. Many species of microalgae such as *Chlamydomonas reinhardtii* (Cakmak et al., 2012), *Chlorella pyrenoidosa* (Tang et al., 2011), *Phaeodactylum tricornutum* (Fajardo et al., 2007), *Scenedesmus obliquus* (Mandal and Mallick, 2009), *Dunaliella parva*, and *Nannochloropsis oceanic* are well-known for biofuel production. These species of microalgae accumulate useful amounts of lipid. There are many reports of large-scale production of biodiesel from microalgal species; for example, Nobre et al. (2013) examined *Nannochloropsis* sp. for biodiesel and co-product syntheses like hydrogen and pigments. They extracted 45% of lipid by the super liquid extraction method (Nobre et al., 2013).

Recent studies have been performed to understand the metabolic network and key enzymes involved in lipid synthesis. These studies elucidate that carbon is accumulated mainly into two primary storage molecules: starch and lipids. Most studies have shown that nitrogen starvation conditions result in high TAG accumulation in the microalgal cell. It has been reported that cyanobacteria are affected differently by nutrient limitation, leading to variation in their lipid productivity, as revealed by the extent of *acc*D gene expression, which was regulated more by nutrients' concentrations than by the organism. The overall expression of *acc*D was found to be upregulated in both *Oscillatoria* sp. and *Microcoleus* sp. under nitrogen limitation but was differentially regulated with both positive and negative induction under phosphorus stress conditions (Kumar et al., 2017a). Furthermore, strategies like knock-down the competitive pathways proven effective increases lipid content of the cell. For example, overexpression of Malic enzyme gene in *P. tricornutum* increases 2.5-fold the total lipid content in dry cell weight (Xue et al., 2015). Moreover, mutants that are unable to synthesize starch accumulate higher

lipid content. To increase biofuel from microalgae, these observations can be exploited using synthetic biology to develop mutants that are deficient in starch synthesis, or knockout, or downregulate key enzymes responsible for their synthesis. Additionally, metabolic engineering, along with approaches like genetic engineering and flux balance analysis, are also used to target key enzymes for enhancing biofuel production (Banerjee et al., 2018).

4.2.2 Various other bioactive products

Microalgae are rich sources of various bioactive compounds such as vitamins, proteins, fatty acids, pigments, etc. These compounds are known for their application in human nutrition, aquaculture feed, bio-fertilizers, etc. (Raposo et al., 2013). Some microalgae and cyanobacteria also produce polysaccharides, which have antiviral, anticancerous, and nutraceutical applications (Huheihel et al., 2002; Gardeva et al., 2009; Dvir et al., 2009).

Fatty acids are essential for human growth and metabolism, but many fatty acids, such as ω-3 and ω-6, cannot be synthesized on their own, so these are needed from external sources. These essential fatty acids have a role in the integrity of tissues where they are present; for example, γ-linolenic acid (GLA) is used in the cosmetic industry as it has skin-revitalizing properties (de Jesus Raposo et al., 2013). GLA has an important role in the synthesis of the cell membrane, prostaglandin for the immune system, and other processes of skin regeneration. Other fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have a role in reducing lipid content and also in nutraceutical, antiinflammatory, and antimicrobial activities (Alvarez and Rodríguez, 2000). Additionally, DHA has an important role in brain development as well as in the functioning of the nervous system. Many marine microalgae have been reported to produce these fatty acids, such as *Isochrysis galbana*, *Arthrospira platensis*, *Spirulina* spp. *Porphyridium cruentum*, and *Odontella* (Raja et al., 2008; Bhakar et al., 2014).

Microalgae are one of the richest natural sources of various pigments, especially carotenoids. These are accessory pigments that also absorb light in the visible spectrum. These pigments protect the microalgal cell from excessive light by quenching excited free radicals. Carotenoids like β-carotene, astaxanthin, lutein, and lycopene are commercially produced using various microalgae (Jacobsen et al., 2019; Saini et al., 2020). Among microalgae, carotenoids are important, as they provide a protective coat for chlorophyll against photo damage. They are also reported for their role in the prevention of many diseases and cancers in humans. To increase the productivity of carotenoids, extensive studies on their metabolic pathways and genes have been carried out. Phytoene synthase (PSY) catalysis is the first step toward the synthesis of lycopene, an intermediate of carotenoids, which is further followed by a cascade of enzymes such as phytoene desaturase (PDS), z-carotene desaturase (ZDS), and carotene *cis-trans* isomerase (CRISCO), etc. (Farré et al., 2010). Enhancement of the expression of these enzymes directly increases the concentration of carotenoids. For example, overexpression of PDS and PSY genes was investigated in microalgae such as *H. pluvialis*, *C. reinhardtii*, and

P. tricornutum, which resulted in an increase of carotenoids' content in mutant cells (Liu et al., 2013; Tran et al., 2009). Additionally, β-carotene oxygenase (BKT) is a key regulating enzyme in the synthesis of β-carotene and astaxanthin. BKT was introduced successfully into *C. reinhardtii* and resulted in the start of production of astaxanthin (León et al., 2007). In the case of pigment, most research into the enhancement of carotenoids has only been done by changing nutrient conditions and some abiotic factors such as light, temperature, etc. There are fewer studies using genetic engineering and synthetic biology, which have great potential in the enhancement of carotenoids' production.

Along with feedstock for biofuel, microalgae also accumulate carbohydrates to a greater extent (Zhao et al., 2013). Many microalgal species like *Chlamydomonas*, *Chlorella*, *Spirulina*, *Dunaliella*, and *Scenedesmus* have a high content of carbohydrates (especially starch) as a proportion of dry weight. For example, *Chlamydomonas* produces more than 50% of carbohydrates, which can be further hydrolyzed using microorganisms (bacteria, fungi) to bioethanol for biofuel production. Choi et al. (2010) reported that carbohydrates obtained from *Chlamydomonas* were hydrolyzed into glucose, which, when fermented further using *Saccharomyces cerevisiae*, produced 235 mg ethanol/g algae (Choi et al., 2010).

4.3 Challenges to developing microalgae as a chassis for synthetic biology

Microalgae are a highly diverse group of microorganisms, capable of growth in various kinds of habitat (freshwater, seawater, terrestrial, hotspot, etc.) and environments. In comparison to higher plants, these microalgae grow faster and produce different kinds of metabolites and biochemicals, as has been discussed in the previous sections. Microalgae have undoubtedly been one of the potential candidates for biotechnological application, but many challenges remain in terms of the use of synthetic biology in these microorganisms to develop them as a chassis for synthetic biology.

BioBricks are one of the most important requirements to develop any organism suitable for synthetic biology (Lewens, 2013). There are a limited number of BioBricks characterized for microalgae, which do not fulfill the needs of synthetic biology for controlled gene expression and their regulation (Ginsberg et al., 2014). Thus, there is a need for development of a system for collection and characterization of a new set of BioBricks for the microalgal system. Many strains of *E. coli* and yeast are being commercialized, which are genetically modified for different purposes, but in algae, a limited number of algal models are available that can be used as a chassis to construct and design new synthetic pathways. Transformation efficiency is another approach that needs to be standardized in microalgae for synthetic biology. Transformation protocols are well-established in cyanobacterial species like *Synechococcus* and *Synechocystis* (Heidorn et al., 2011), but they need to be improved further.

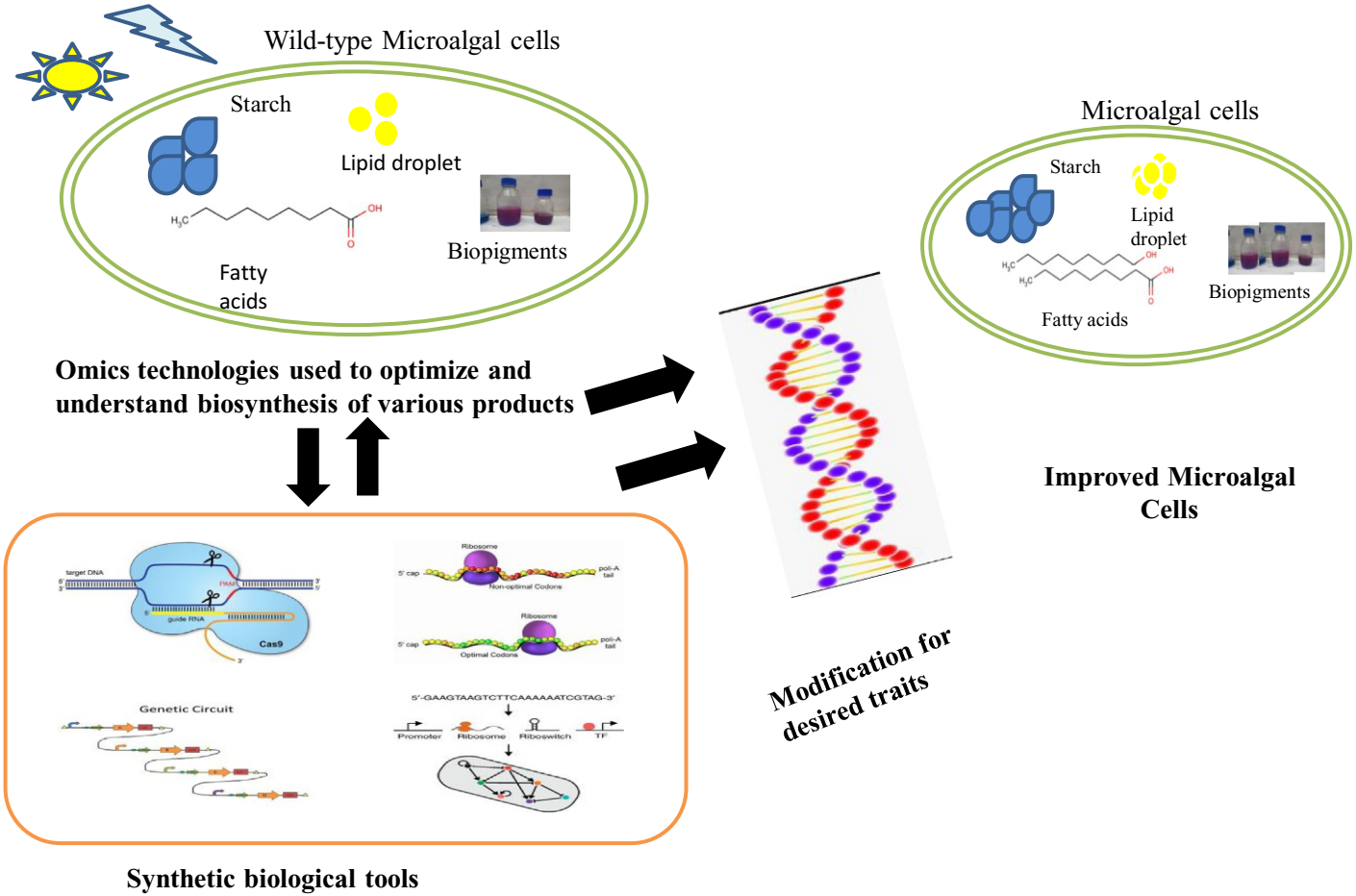

Fig. 4.1

Integration of synthetic biological tool in microalgal research and their value added product.

Oxidative stress is also a challenge that must be overcome to develop microalgae as a chassis. All algae, including microalgae, perform oxygenic photosynthesis by splitting water molecules to produce oxygen during photosynthesis. However, it will be a problem to synthesize various oxygen-sensitive enzymes such as nitrogenase (Lee et al., 2010), hydrogenase (Fay, 1992; Unay and Perret, 2019), and other oxygen-sensitive commodities. In broader terms, the oxygenic nature of microalgae may be a problem in integrating pathways of anaerobic microorganisms to express in the algal cell. To overcome this challenge, alternatives have been suggested in the case of the cyanobacterial cell. As in cyanobacteria, there are specialized anoxygenic cells, known as heterocysts, which are used for nitrogen fixation that can be exploited to avoid this problem (Wang et al., 2012,b). Another alternative is the Mehler reaction, which can be used to overcome oxidative stress as it shows the scavenging nature on reactive oxygen species from intracellular chloroplast and cyanobacteria. Therefore, it will be possible to produce oxygen-sensitive enzymes from microalgae (Asada, 2006).

4.4 Future perspective

An important goal of using photosynthetically active organisms for synthetic biology is that they use sunlight as a source of energy to synthesize biofuel and chemical compounds. However, the photosynthetic efficiency of microalgae has not reached its full potential: these microalgae have solar conversion efficiency around 10%–15%, which is relatively low. One of the well-studied strategies to increase photosynthetic efficiency is to have truncated photosystem antenna, which helps in light penetration in photosynthetic organisms. For example, in green algae, *C. reinhardtii*, the truncated light-harvesting antenna complex (LHS) showed higher photosynthetic efficiency under intense sunlight in comparison to normal light-harvesting antennae (Blankenship et al., 2011; Ort et al., 2011). Synthetic biological tools can be used to enhance photosynthetic efficiency and increase the productivity of microalgae. Moreover, photo-systems I and II in microalgae absorb light mainly in the visible spectrum only, but the integration of bacteriochlorophyll may widen the light-harvesting spectrum of the microalgae and enhance productivity (Blankenship et al., 2011). More studies are needed in this area in order to understand the molecular and regulatory mechanism of the light-harvesting system of microalgae.

4.5 Conclusion

Microalgae, along with cyanobacteria, have the potential to become a green chassis for production of renewable fuel and chemical commodities. Presence of their genome sequence, relative knowledge about genetic workflow, and advanced genetic tools make them desirable for synthetic biology. Many positive results achieved recently, regarding not only the understanding of their molecular mechanism but also the development of various products including biofuel and recombinant proteins, have reconfirmed their potential as algal cell factories. However, the use of synthetic biology in microalgae is in its early days as many

challenges and bottlenecks need to be addressed and improvements have to be made regarding tools for genetic manipulation, which enhance their photosynthetic ability, CO_2 sequestration efficiency, etc. Metabolic modeling and genome-scale reconstruction may help to create desirable strains and products, and functional genomics, which includes transcriptomic, proteomic, and metabolomics, will aid in understanding their molecular mechanism so that it directly benefits the use of synthetic biology in it. These approaches will be helpful for proper functional annotation of gene and metabolic pathways of the microalgae, as these are currently very poorly annotated. Moreover, advancements in synthetic biology may unlock the vast opportunities from abundant light and CO_2 resources for biotechnological applications.

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PART 2

Microalgae-based processes

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CHAPTER 5

Photobioreactor design

Jose C. Merchuk

Ben-Gurion University, Beer-Sheva, Israel

Chapter outline

5.1 Chemical reactors versus photobioreactors 101
5.1.1 The chemical reactor 101
5.1.2 Microalgae and seafood 103
5.2 Kinetics of photo-biosynthesis 106
5.2.1 *P-I* charts 106
5.2.2 Other kinetic models 106
5.2.3 PSF 106
5.3 Fluid dynamics relevance 108
5.3.1 The effects of fluid dynamics on photosynthesis 108
5.3.2 Photosynthesis and ordered mixing 110
5.4 Ingenuity and inventiveness 116
5.5 Advanced models 122
5.6 Conclusions 124
Acknowledgment 124
References 125

5.1 Chemical reactors versus photobioreactors

5.1.1 The chemical reactor

Chemical engineering started to become a scientific discipline during the first part of the 20th century. Chemical processes existed already many centuries before, but the design of chemical production plants was the realm of experts in specific products, and the expertise was based on empirical experience. Chemical engineering emerged from the unification of operations (like liquid or solid transportation, mixing, filtration heat transfer, etc.), which, while used in many different processes, are essentially the same phenomena, i.e., transfer phenomena. When chemical reactions are involved, however, the complexity of the process increases noticeably. The quest to find a common frame for the many complexities appearing in the interactions of chemical reactions with heat transfer, mass transfer, and flow led to the definition of one of the most important subareas in the profession: chemical reactor engineering.

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Conventionally, chemical reactors are grossly classified by their mode of operation in two categories: batch and flow reactors. Those two categories became identified in hardware language as stirred tanks and tubular reactors, respectively. The basic textbooks start by defining the "ideal reactors" in each of those categories. A more comprehensive form of definition would be to use the term "flow models" in each case. In this chapter, we will define model as a series of assumptions about the way a phenomenon actually takes place. It should be noted that this definition is essentially different from the concept associated to the word "model" in biological sciences, where the term may refer to an organism or even to a molecule. The ideal batch reactor, in terms of a model in the sense mentioned above, would be one with ideal mixing (zero time for homogenization: particles of any age in the vessel can meet and interact). On the other hand, the ideal plug-flow reactor would be one with ideal segregation, where encounters between particles of different ages could never take place. Here age is defined as the time of residence in the reactor of a particle (Fig. 5.1).

We refer to an "ideal model" as a phenomenological model based on simplifying assumptions while maintaining its dominant features. This approach is particularly useful when discussing chemical reactors. When the well-mixed system assumption is applied, the relations between the system variables, expressed in terms of mathematical equations, are also simplified, yet providing satisfactory approximations to the actual process. Those equations can be easily found in standard textbooks and are not shown here. If the flow characteristics differ distinctly from well-mixed flow, more complex models must be used, possibly including adjustable parameters that characterize each specific device and operation mode. Examples of the above include "the axial dispersion model" or "the tanks-in-series model" for flow reactors, or "dead zones" for batch reactors. Many other variations are possible, and many of them have been proposed and tested for specific reactors. Extremely useful tools in this area are the mixing time

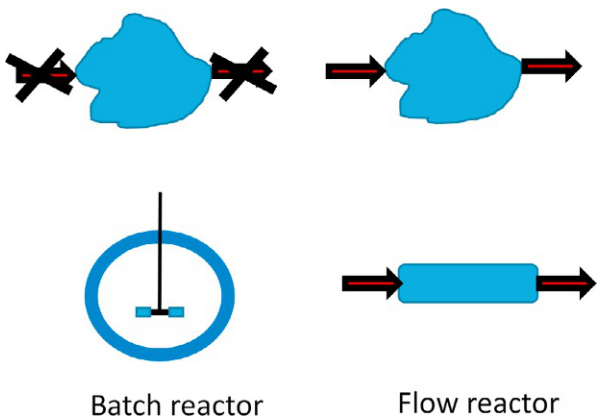

Fig. 5.1
Types of chemical reactors. The main point is the flow and not the shape.

(t_m) and especially the residence time distribution (RTD) (Danckwerts, 1953; Bischoff and McCracken, 1966). Both are relatively easy to measure, and are the most effective means for flow characterization in chemical reactors.

In most common reactors or bioreactors, the information needed for mathematical modeling involves three categories: (a) stoichiometry of the chemical reaction or reactions; (b) the corresponding kinetics; and (c) fluid dynamics (commonly expressed in terms of residence time distribution). Basic knowledge of the above should be sufficient for mathematical representation of the process, thereby paving the way for simulation and reactor design. The design of a photobioreactor is, however, more complex because residence time distribution is not enough, and more information on fluid dynamics is required.

5.1.2 The photobioreactor

It was observed above that the information needed for chemical reactor design is insufficient in the case of photochemical or photosynthetic reactions. Many early studies were collected in a now classic book edited back in 1953 by Burlew (1953). Since light is the main substrate for the process (light is considered here a substrate, since it is the source of energy for biosynthesis), it is obvious that it should be one of the most important variables in the kinetic expression: the rate of photosynthesis will be heavily influenced by light intensity. Obviously, under certain conditions many other variables may have influence on the kinetics of biomass generation: CO_2 concentration in the gas, temperature, and various nutrients. But in large-scale photobioreactors, it is usual for all the substrates necessary to be proportioned to the liquid in sufficient concentrations, to ensure that they do not control the kinetics, and that temperature is generally homogeneous because the time constants for heat transfer phenomena are much lower that the time constant for growth (the inverse of the specific growth constant, μ).

However, in the present review, which focuses on production of biomass in photobioreactors, we concentrate on light effects. Light, whether natural or artificial, is very different from any other substrate, because it decays with distance from its source, and is independent of fluid dynamics. In simpler words, light cannot be mixed. Thus, the most important variable in the kinetics of the process, light intensity, changes with the geometric position in the reactor, but is not influenced by manipulation of any of the operation variables of the process.

A very simplified scheme that helps us to understand the phenomenon is shown in Fig. 5.2 (Merchuk et al., 2019). The figure presents the profile of Illuminance (I) as it decreases exponentially from the illuminated face of the reactor. A one-dimensional geometry has been adopted for simplicity. The only space coordinate is z, the distance from the light source.

On the same scheme, the rate of growth of a photosynthetic organism, μ, is also shown. Three illumination zones in a photobioreactor can be identified in the scheme, and each zone will have a

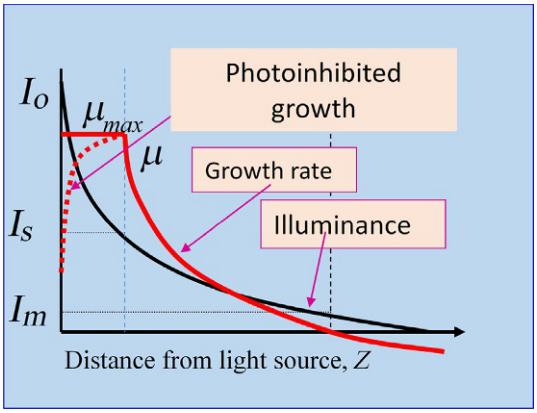

Fig. 5.2

Illumination zones in a photobioreactor. Light saturation zone near the illuminated wall: maximal growth rate (or, alternatively, photoinhibition). Illuminance between I_s and I_m: μ is linear on I. $I < I_m$: the culture dwindles. *Adapted from Merchuk, J.C., Garcia-Camacho, F., Molina-Grima, E., 2019. 2.25- Photobioreactors–Models of photosynthesis and related effects. In: Murray Moo-Young (Ed.), Comprehensive Biotechnology.*

different regime of biomass growth. The first zone extends from the illuminated wall to the point where the illuminance reaches I_s, which renders the maximal growth rate. Any value of I above I_s will not lead to an increase in the biomass growth rate, and may even yield a decrease in the growth rate if photoinhibition occurs. This may be called the light saturation zone. Further on, within the zone where the irradiance is bounded between I_s and I_m, the dependence of μ on I is generally assumed to be linear. I_m is the irradiance that does not lead to any perceptive photosynthetic growth, but just balances maintenance, a phenomenon that continuously consumes biomass. For $I < I_m$ the growth rate is below the rate of consumption by maintenance, and biomass concentration may dwindle. If we now envisage a photobioreactor where the distance of every photosynthetic cell from the light source remains constant, as in an immobilized bed reactor or an ideal plug-flow reactor, the average biomass growth rate will result from the integration of the rates over the whole volume of the photobioreactor for a steady-state situation:

$$\mu_{av} = \frac{1}{z_f}\int \mu(I(z))dz \tag{5.1}$$

where z is the distance from the illuminated surface, I is the local value of the illuminance, z_f is the distance of reference, and I is related to z via the optical decay equation appropriate to the system, $I(z)$.

The Lambert-Beer equation is frequently taken as a first approximation for $I(z)$. Here a one-dimensional formulation has been used for simplicity, which makes Eq. (5.1) rigorously correct only for flat surfaces. This simplification is done for didactical purposes, but can be

justified in dense cultures where the drop in $I(z)$ is very sharp, taking I to practically zero in just a couple of centimeters. However, the aim here is to present just the principle rather than the details of the calculation.

In practice, in any real photobioreactor where the photosynthetic cells are suspended in a liquid (except for an ideal plug-flow reactor), the cells move with the liquid following turbulent paths, the distance from the light source changes with position, and the local value of the growth rate μ changes with position accordingly. Therefore, any application of the conventional models of chemical reactors, designed to predict the yield of a photochemical reaction, will fail. This is probably the reason behind the fact that most of photobioreactors are designed on an empirical basis. In an attempt to address this problem, we proposed the concept of "light history" (Merchuk et al., 1996), inspired by the idea of "trajectory length distribution"—a concept that has not been appreciated sufficiently in the literature, in our opinion—in a paper by Villermaoux (1996). The concept of light history was further extended and applied (Wu and Merchuk, 2001, 2002, 2004), and is now widely used in the scientific community. The idea is based on the observation that every fluid element moves along a specific trajectory within the reactor and the photosynthesis rate changes along its flow history as a function of its distance from the light source, z, which in most cases is the surface of the reactor. In other words $I = I(z)$, while the growth rate in its turn depends on illuminance, $\mu(I)$.

The integral of the specific rate of growth in a photosynthetic element traveling along its trajectory will give the biomass production in this element, and the sum of the productions in all elements results in the overall photobioreactor yield. This is in fact a Lagrangian approach. Eq. (5.2) expounds the nature of the strong relation between photosynthesis and fluid dynamics that has been recognized for a long time. It can be formulated in a general way (for the presently adopted one-dimensional geometry), as:

$$\eta_{av} = \sum_{i=1}^{i_f} \int_{\widetilde{t}=0}^{t} \mu\left\{\alpha, I\left[z_i\left(\widetilde{t}\right)\right]\right\} d\widetilde{t} \tag{5.2}$$

where i is the path line followed by the photosynthetic element, and the number of paths is i_f. Each path is expressed as a function $z_i(t)$ of the distance to the light source along the time considered and I is a function of z, and μ in turn is a function of several variables which do not change with position, lumped as α here, and of I, which is our main concern here. The total rate of biomass production μ_{av} is the sum of the production over all the trajectories i, assumed to be all of the same duration, $\widetilde{t}$.

Thus, paradoxically, the nondependence of illumination on flow becomes the main reason that makes fluid flow so important in the integration of kinetics and flow for photobioreactor design. This will be discussed in the next sections.

5.2 Kinetics of photo-biosynthesis

5.2.1 P-I charts

Describing kinetics of photosynthetic growth is an extremely complex task because it must include various interacting phenomena, and it depends on the timescale due to the effects of acclimation and adaptation. A relatively extended exposition of the matter has been presented elsewhere (Merchuk et al., 2019). The most basic representation of photosynthesis kinetics is the so-called *P-I* chart or saturation curve: the dependence of the photosynthesis rate on illuminance, with easily measurable parameters, usually called α, the initial photosynthesis rate (initial slope), and P_m, the maximal photosynthesis rate. These empirical values are parameters of the curve, and can be associated to the growth process and to the behavior of the culture, which depend on its physiological state. P is usually given in terms of biomass produced per unit time and unit volume (or mass) of the culture, or per unit illuminated surface. Each *P-I* chart is valid only for a photo-acclimated system—that is, a system that has been kept for a sufficient time at each of the irradiances before P was measured.

5.2.2 Other kinetic models

A detailed table of models of several degrees of sophistication has been published recently elsewhere (Merchuk et al., 2019). They range from very simple representations of the *P*/*I* chart as an exponential and Monod-type equations to much more complex forms.

There is also a group of much more sophisticated models that can be called physiological, aiming at the representation of the dynamic behavior of photosynthetic cells, and proposing approximations to the mechanism operating inside the cells, which depends on their capacity for adaptation to different illumination intensities. Those models aim to express the dynamics of a photosynthetic culture taking into account a considerable number of variables in addition of the obvious (carbon source and light), and among them various substrates that algae require for growth, such as nitrate and phosphate, and also intracellular concentrations. Here we will concentrate on a model built around a different concept: the photosynthetic unit model, or PSF.

5.2.3 PSF

The photosynthetic unit, or PSU, is an imaginary factory that opens its doors to accept a certain amount of radiation energy, closes to elaborate it, and yields a certain amount of biomass. In biological terms, it lumps together photosystem I, photosystem II, and a series of biochemical processes following them that end in the synthesis of a polysaccharide. When the product is finished and delivered, the doors of the factory open again to restart the process. If for some reason an excess of energy enters during the closed state, the operation becomes inhibited for some time before rehabilitation. Since many such units exist, the model is presented as a

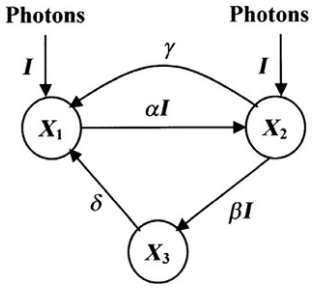

Fig. 5.3

The scheme shown in the figure accounts for the influence of *I* on the biomass production rate and for the inhibition of the photosynthetic cell as a reaction to an excess of illumination. It does not take into account photoadaptation (Wu and Merchuk, 2001). *Based on Eilers, P.H.C., Peeters, J.C.H., 1988. A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. Ecol. Model. 42, 99–215.*

dynamic system where open, closed, and inhibited factories coexist. The mathematical formulation of the model consists of differential equations that represent the rates of the change in time of the fraction of those factories that can be found in each of the states: open, closed, and inhibited. Fig. 5.3 provides a schematic representation of the model, following the formulation by Eilers and Peeters (1988), In the figure, X_1, X_2, and X_3 represent the fraction of the total units (or factories) that are open, closed, or inhibited, respectively. Each of the arrows in the scheme represents a step in the process. The whole system of differential equations will not be presented here. The constants α, β, χ, and δ are parameters that correspond to the rates of the passage from one state to another. Wu and Merchuk (2001) slightly modified the system, adding the biomass production rate and maintenance losses, and evaluated those kinetic parameters. In spite of being apparently too elementary, the model is able to produce acceptable approximations to the actual extremely complex photosynthetic phenomena.

A more sophisticated version of this model, which takes into account photoadaptation, was proposed later by Garcia-Camacho et al. (2012). Photosynthetic cells change biomass composition as the illuminance that they perceive changes. Because of this, data reported on *P-I* are collected usually after keeping the culture at a constant illuminance for a considerable time. During this time, the cells adapt themselves to those conditions, a fact that is detectable as a change not only in biomass synthesis rate, but also in the cell composition. Chlorophyll a (Chla) concentration in the cells changes: at higher illuminances, the Chla content usually diminishes and vice versa. This is called photoacclimation or photoadaptation. In the model of Garcia-Camacho et al. (2012), the net process of excited PSUs disappearance rate (photoinhibition), and that of damaged PSUs repair have been redefined in the context of quenching, photochemical, and nonphotochemical. Those are explicitly shown in Fig. 5.3. In addition, the phenomenon of photoacclimation in microalgae has been extensively incorporated, improving the model prediction capabilities significantly. As to the new mathematical formulation, the

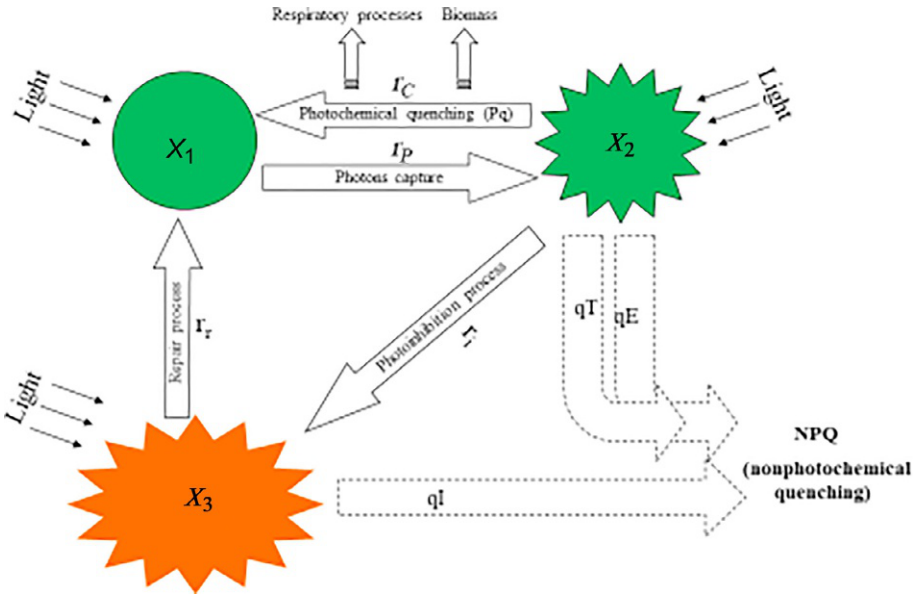

Fig. 5.4
The scheme of the modified PSF kinetic model by Garcia-Camacho et al. (2012). Both photochemical and nonphotochemical quenching are formulated, and photoadaptation is taken into account.

reader can find it in the source. Several sets of experimental results reported previously (Fisher et al., 1996) were successfully interpreted using the novel formulation: (1) photosynthetic response of cells photoacclimated to changes in constant continuous irradiances (photoacclimated cells); (2) short-term photosynthetic response of cells nonphotoacclimated to different constant continuous irradiances (nonphotoacclimated cells); (3) kinetics of the photoacclimation response; (4) photosynthetic response under intermittent light; and (5) respiration. A comparison of several variations on the three-states kinetic model has recently been made by Gao et al. (2018b). The model shown in Fig. 5.4 seems an excellent compromise between the extremely complex picture of photosynthesis as understood today in biology and the engineering need for relatively simple mathematical forms that make possible the integration of the model with the fluid dynamics in the reactor, as will be presented in the next sections.

5.3 Fluid dynamics relevance

5.3.1 The effects of fluid dynamics on photosynthesis

It is generally accepted, based on empirical evidence, that an increase in turbulence enhances photosynthetic production, as long as the photosynthetic cells are sturdy enough to endure hydrodynamic shear effects. Many algae are stress-tolerant, but this is not a general rule. Some photosynthetic microorganisms are especially shear sensitive, as in the case of marine

dinoflagellates. This case has been reviewed lately in depth, studying the effects of shear stress on microalgae and the impact of environmental factors on shear stress tolerance of microalgae (Wang and Lan, 2018; Rodríguez et al., 2009).

The dynamics of the photochemical reaction was analyzed previously by Lam and Bungay (1986). They studied this matter from the point of view of process dynamics. They describe the chain of processes that lead from photon capture to organic molecule synthesis reaching up to cell adaptation, covering an extremely extended range of time constants. Some researchers consider that the photon capture rate should be a key feature of the process, as will be commented on later. On the other hand, the timescale of CO_2 fixation in the process, which corresponds to biochemical dark reactions in the cell, is of the same order of magnitude as the time constants for bioreactor dynamics. This allows, from the point of view of process dynamics, the interaction of these two processes (processes with very different constant times cannot affect each other—as a gross example, the slow movements of tectonic plates will not affect the mixing in a cup of tea, and vice versa). This is graphically described in Fig. 5.5, where the ranges of time constants for the processes of photon capture, electron transfer, CO_2 fixation, and cell growth, which comprise phenomena of radiation physics, physical chemistry, biochemistry, and cell physiology, are represented along a logarithmic timescale, in parallel to the range of bioreactor dynamics time constants (Merchuk et al., 2019).

Mixing is known to improve the yield in photosynthetic processes in two different ways. First, it does so simply by securing the suspension or fluidization of the photosynthetic cells. Cells

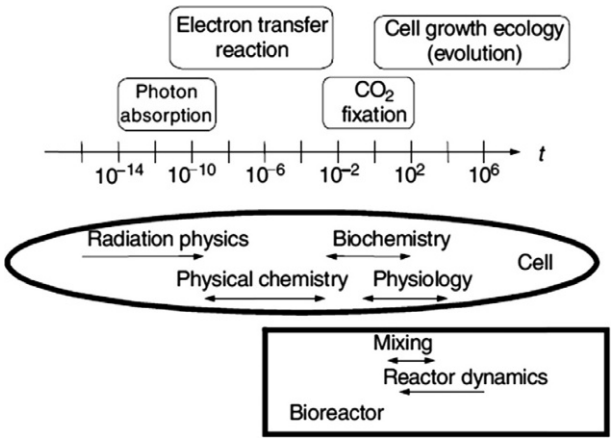

Fig. 5.5

Ranges of time constants for the processes of photon capture, electron transfer, CO_2 fixation, and cell growth, which comprise phenomena of radiation physics, physical chemistry, biochemistry, and cell physiology, are represented along a logarithmic ruler, in parallel to the range of bioreactor dynamics time constants. CO_2 fixation (biochemistry) has constant times of the order of those of reactor fluid dynamics. *Adapted from Lam, H.L.Y., Bungay, H.R., 1986. Frequency response analysis of oxygen evolution by algae. J. Biotechnol. 4, 125–142.*

usually have a tendency to settle down in quiet liquid. Liquid movement will stir and suspend them, thus avoiding a serious problem, because settled cells will not have a satisfactory exposition to nutrients and light. Second, because of the shape of the *P*/*I* saturation curve, it is important that the photon flux is distributed as homogeneously as possible among the photosynthetic cells. Since all photosynthetic cells have a certain maximal number of photons that they are able to trap and elaborate per unit time, it follows that both cells that are exposed to an excess of illuminance, as well as those poorly illuminated, will perform below their optimal photosynthetic rate. That is why it would be desirable to have the light distributed homogenously among all cells. However, light cannot be manipulated easily. Since it is difficult to bring light to each cell, an intuitive solution is bringing the cells to the light, and this can be accomplished by adequate mixing. That is the motivation of focusing the design on ordered rather than on random mixing.

5.3.2 Photosynthesis and ordered mixing

In nature, photosynthesis takes place in a circadian cycle, but the influence of light–dark cycles of quite different frequencies have been studied. The existence of a high frequency "flashing effect" was reported long ago. Emerson and Arnold (1932) measured a sensible increase in the photosynthesis rate when comparing continuous and flashing illumination. They found that a frequency of 0.02 Hz was compatible with the time required by the Blackman reaction, which involves the decomposition of a peroxide formed in a preceding photochemical reaction by the enzyme catalase, which is considered as the limiting step in the process when photosynthesis is saturated with both light and carbon dioxide. Many years later, Laws et al. (1983) designed an ingenious device in order to obtain light–dark cycles of high frequency in a relatively large photobioreactor (Fig. 5.6). They inserted special foils with a cross profile similar to that of airplane wings, and the pressure difference generated between the liquid flowing above and below the foils was enough to generate a vortex with a frequency of approximately 0.5–1 Hz, which allowed them to take advantage of the flashing light effect if the culture was optically dense enough. Fig. 5.5 shows the principle of their invention. They claimed that under certain set of operation variables, the production obtained with their device more than doubled.

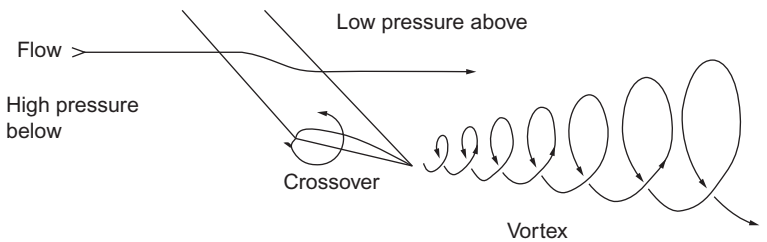

Fig. 5.6
Design of a single foil indicating mechanism of vortex production (Laws et al., 1983).

At a much smaller scale, Terry (1986) measured photosynthesis in cycled light periods and studied quantitatively the dependence of photosynthesis on flashing rate. This work was focused not on the direct design of a photobioreactor, but on the understanding of the phenomenon. He used a rotating, sectored disk to create intermittent light with frequencies ranging from 0.25 to 7 Hz, and evaluated photosynthesis following the O_2 generated at illuminances I from 250 to 1750 [μEinstm^2/s]. He concluded that at high flash frequencies, the photosynthetic rate was determined by the average intensity received by the cells (defined as full light intensity integration), while at low frequencies the cells responded to the instantaneous intensity (no light intensity integration). As flash frequency increased, complete integration of the illumination received over a period of time was approached asymptotically. Later, Martín-Girela et al. (2017) used higher frequencies (0.1, 1 and 10 kHz) in a laminar immobilized algae consortium, and reported better results for the highest frequencies.

Many of the experiments studying the flashing light effect were done in the low range of time constants that is close to the range of physical chemistry shown in Fig. 5.3. This is, however, much lower than the time constants of mixing and bioreactor dynamics in the same figure. The device proposed by Laws et al. (1983) mentioned above is indeed in the range of frequencies that can be reached in a large-scale device, but to our knowledge, no further applications of these mixing foils have been published. Lee and Pirt (1981) showed that, under certain conditions, the growth rate of algae in short alternating light and dark cycles was the same as that when the culture was exposed to a continuous light source, with the consequential reduction of the energy needed. Merchuk et al. (1998) showed similar results and used this output to explain the superior biomass yield of an airlift reactor when compared with a bubble column of equal external dimensions. The interpretation considered the possibility of strong illumination even reaching photoinhibition in the most strongly illuminated zones, while other parts in the photobioreactor may be practically in the dark because of self-shading in a dense culture.

Wu and Merchuk (2003) formulated a mathematical model describing this case, shown schematically in Fig. 5.7, using the three-state kinetic model (Photosynthetic Factories, PSF) for photosynthesis in a photobioreactor that was originally proposed by Eilers and Peeters (1988). The symbols X_1, X_2, and X_3 (see Fig. 5.3) are the three possible states of the PSF: open, closed, and inhibited. Fig. 5.7 shows the possible changes in a light/dark environment.

The model was solved analytically and the constants were fitted to experimental data obtained in a thin film tubular reactor. The theoretical prediction made that the introduction of light-dark cycles may enhance the growth was confirmed by the experimental results. The model also allows the prediction of the potential collapse of cultures in photobioreactors, under either light-deficit or light-excess conditions, as well as the influence of mixing on these critical phenomena. One of their figures, which most directly displays the main point in their

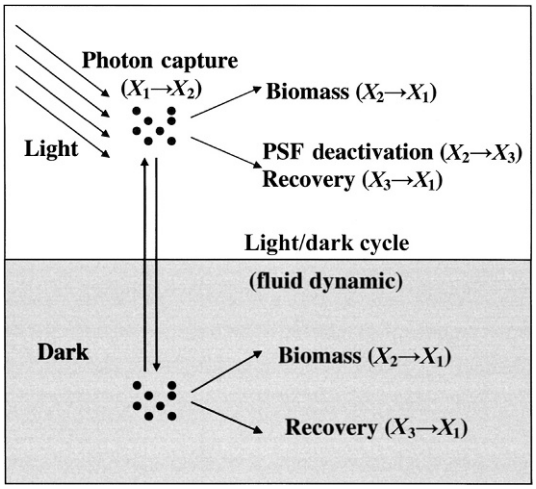

Fig. 5.7
Application of the three-state kinetic model of photosynthesis in a photobioreactor. *From Wu, X., Merchuk, J.C., 2001. A model integrating fluid dynamics in the photosynthesis and photoinhibition process. Chem. Eng. Sci. 56, 3527–3538.*

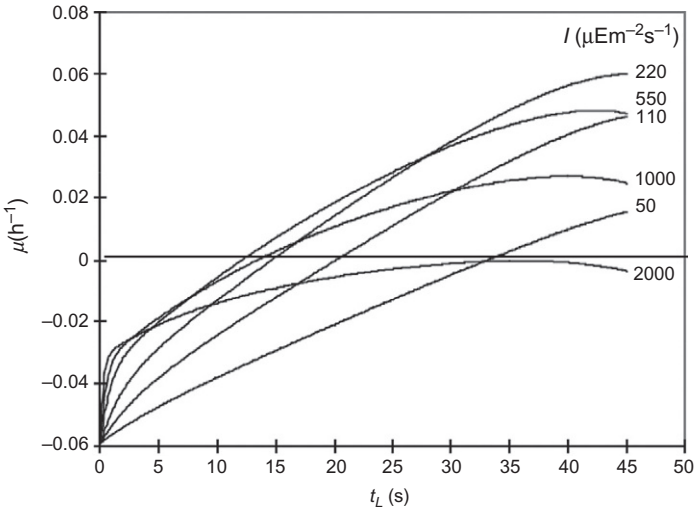

Fig. 5.8
The effect of photoinhibition on growth of a culture under intermittent illumination (Wu and Merchuk, 2001).

experimental work, is shown here as Fig. 5.8. The net rate of growth of a culture is shown as a function of the photoperiod t_L for a constant total cycle length of 60 s and a series of illuminances I. At the lowest I, 50 (μE m^{2}s^{-1}), the culture is able to succeed in growing when t_L is around 35%. Below that, the culture dwindles because of maintenance costs. At higher I, the t_L required for survival of the culture decreases consistently. However, this reverses at $I = 1000$

($\mu E\ m^2\ s^{-1}$) due to the effects of photoinhibition. At $I = 2000$ ($\mu E\ m^2\ s^{-1}$), we find again in the figure a culture that dwindles and disappears, this time because of excess rather than deficit of light.

The results obtained from a mathematical simulation of the system are shown in Fig. 5.9, which shows what was called by the authors an "island of existence" or island of growth in a photobioreactor. This is an area on the plane illuminance/cycle-time (which is equivalent to illuminance/mixing), which can be seen in Fig. 5.9. The closed line is the common place of all conditions that lead to zero biomass growth. Fig. 5.8 also shows that for each I, there is a clearly defined range of t_l/t_c (mixing) that allows growth. Outside this range, the culture would decline and die because of photoinhibition. The contour defining this range represents a combination of the two variables that provides the amount of effective photons required to balance maintenance. Higher amounts of light energy absorbed will allow actual growth, $\mu > 0$.

A further step in this field was the simulation of a bubble column photobioreactor. Wu and Merchuk (2002) applied the same kinetic model to a bubble column, describing the distribution of trajectories of the cells on the basis of available knowledge of fluid flow in bubble columns (Joshi and Sharma, 1979; Deckwer, 1992). The liquid was assumed to follow a circulation cell of the type defined by Joshi and Sharma (1979), and the cycle time t_c was obtained using the surface renewal model proposed by Danckwerts (1951). Wu and Merchuk needed the

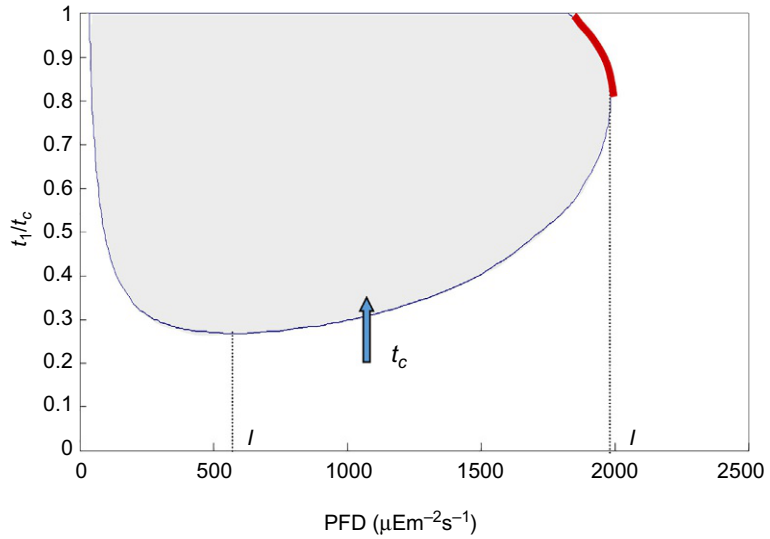

Fig. 5.9

The island of existence of a photobioreactor as defined by Wu and Merchuk (2001). The area shaded represents the surface where a photobioreactor could operate in stable conditions. The red (*gray color in print version*) line indicates the upper limit of the fraction of light in a cycle that would lead to the collapse of the system because of photoinhibition. *From Wu, X., Merchuk, J.C., 2001. A model integrating fluid dynamics in the photosynthesis and photoinhibition process. Chem. Eng. Sci. 56, 3527–3538.*

distribution of times in each zone of illumination for the purposes of the simulation. This distribution was discretized considering three fractions, F_i, with three different mean cycle times: one equivalent to all the elements having mean cycle time shorter than t_1, the second equivalent to all elements having mean contact times between t_1 and t_2, and the third one, t_3, equivalent to all the elements with mean contact times longer than t_2 (Fig. 5.10). It was assumed further that the cells traveling along the different trajectories return to the starting point and mix together, such that after the longest of the mean times (t_3 in this case), the second fraction has completed three cycles and the first (and fastest) fraction 12 cycles, and all the elements have come together. At this moment, the updated biomass concentration is evaluated and a new series of cycles begins. This light history of the photosynthetic cells was integrated with the kinetics used by Wu and Merchuk (2001) using the classic Lambert-Beer equation for the decay of illuminance according to the distance from the illuminated wall.

The frequency of the photoperiods has also been used as a criterion for the scale-up of tubular reactors with satisfactory results (Molina Grima et al., 2000). In the field of airlift bioreactors, the concept of helical flow promoters (HFP) was proposed by Gluz and Merchuk (1996) with the aim of incrementing the secondary flow by adding inclined baffles at the top of the down comer, obtaining shorter periodic flow cycles. This idea was recently extended to tubular photobioreactors (Qin et al., 2018). They used discontinuous double inclined ribs, and studied their influence on heat transfer. Those ribs are multiple, located periodically along the entire reactor, in contrast to the short single HFP mentioned above. The number of ribs in a cross section was discussed in terms of the flow structure, cycle frequency, and efficiency, using computational fluid dynamics. Fig. 5.11 shows a scheme of their system.

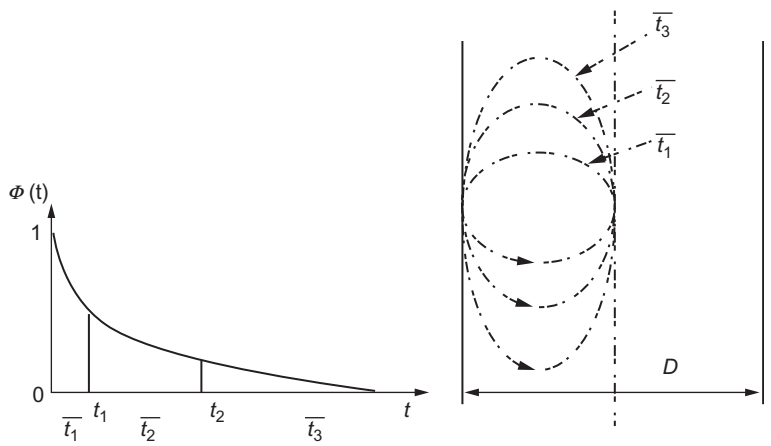

Fig. 5.10

The mean discrete distribution adopted for the circulation trajectories in a bubble column. After the longest of the times t_3, the second fraction has completed three cycles and the first (and fastest) fraction, 12 cycles, and all the elements of different ages coincide and mix. *From Wu, X., Merchuk, J.C., 2002. Simulation of algae growth in a bench scale bubble column. Biotechnol. Bioeng. 80, 156–168.*

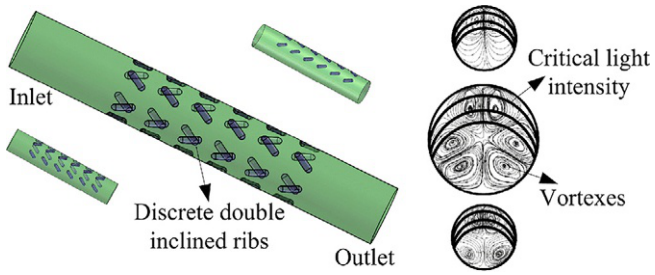

Fig. 5.11
Scheme of the inclined discontinuous baffles designed by Qin et al. (2018).

There is some similarity between this work and the one of fluid dynamics evaluation and energy consumption minimization in a twisted tubular photobioreactor that was done by Gómez-Pérez et al. (2017), using static mixers as promotors of secondary flows and cyclic circulation in the tube. CFD was used to predict the flow lines inside the photobioreactor (Fig. 5.12).

Following the recognized importance of mixing and illumination cycles in photosynthesis described above, a large amount of diverse designs have been proposed, which is the theme of the next section.

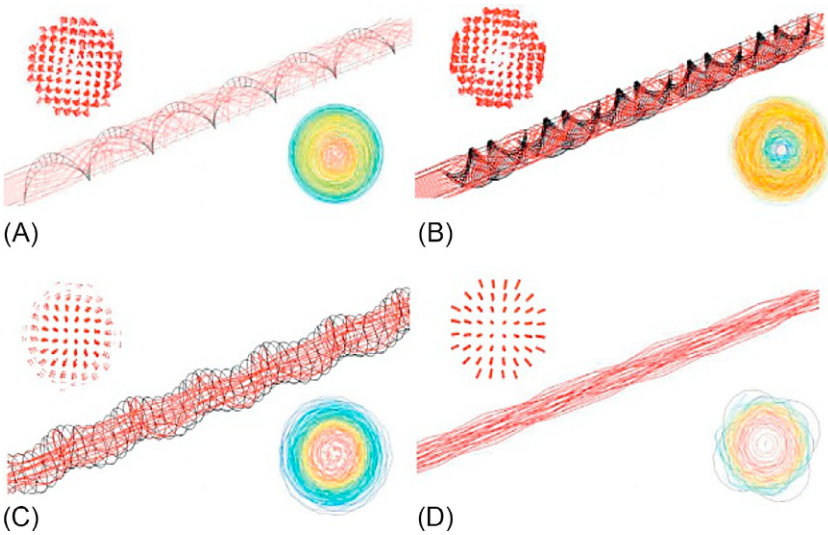

Fig. 5.12
Fluid dynamic simulation in a tubular photobioreactor. The velocity field is presented using arrow direction vectors and string lines that follow the fluid behavior in cross-sectional view. Several configurations are compared: (A) tubular PBR with helical mixer, (B) tubular PBR with static mixer, (C) spiral tubular PBR, and (D) twisted tubular PBR. See details on source. *From Gómez-Pérez, C.A., Oviedo, J.J.E., Ruiz, L.C.M., Van Boxtel, A.J.B., 2017. Twisted tubular photobioreactor fluid dynamics evaluation for energy consumption minimization. Algal Res. 27, 672.*

5.4 Ingenuity and inventiveness

The variables that exert influence on the performance of a photobioreactor are many, and we have stressed here only some of them. But the knowledge already available on the relevance of those variables on photosynthesis brought to a wide range of inventions that reveal, on one hand, the ingenuity of the developers, and on the other hand an economic and social driving force that stresses the relevance of algal biomass production. The importance of mixing and attenuation of light, which was described above, has triggered many novel devices of different types.

Most probably, the first photobioreactors were natural water reservoirs where algae grew, which evolved into artificial ponds and then to raceways, circular tanks, and other devices where suspended algal culture could grow and be collected. The designs proposed, some of which can be seen in Fig. 5.13, taken from Merchuk et al. (2019), are many. A good review of systems used to cultivate microalgae for biofuel production was previously published by Kunjapur and Eldridge (2010). The main problems that must be addressed are light, CO_2, feed, and O_2 ventilation. The raceway offers the advantage of being shallow vessels with a large ratio of surface area to volume (light effectiveness), and liquid flow controlled by a mechanical actioned paddle wheel. The simplest design will provide those, but would most probably be limited by the low concentration of CO_2 in the air. Bubbling the gas in a shallow liquid is not effective. The solution was to provide a sump where the CO_2 could be bubbled in deeper liquid and a reasonable fraction of it could be absorbed before it disengaged the liquid. Zeng et al. (2016) did a comprehensive study of the flow in a raceway pond. The local values of the liquid velocity were measured to validate the reliability of a CFD model, including the zone around a modified paddle wheel that they tested (Fig. 5.14).

A conventional stirred tank would provide the possibility of dispersing the CO_2 for better absorption, at a cost of a much smaller ratio of surface area to volume and more energy expenditure by the mixer. The bubble column and the variations of airlift reactors (concentric tubes, split cylinder, and external loop) offer enough height for CO_2 absorption and are of simple design and construction, but have a smaller ratio of surface area to volume (Wu and Merchuk, 2002; Wu and Merchuk, 2003; Olivieri et al., 2016, among others). This is balanced in the flat-plate type photobioreactors, which offer much larger surface area and a short light path, but may be more complex to maintain. Tubular bioreactors, where the liquid is circulated by a pump or an attached airlift, have also been intensively tested (Molina Grima et al., 2000). A typical issue in this type of device is that the arrangement of long tubes causes large pressure losses due to the many U-bends. Wongluang et al. (2013) used CFD (Fig. 5.15) modeling of the flow in a conventional tubular loop photobioreactor to reveal the existence of substantial dead zones in the typically used U-bends and 90° elbows. They proposed alternative designs to overcome the energy losses (Fig. 5.15).

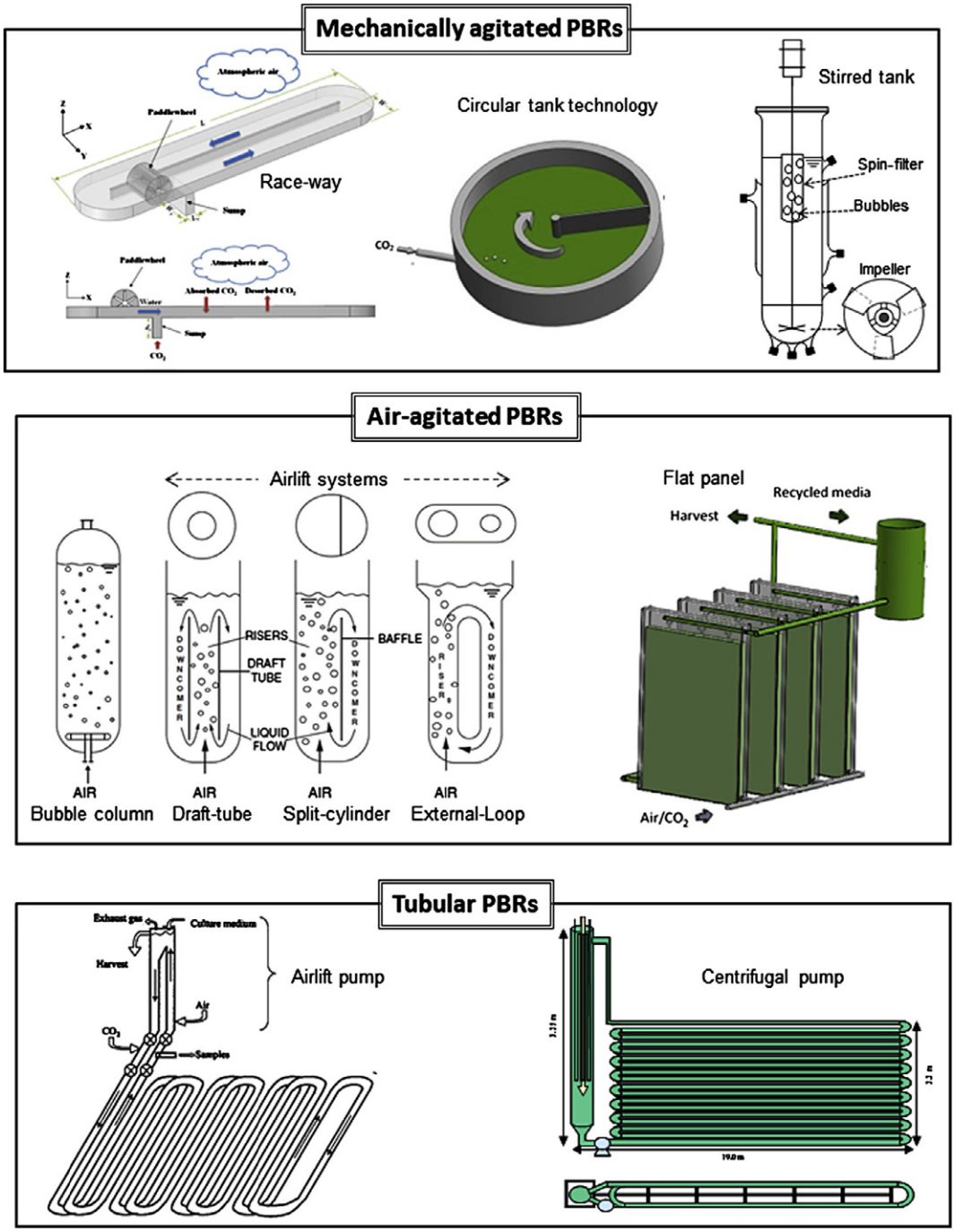

Fig. 5.13

Most common type of photobioreactors. *From Fig. 1 in Ali, H., Park, C.W., 2017. Numerical multiphase modeling of CO2 absorption and desorption in microalgal raceway ponds to improve their carbonation efficiency. Energy, 127, 358–371, Elsevier; From Fig. 5 in Chisti, Y., Moo-young, M., 2002. Bioreactor. Encyclopedia of Physical Science and Technology. Elsevier, pp. 248–255; From Fig. 1 in Camacho, F.G., et al., 2011. Photobioreactor scale-up for a shear-sensitive Dinoflagellate microalga. Process. Biochem. 46(4), 936–944, Elsevier; From Fig. 1 in Molina, E., et al., 2001. Tubular photobioreactor design for algal cultures. J. Biotechnol. 92(2), 113–131, Elsevier; From Figs. 3 and 6 in Kiran, B., et al. 2014. Perspectives of microalgal biofuels as a renewable source of energy. Eng. Convers. Manag. 88, 1228–1244, Elsevier.*

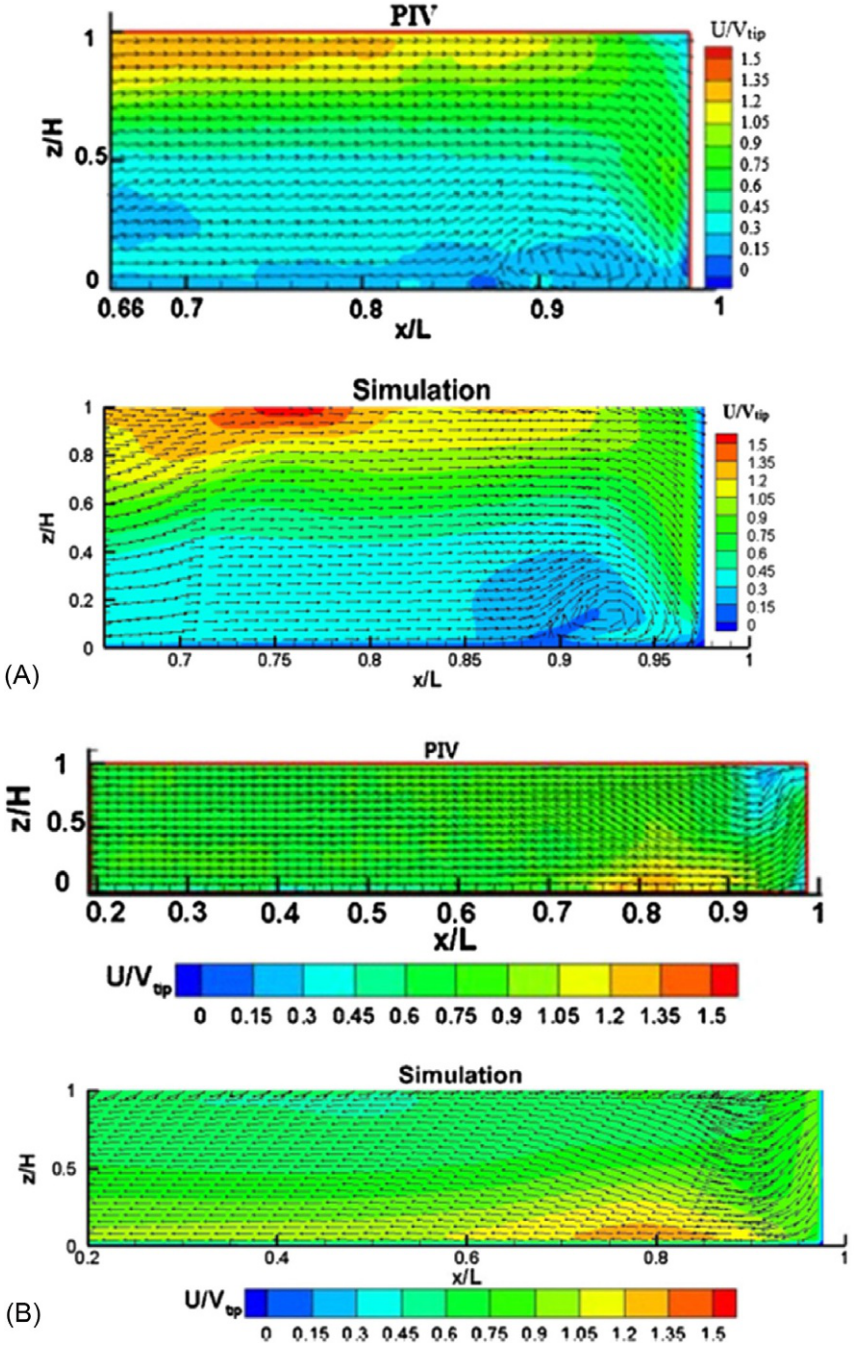

Fig. 5.14

(A) and (B) Contours of average velocity with PIV and simulation based on k–ε turbulence model in two different planes. *Reproduced with permission from Zeng, F., Huang, J., Meng, C., Zhu, F., Chen, J., Li, Y. 2016. Investigation on novel raceway pond with inclined paddle wheels through simulation and microalgae culture experiments. Bioprocess Biosyst. Eng. 39, 169–180.*

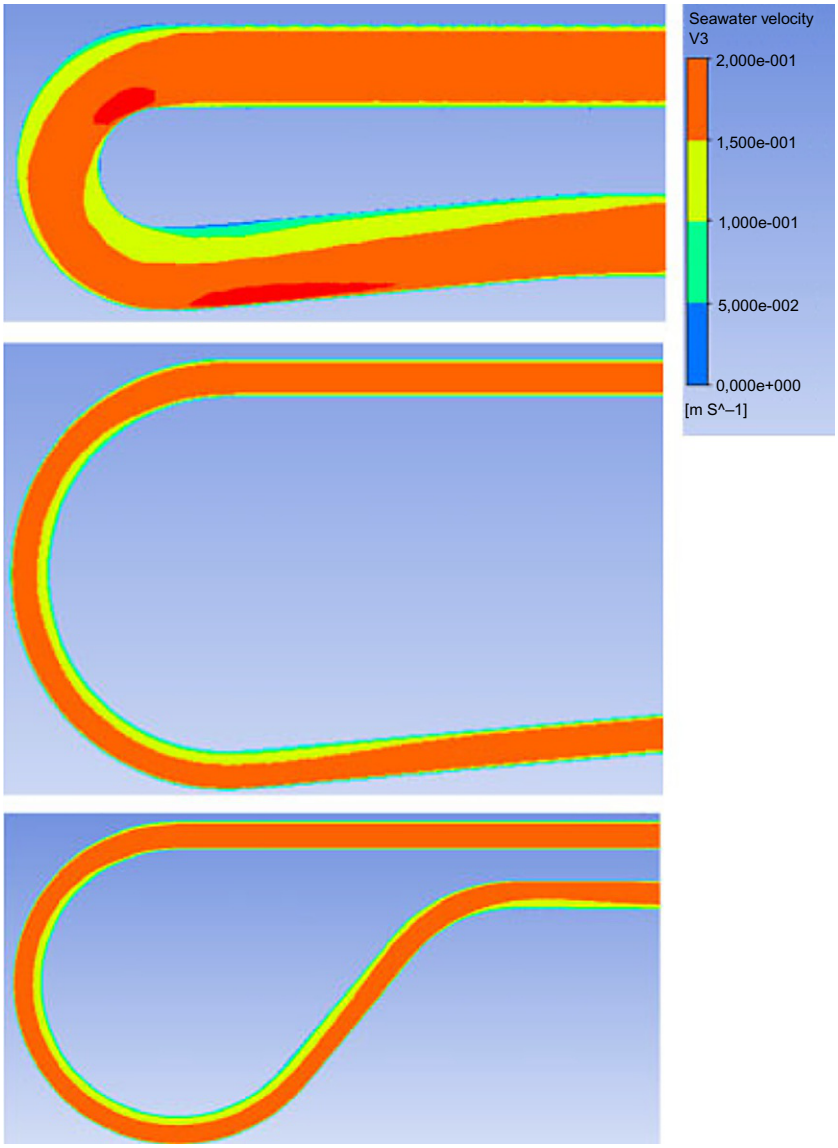

Fig. 5.15
Velocity profiles and dead zones in various configurations of U bends. *From Wongluang, P, Chisti, Y and Srinophakun, T. 2013. Optimal hydrodynamic design of tubular photobioreactors. J. Chem. Technol. Biotechnol., 88: 55–61.*

As in all the devices where the light path is short, or in other words, where the ratio of external surface to volume is high, the control of temperature becomes critical, and extensive cooling must be applied. One of the possibilities is submerging the photobioreactor in a pool of controlled temperature (Molina Grima et al., 1999), but this is practical only for a relatively small scale. A tubular photobioreactor claimed as the largest installation existing is shown in Fig. 5.16 (Pulz, 2001).

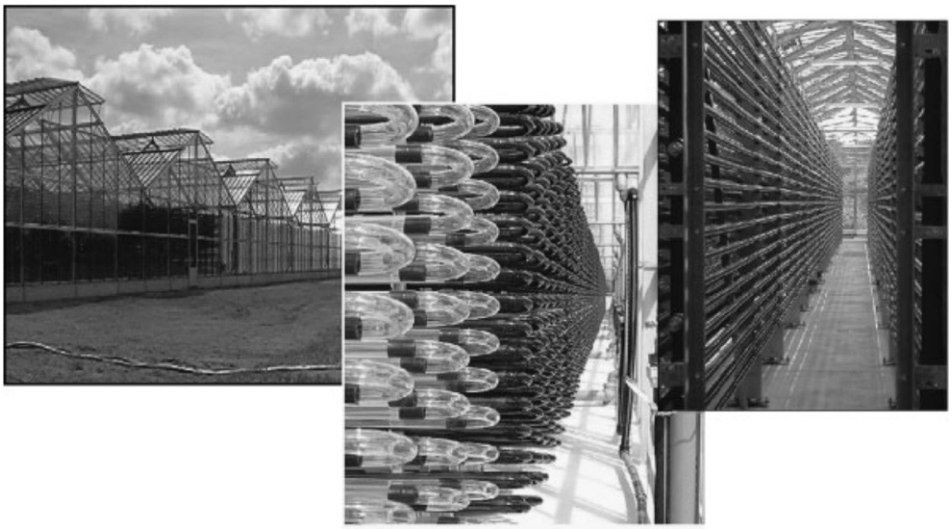

Fig. 5.16
Large-scale tubular photobioreactor (Pulz, 2001).

The variety of designs proposed for bioreactors is quite large. Inclined tubes have been proposed (Merchuk et al., 2007). Thin films falling over inclined planes, in several configurations, were proposed by Doucha et al. (2005). Floating bioreactors where the sea waves provide the energy for photobioreactor mixing were studied, including the response of the photobioreactor to wave conditions (Zhu et al., 2019).

Spherical photobioreactors have been proposed and their fluid dynamics simulated (Sato et al., 2006). A wide variety of biofilm-based photobioreactors, where most of the photosynthetic cells are immobilized and remain and grow in the film, have been described and can be seen elsewhere (Merchuk et al., 2019). Those types of photobioreactors claim the advantage of smaller costs of water separation from the product, obtaining a very concentrated biomass simply by scraping the film. An ingenious device has been developed for the production of marine microalgae, which are cultivated using a brackish/marine water source, and may present the best sustainable option to respond to the food crisis looming over the future; an example is shown in Fig. 5.17 (Moomaw et al., 2017).

Deprá et al. (2019) recently proposed a hybrid photobioreactor combining a spiral tubular illuminated zone and a bubbling zone for gas exchange, and performed a hydrodynamic characterization, studying growth kinetics, removal of carbon dioxide, oxygen release rates, carbon and energy balances, and the surface area required (Fig. 5.18).

Fig. 5.17
Photobioreactor installation under the Aurora Borealis, in Iceland. Moomaw et al. (2017).
Picture by I. Berzin.

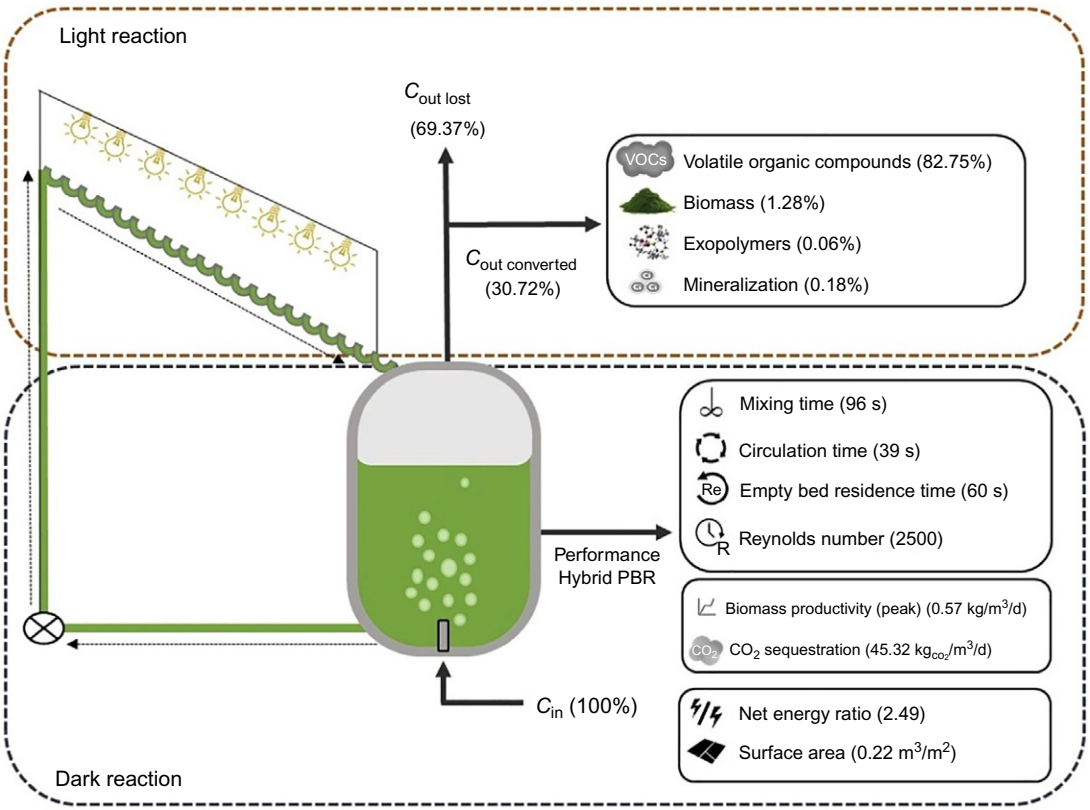

Fig. 5.18
Scheme of the hybrid reactor tested by Deprá et al. (2019).

A novel photobioreactor has been proposed for microalgae production and simultaneously shading system in buildings, combining environmental and biotechnology science with architecture (Pagliolico et al., 2016).

5.5 Advanced models

However, ingenuity and inventiveness are not a good enough substitute for deep understanding of the phenomenon at hand. They may even give excellent productivity, but the problem of photobioreactor design and scale-up is only palliated, and at the cost of expensive experiments and time. Only a good knowledge of the mechanistic aspects of photosynthesis in a bioreactor can grant a successful design of a photobioreactor de novo, without trial and error procedures and minimizing the stages in the scaling from lab to plant. This can only be done by integrating our knowledge on the kinetics with fluid dynamics and light. This approach was presented in Eq. (5.2) in a very simplified one-dimensional form. With the present extended development of computation capacity, CFD has become a very strong instrument for the prediction of fluid trajectories, and therefore of light history of every photosynthetic element in the reactor. An excellent review of such CFD applications has recently been published (Gao et al., 2018a).

Several examples of this approach, which uses the measured (or calculated) flow patterns together of a kinetic of growth, based on the light history of the photosynthetic cells, can already be found in the literature; Gao et al. (2015, 2017) studied the performance of a Taylor-Couette photobioreactor. The toroidal flow was driven by an internal rotating cylinder. One of the conclusions derived from their experiments was that the cyclic circulation of the cells provoked by the toroidal flow improved the photosynthesis. As a sequence of this work (Gao et al., 2018a), they used Wu and Merchuk's (2001) version of the three-states PSU model in an airlift for algal growth, integrating it with the flow trajectories that they calculated using a three-phase Eulerian approach with CFD. Fig. 5.19 is taken from the results, showing the distribution of the three possible states proposed in the model: PSF in the different places inside the photobioreactor. The resting, or open state, (X_1 in Fig. 5.7), the active or closed state (X_2 in Fig. 5.7), and the inhibited state (X_3 in Fig. 5.7).

Their final results are presented as a table comparing the deviation of four different simulations from the experimental results of biomass generation mentioned above. Four calculations are mentioned: that by the authors (Gao et al., 2018b), using their Eulerian approach, two by Luo and Al-Dahhan (2004, 2011) that use a Lagrangian approach, and one by Wu and Merchuk (2001) using a Lagrangian approach but obtaining the trajectories not from experimental measurements but modeling them on the base of the circulation times in each zone of the airlift: riser, down comer, and gas separator. All of the results correspond to airlift photobioreactors of similar dimensions.

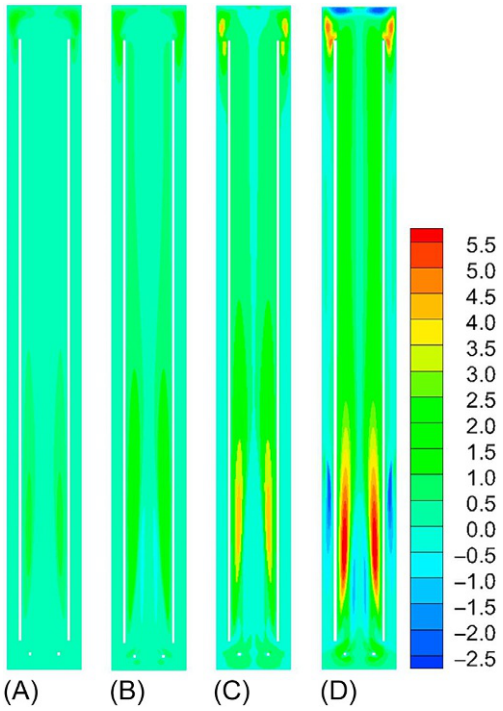

Fig. 5.19

Fraction of photosynthetic units in the (A = X_1) resting, (B = X_2) active, and (C = X_3) inhibited states as a function of time. The radial position of the light/dark boundary, based upon the photocompensation point (I_m in Fig. 5.2), is shown in (D). See there for more details. For X_1, X_2, and X_3, see Fig. 5.7 in this work. *Taken from Gao, X., Kong, B., Vigil, D., 2018a. Simulation of algal photobioreactors: recent developments and challenges. Biotechnol. Lett. 40, 1311–1327. From Luo, H.P., Al-Dahhan, M.H., 2011. Verification and validation of CFD simulations for local flow dynamics in a draft tube airlift bioreactor. Chem. Eng. Sci. 66, 907–923.*

In order to quantify the accuracy of each simulation approach, the absolute errors in the prediction of dry biomass concentration for several flow conditions were calculated. The results presented indicated that in these specific geometry and operation ranges, both approaches can be used. The deviations from experiments in the calculations of Gao et al. (2018b) are smaller than those in the papers by Luo and Al-Dahhan (2004, 2011). However, the deviations are still smaller in the case of the calculations by Wu and Merchuk, despite being based on a mere modeling of the trajectories (Table 5.1).

Nauha and Alopaeus (2012) presented a different treatment of a similar bubble column. They find that their modeling approach, which combines computational fluid dynamics with a compartmental model, depicts simultaneously and satisfactorily the influence of many parameters on the growth of algae. An alternative description of a bubble column

Table 5.1: Comparing simulation results through circulation time approach, Lagrangian approach, and Eulerian approach with experimental data.

Absolute error	Circulation time (Lagrangian) Wu and Merchuk (2004)	Lagrangian approach Luo and Al-Dahhan (2004)	Lagrangian approach Luo and Al-Dahhan (2011)	Eulerian approach Gao et al. (2018b)
$Ug = 0.054\,cm/s$	3.3%	65.0%	13.3%	7.9%
$Ug = 0.16\,cm/s$	3.8%	NA	NA	13.1%
$Ug = 0.33\,cm/s$	13.5%	74.1%	42.4%	19.5%
$Ug = 0.82\,cm/s$	9.6%	77.9%	NA	19.0%

From Gao, X., Kong, B., Vigil, R.D., 2018b. Multiphysics simulation of biomass growth in an airlift photobioreactor: effect of fluid mixing and shear stress. Bioresour Technol. 251, 75–83.

photobioreactor was proposed by Olivieri (2016), in a model aiming to assess the photosynthetic performance of tubular, bubble column, and flat photobioreactors. It seems, therefore, that the direction taken in the latest published research is a basis for optimism, but a considerable amount of work remains to be done on this front.

5.6 Conclusions

The design de novo of photobioreactors is a titanic task due to the multiple variables, and especially because of the nature of light, which differs very much from chemical variables (concentrations). Because of the dependence of light on the distance from the illumination source and the difficulty of homogenizing illuminance, the correct approach is that of the light history or light trajectories, and from this fact stems the need for integrating fluid dynamics with kinetics to predict photo-biosynthetical production. In addition, the nonlinearity of illuminance with distance, which the possibility of photoinhibition complicates further, calls for the use of sophisticated kinetic models. Much of the published literature on photobioreactor design is empirical, and sometimes based on unjustified simplifications, which although producing impressive results, are difficult to scale-up. Lately, nevertheless, a series of models integrating advanced kinetic models and fast CFD have shown the way to a more scientific design that may provide a stronger basis for the design, which may bring, if not a completely de novo design, then at least a minimization of scale-up experiments on the way to large-scale biomass production plants.

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CHAPTER 6

Microalgae production systems

Francisco Gabriel Acién Fernández, José María Fernández-Sevilla, Bernardo Llamas Moya, Emilio Molina Grima

Department of Chemical Engineering, University of Almería, Almeria, Spain

Chapter outline

6.1 Introduction 127
6.2 Major factors on microalgae production 129
6.2.1 Microalgae requirements 130
6.2.2 Photobioreactor capacity 135
6.3 Photobioreactor types 146
6.3.1 Raceway reactors 146
6.3.2 Tubular reactors 150
6.3.3 Other designs 154
6.3.4 Thin-layer reactors 156
6.4 Future trends 158
References 158

6.1 Introduction

Microalgae are used in high-value applications from pharmaceutical to nutraceutical, cosmetic and food industries (Molino et al., 2018; Novoveská et al., 2019). Additionally, these microorganisms have been also proposed as potentially useful for other applications such as biofuels production, wastewater treatment, and CO_2 mitigation, although these are not still commercial (Chisti, 2007; Acien et al., 2012). Whatever the application, the core of the process is the photobioreactor on which the process is performed, which must be designed according to (i) the microorganism to be produced, (ii) the quality of biomass required, and (iii) the overall production capacity. In this sense, largely different photobioreactors are used at commercial scale, from little bags used in aquaculture to produce small amounts of high-quality biomass of marine strains, to tubular photobioreactors developed for the production of tons of high-value and sensitive strains such as *Haematococcus* and *Nannochloropsis*, or large open raceways used for the production of hundreds of tons of extremophile strains such as *Spirulina* or *Dunaliella* (Acién et al., 2017). These are examples of photobioreactors because they

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use light for the production of autotrophic microorganisms. Microalgae can also use organic compounds as an energy source, then it growing in heterotrophic mode, or additionally also in mixotrophic growth if the light is also provided at the same time. In this case the utilization of conventional fermenters is recommended (Venkata Mohan et al., 2015). For the production of autotrophic microalgae, it is also possible to use artificial illumination instead of natural sunlight. However, due to low light conversion efficiency of the photosynthesis process (below 10%), it is only suitable at very special conditions, such as energy free supply or producing very high-value biomass (Tredici and Zittelli, 1998).

Focusing on autotrophic growth, the most extended types of photobioreactor are open raceways and tubular photobioreactors. Open raceways are the most extended technology, but they are only suitable to produce fast-growing strains such as *Chlorella*, or alternatively strains growing under extreme conditions such as high alkalinity (*Spirulina*) or high salinity (*Dunaliella*). At these extreme conditions, only the strains capable of growing do so, thus reducing contamination problems. However, these growth conditions also become inadequate for the target strains, then reducing their performance and thus their productivity. To improve the biomass productivity and to be able to produce any microalgae strain, the utilization of tubular photobioreactors is recommended. However, these reactors are more expensive and more difficult to scale up. Only recently have large facilities based on tubular photobioreactors been developed. In addition to these main two photobioreactor designs, other types such as bubble columns, flat panels, and thin-layer cascade, among others, have been proposed. However, none of them is currently used at industrial scale, mainly due to the difficulties of scaling up. Thus, the scale of production is the other major factor determining the type of reactor that can be used.

Whatever the reactor type, its function is to provide adequate conditions for the growth of selected microalgae. The higher the capacity of the reactor to adjust the operational conditions to the requirement of the strain, the higher the biomass productivity achievable will be. Major requirements in the production of phototrophic microalgae are the supply of light and nutrients (carbon, nitrogen, phosphorous, etc.), in addition to providing the adequate culture conditions (pH, temperature, dissolved oxygen) (Tredici and Zittelli, 1998; Posten, 2009; Acién Fernández et al., 2013). At small scale, it is quite easy to provide these inputs and to guarantee the overall availability of all of them at the entire volume of the reactor. However, at large scale this is much more difficult to do, and existing gradients of properties greatly reduce the adequacy of culture conditions along the reactor, thus largely reducing the biomass production capacity. Designing adequate reactors capable of providing the requirements of microalgae is a challenge, especially achieving this at a reasonable cost (Norsker et al., 2011; Acién et al., 2012). In the next section, the major factors influencing the performance of microalgae-related processes are discussed.

6.2 Major factors on microalgae production

The core of any microalgae-related process is the biological system to be used, whatever microalgae strain performing at its maximal capacity if providing the optimal conditions such as irradiance, temperature, pH/CO_2, and dissolved oxygen (Fig. 6.1). To provide these conditions at laboratory/small scale is relatively easy, thus the performance of microalgae strains at laboratory conditions must be maximal. Other factors such as nutrients' availability are also important, but these can be provided more easily, including at large scale. Once the biological system is defined, the next step is to select/design the adequate photobioreactor to be used. At large scale, specifically designed photobioreactors must be used. The objective of any reactor is to provide the optimal conditions required by the microalgae strain at minimum cost, thus different design have been proposed, but they are all designed according to the same fundamental principles. The capability of any photobioreactor to provide the required optimal conditions will be a function of its geometry, fluid-dynamic, mass transfer, and heat transfer capacity (Fig. 6.1). The photobioreactor must also be designed taking into account the environmental conditions prevailing in the selected location, such as solar radiation and temperature, not only at one specific time but throughout the year, and accounting for the daily cycle (Fig. 6.1). In the next section, these factors are discussed.

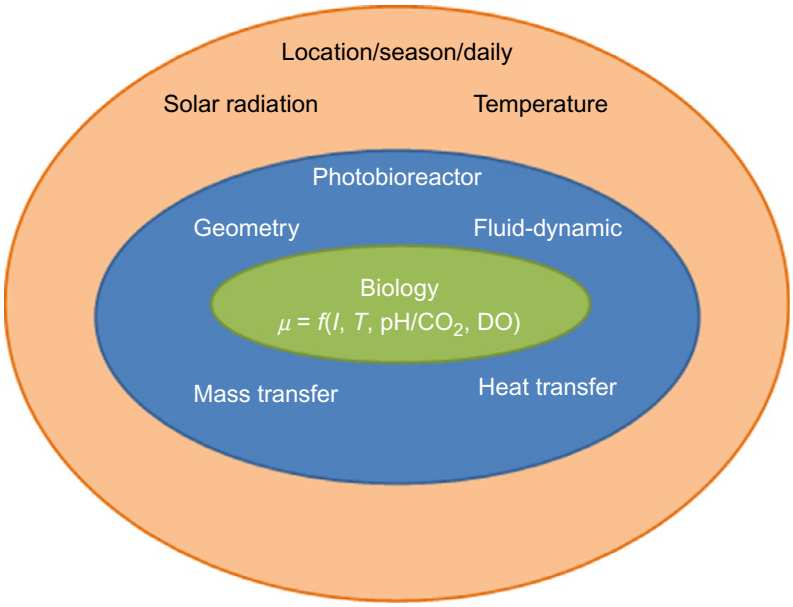

Fig. 6.1
Major parameters determining the productivity of microalgae-related systems.

6.2.1 Microalgae requirements

6.2.1.1 Light

Light is the driver of photosynthesis reaction, thus it is the major factor determining the photosynthesis rate in microalgae cultures, and ultimately overall biomass productivity. Light is utilized by the photosynthetic apparatus to transform inorganic into organic components, thus it is the energy source for this process and for the overall metabolism/growth of microalgae biomass. From the entire spectrum of sunlight, the photosynthetic apparatus only uses that between 400 and 700 nm (photosynthetically active radiation, PAR) to perform photosynthesis; other wavelengths are not useful. When analyzing the response of photosynthesis rate versus irradiance, typical behavior as shown in Fig. 6.2 is observed. There is a minimum of light required to perform photosynthesis, known as compensation irradiance (Ic); below this value, respiration takes place. Above this compensation value, the photosynthesis rate increases with the light to achieve a maximum at light saturation irradiance (Is), at irradiances upper than inhibition irradiance the photosynthesis rate reduces by photoinhibition (Ii). The values of these light characteristic parameters are different for different microalgae, but in general it can be summarized that compensation irradiance ranges are 10–20 $\mu E/m^2$ s, whereas the saturation of photosynthesis is achieved at 200–400 $\mu E/m^2$ s, and the inhibition is relevant at irradiances higher than 1000 $\mu E/m^2$ s (Costache et al., 2013,b; Ippoliti et al., 2016a).

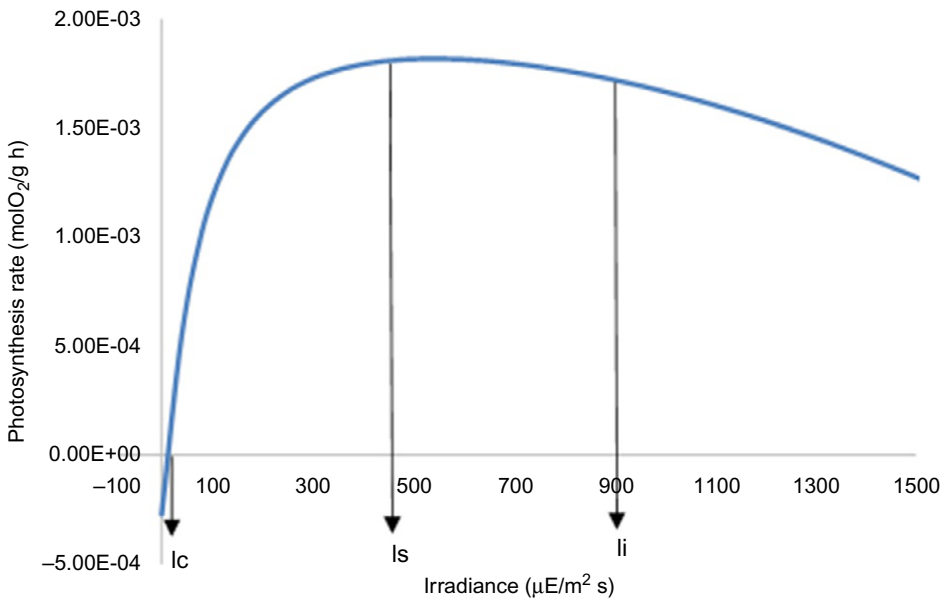

Fig. 6.2
Variation of photosynthesis rate with the irradiance to which the microalgae cells are exposed.

Photosynthesis is the first step in the biomass production process, the fundamental of photosynthesis reaction indicating that for the production of carbohydrates up to 1.06 gO_2/gCH_2OH are produced, whereas up to 1.46 gCO_2/gCH_2OH are required (Eq. 6.1). These are theoretical values, because the production of biomass is much more complex, and the synthesis of proteins, carbohydrates, and lipids, in addition to other components of the biomass, must also be considered. Thus, considering the elemental composition of the biomass, up to 50% of the biomass is carbon, 7% is nitrogen, and 1% is phosphorus; this means that the requirement of CO_2 is up to 1.83 gCO_2/gbiomass and the production of oxygen up to 1.33 gO_2/gbiomass, whereas up to 0.31 gNO_3^-/gbiomass and 0.03 gPO_4/gbiomass are required, respectively. To modelize the growth of any microalgae strain, a direct conversion of photosynthesis rate to biomass production cannot be used; experimental measurements must be taken. On these terms, the growth rate can be modeled on the basis of irradiance (Eq. 6.2), the hyperbolic equation being one of those most extensively used, although other equations can also be utilized (Molina Grima et al., 1996) (Fig.6.3A). The hyperbolic model allows estimation of the growth rate as a function of specific maximal growth rate (μ_{max}), irradiance at which the cells are exposed to (I), irradiance constant (Ik), and form parameter (n). The irradiance constant is the irradiance required by the cells to achieve half of the maximal growth rate, in equivalence with Monod's model. Experimental data already reported in the literature indicate that the specific maximal growth rate range was 0.5–1.5 day^{-1}, whereas the irradiance constant range was 50–100 $\mu E/m^2$ s, the value of the form parameter being close to 2 (Fernández Sevilla et al., 1998; Bazaes et al., 2012; Sepúlveda et al., 2015).

$$CO_2 + H_2O \rightarrow CH_2OH + O_2 \tag{6.1}$$

$$\mu(I) = \frac{\mu_{max} \cdot I^n}{Ik^n + I^n} \tag{6.2}$$

However, in dense microalgae cultures the cells are exposed not to unique irradiance, but to different irradiances according to their position inside the reactor, which changes with time according to the fluid-dynamic and light profile inside the reactor. To determine the light availability inside any microalgae reactor, the concept of average irradiance has been defined (Molina-Grima et al., 1996), and this is the volumetric integral of irradiance in any microalgae culture. It can be calculated by using the simplified Eq. (6.3), as a function of irradiance on the reactor surface (I_o), extinction coefficient of the biomass (K_a), biomass concentration inside the culture (C_b), and light path inside the reactor (p) (Molina Grima et al., 1996). In this way, the growth rate can be modeled on the basis of average irradiance. Experimental values of the extinction coefficient of the biomass range from 0.05 to 0.15 m^2/g, mainly changing as a function of cell size and pigment content, whereas the other parameters are mainly functions of design and operation conditions of the culture system (Morales-Amaral et al., 2015; Ippoliti et al., 2016b).

$$Iav = \frac{I_o}{K_a C_b \cdot p} \cdot (1 - \exp(-K_a C_b \cdot p)) \tag{6.3}$$

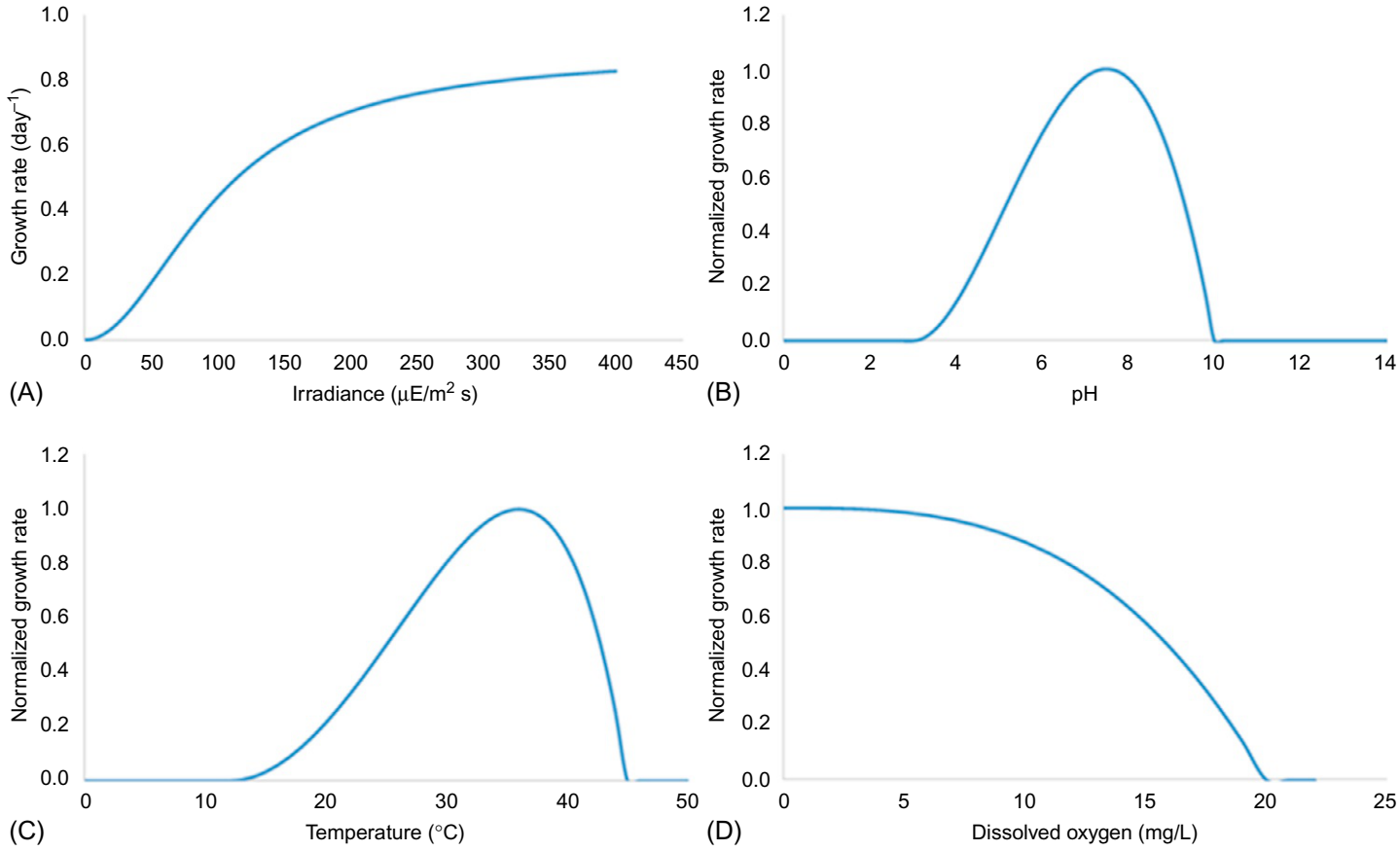

Fig. 6.3

Variation of growth rate as a function of most relevant factors influencing the performance of microalgae cultures: (A) irradiance, (B) temperature, (C) pH, (D) dissolved oxygen. Simulations performed using the proposed models.

The utilization of instantaneous or average irradiance to modelize the growth rate in any culture will depend on the light regime to which the cells are exposed inside the culture, it being a function of mixing inside the reactor. To define this light regime, the frequency (ν) to which the cells are exposed inside the reactor is defined as a function of flash time (t_f), dark time (t_d), and total cycle time (t_c) (Eq. 6.4), the duty-cycle of the light regime (ϕ) being also a function of the same parameters (Eq. 6.5) (Terry, 1986). The concept of the light integration factor is then defined as a function of experimental growth rate (μ_{exp}), the duty-cycle (ϕ), the instantaneous growth rate at irradiance in each position ($\mu(I)$), and growth rate at "mean irradiance" ($\mu(\phi I)$), as described by Eq. (6.6) (Terry, 1986). According to this concept, the light integration factor is equal to 1 when the light distribution is uniform or well-mixed enough for cells to respond to the mean irradiance (ϕI), while it is equal to 0 in an environment segregated enough to experiment with different local growth rates, depending on the local irradiance. It has been reported that relatively high frequency (10 Hz) is sufficient in most cases in order to take advantage of the flashing light ($\Gamma = 1$) at moderate external irradiances (I_o) (Brindley et al., 2011). However, it is not easy to obtain those high frequencies in most of the culture systems currently used. Experiments carried out under real light regimes show that it is possible to obtain integration ($\Gamma = 1$) even in situations of low mixing, but only if diluted cultures are used (Brindley et al., 2011).

$$\nu = 1/(t_f + t_d) = 1/t_c \tag{6.4}$$

$$\phi = t_f/(t_f + t_d) \tag{6.5}$$

$$\Gamma = \frac{\mu_{exp} - \phi\mu(I)}{\mu(\phi I) - \phi\mu(I)} \tag{6.6}$$

6.2.1.2 Temperature

In addition to light availability, the growth of any microalgae strain is largely influenced by the culture conditions and how they fit to the optimal ones required by the cells. The influence of temperature is one of the most relevant because below the optimal value the performance of microalgae reduces, approximately by half every 10 degrees, but above the optimum value the culture can die by overheating, usually at 10°C above the optimum value (Fig. 6.3B). The influence of temperature on the performance of microalgae strains has traditionally been modeled using the Arrhenius equation, but the Cardinal model (Eq. 6.7) can also be used, and provides a better understanding of the maximal, minimal, and optimum values for any microalgae strain (Bernard and Rémond, 2012). For strains supporting warm conditions, optimal temperatures of 30–35°C and minimum and maximal temperatures values of 7–10°C and 40–45°C, respectively, have been reported (Costache et al., 2013,b; Ippoliti et al., 2016a). However, in the case of strains adapted to cold conditions, optimal temperatures of 18–20°C, and minimum and maximal temperatures values of 2–5°C and 25–30°C, respectively, can be found (Grimaud et al., 2014).

$$\overline{\mu(T)} = \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]} \quad (6.7)$$

6.2.1.3 pH/CO_2

The pH is the other relevant factor determining the performance of any microalgae strain. pH has a double effect because it not only influences the physiological status of the cells, but also determines the CO_2 availability into the culture. To optimize the productivity of microalgae cultures, it is recommended to maintain a concentration of CO_2 in the culture higher than $65\,\mu mol\,L^{-1}$, corresponding to a pH value of 8.5 (Weissman and Goebel, 1987). In the microalgae field, it is common to supply CO_2 to control the pH and to provide the inorganic carbon required by the cells for growth. Due to the existence of a bicarbonate buffer, the concentration of CO_2 into the culture is determined by the total inorganic carbon concentration into the culture medium and the pH. Thus, the pH determines the concentration of the three carbon species, such as CO_2, HCO_3^-, and CO_3, as a function of equilibrium constants (Camacho-Rubio et al., 1999; de Godos et al., 2014). Whatever pH control method is utilized, the influence of pH on the performance of microalgae cells can also be modeled using the Cardinal model (Eq. 6.8) (Ippoliti et al., 2016a) (Fig. 6.3C). Although it has been reported that some strains can grow under extreme acidic conditions below 3 (Merola et al., 1981), or alkaline conditions above 10 (Vadlamani et al., 2017), the optimal range for most microalgae strains is close to neutral pH. For different strains it has been reported that minimum, optimum, and maximal value ranges were 4–5, 7–8, and 9–10, respectively (Costache et al., 2013,b; Ippoliti et al., 2016a).

$$\overline{\mu(pH)} = \frac{(pH - pH_{max})(pH - pH_{min})^2}{\left(pH_{opt} - pH_{min}\right)\left[\left(pH_{opt} - pH_{min}\right)\left(pH - pH_{opt}\right) - \left(pH_{opt} - pH_{max}\right)\left(pH_{opt} + pH_{min} - 2pH\right)\right]} \quad (6.8)$$

6.2.1.4 Dissolved oxygen

The dissolved oxygen concentration is another relevant factor influencing the performance of microalgae cells. The concentration of dissolved oxygen in equilibrium with air is determined by the temperature and ionic strength of the culture medium, ranging from 8 to 9 mg/L at ambient conditions for freshwater and seawater, respectively. However, during photosynthesis oxygen is produced, which is accumulated into the culture medium if adequate mechanisms to remove it are not implemented. Excess of dissolved oxygen reduces the effectiveness of the photosynthesis process by causing inhibition of the product, the fundamental of this phenomenon being the conversion of RuBisCO from carboxylase to oxidase when dissolved oxygen concentration into the culture medium exceeds the saturation constant of this enzyme.

Thus, at dissolved oxygen concentrations higher than 12 mg/L, equivalent to 150 %Sat., a relevant reduction of photosynthesis rate for different microalgae strains has been reported, with the photosynthesis rate approaching zero at dissolved oxygen concentrations of 20 mg/L, equivalent to 250 %Sat. (Reis and Da Silva, 2016; Mendoza et al., 2013b). To modelize the influence of dissolved oxygen concentration on the performance of microalgae cells, inhibition by product model can be used (Eq. 6.9) (Costache et al., 2013,b; Ippoliti et al., 2016b) (Fig. 6.3D). Reported values of characteristic parameters of this model range from 12 to 15 mg/L for maximal dissolved oxygen concentration tolerable (DO_{2max}), and 2–3 for the form parameter (m) (Costache et al., 2013,b; Ippoliti et al., 2016b).

$$\overline{\mu(DO_2)} = 1 - \left(\frac{DO_2}{DO_2 max}\right)^m \tag{6.9}$$

6.2.1.5 Overall growth model

From this analysis of major factors influencing the growth rate of microalgae strains, an overall model taking into account the considered factors can be obtained. Disregarding interactions between the different major factors, a general growth model can be obtained by multiplying the growth rate as a function of irradiance by the normalized growth rate as a function of different culture parameters (Eq. 6.10) (Ippoliti et al., 2016b).

$$\mu(I, T, \text{pH}, DO_2) = \mu(I) \cdot \overline{\mu(T)} \cdot \overline{\mu(\text{pH})} \cdot \overline{\mu(DO_2)} \tag{6.10}$$

This model allows determination of the performance of any microalgae strain in a real reactor if data from laboratory (characteristics parameters of the strain) and from the real reactor (real conditions existing in the reactor) are known. Models of this type are required to identify the major factors reducing the performance of outdoor cultures in real reactors, and to evaluate the reliability and profitability of improving strategies/equipment to respond to these factors. Thus, this model is useful not only for the design of photobioreactors but also for optimizing the operation conditions of any photobioreactor for microalgae production.

6.2.2 Photobioreactor capacity

Photobioreactors must be designed to provide the optimal conditions required by the selected strains at a minimal cost. Thus, specific culture conditions such as light availability, temperature, pH/CO_2, and dissolved oxygen concentrations must be provided. Additionally, other requirements such as low cost/energy consumption, reliable capacity, and stable production are also required. The capacity of any photobioreactor type to accomplish these requirements will be a function of its design/operation, such as geometry, fluid-dynamic, mass transfer, and heat transfer capacity.

6.2.2.1 Geometry

Microalgae reactors are usually divided into categories according to their purposes. Thus, tubular reactors are composed of a bubble column, an impulsion system, and a solar receiver. In raceway reactors the same sections can also be considered, especially if the final design includes a sump, thus the entire channel and the sump corresponding to the solar receiver and bubble column, respectively. The reason for that is because in any photobioreactor, the solar receiver must be designed to collect the solar radiation optimally, the design of this part not being adequate to optimize the capacity of the system to provide the other requirements (temperature, pH/CO_2, dissolved oxygen). To optimize the capacity to provide the other requirements of the cells, the bubble column system must be designed/optimized separately. Only in some specific designs, such as bubble column and flat panel reactors, is the entire volume of the reactor utilized for both capturing solar radiation and providing the requirements of the cells, but these designs are rarely used at large scale.

The design of the solar receiver strongly influences the capacity of the system to collect the solar radiation and to distribute it into the culture, thus affecting the average irradiance inside the culture to which the cells are exposed. Although the solar radiation available is a function of location at which the reactor is installed, it also changes over annual and daily cycles, the net amount of light available on the reactor surface being also a function of the geometry of the reactor. In the case of raceway reactors, because they are horizontally arranged, the solar radiation available is that arriving from the sun, no more corrections being requested; the irradiance on the surface of the photobioreactor is directly that measured by adequate sensors such as pyranometers. In the case of tubular reactors or flat panels, the irradiance on the reactor surface is different to that arriving from the sun in a horizontal surface, it being a function of the arrangement of the tubes/flat panel, orientation, etc., and it must then be corrected. For this to occur, irradiance correction factors (α) taking into account the real irradiance on the surface as a function of the solar radiation on a horizontal surface must be calculated (San Pedro et al., 2014). This correction factor can be obtained using simulation software (which used the conventional equations of the solar energy field), but it can also be obtained by experimental measurements. In photobioreactors with horizontal tubes vertically arranged, the values of this irradiance correction factor ranged from 0.3 to 0.5 as a function of season and hour of the day (San Pedro et al., 2014; Romero Villegas et al., 2017). In the case of flat plate photobioreactors, the irradiance correction factor range was 0.4–0.8 as a function of height and separation between the panels (San Pedro et al., 2016; Romero-Villegas et al., 2018). Thus, geometry influences the real irradiance on the photobioreactor surface.

Once the irradiance on the reactor surface is known, the average irradiance inside the culture can be calculated by using Eq. (6.3), to calculate the average irradiance inside the culture it is mandatory to know the light path of the culture, then the distance that the light must cross inside the culture. In raceway reactors, the light path corresponds to the water depth, which ranges from 0.2 to 0.4 m, whereas in the case of tubular photobioreactors the light path

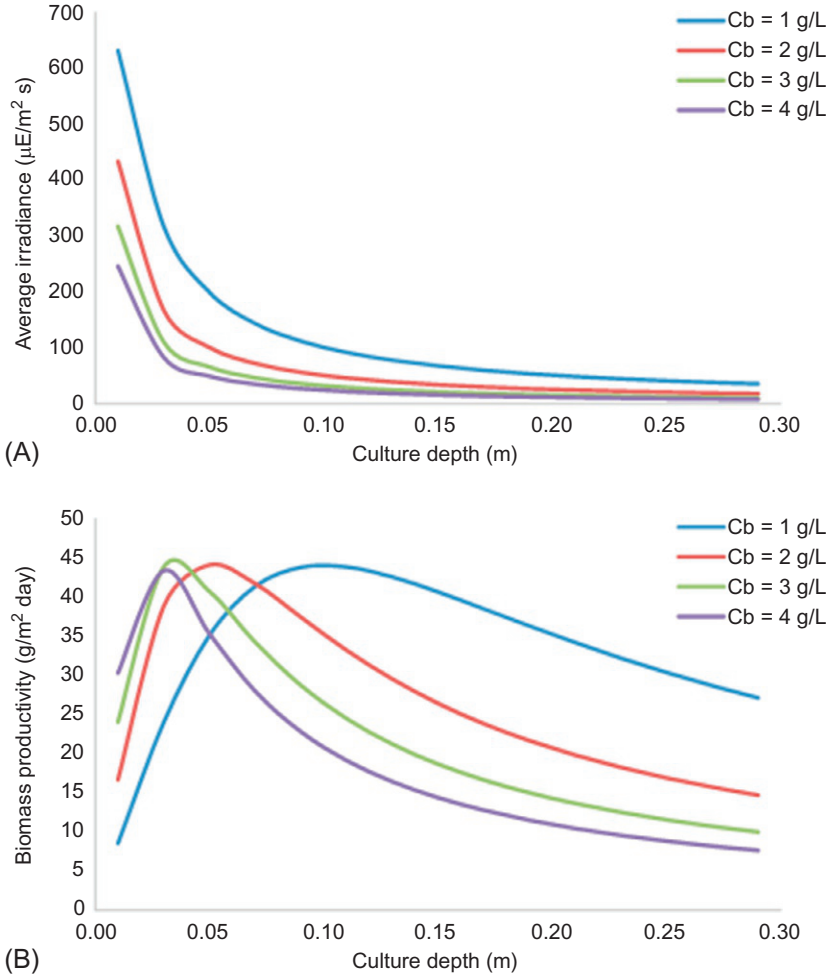

Fig. 6.4

Influence of culture depth and biomass concentration on the average irradiance inside the culture and biomass productivity of microalgae cultures. Data simulated using the growth model of *Scenedesmus* sp. previously reported, and considering an irradiance on the reactor surface of 1000 μE/m^2 s (Costache et al., 2013).

corresponds to the tube diameter, values from 0.03 to 0.10 m usually being used. The light path, in addition to biomass concentration, determines the average irradiance inside the reactor, and ultimately the achievable biomass productivity. As shown in Fig. 6.4A, the average irradiance exponentially decreases with the culture depth, also decreasing with the increase of biomass concentration inside the culture. At water depths greater than 0.2 m, only if the biomass concentration is lower than 1 g/L can the average irradiance inside the culture be higher than 100 μE/m^2 s, which is required to maximize biomass productivity. However, on reducing the culture depth up to 0.03 m the average irradiance is higher than 100 μE/m^2 s, including at biomass concentrations upper than 4 g/L. Regarding biomass productivity, Fig. 6.4B shows

how the culture depths have a direct influence on the biomass concentration at which maximal productivity is achieved; the higher the culture depth, the lower the optimal biomass concentration. It is clear that to achieve optimal biomass concentrations greater than 1 g/L, the culture depth must be lower than 0.10 m.

It is important to note that using Eq. (6.3), a fully dispersed light distribution of light on the reactor surface is considered, also light impinging normally to the surface. A more accurate estimation of the light path can be obtained if considering the real position of the sun each time and the direct and diffuse components of that radiation. It is possible to do that by considering the classical equations of the solar energy field and geometry of the systems, as already performed for both horizontal (Acien Fernandez et al., 1997) and vertically arranged tubes (Garcia Camacho et al., 1999). However, deviation between simple Eq. (6.3) and rigorous calculations considering the real trajectories of light inside the reactors is not large; moreover, taking into account the variation of the trajectory of the light path during the day, the utilization of simple Eq. (6.3) is recommended.

6.2.2.2 Fluid-dynamic

In any photobioreactor, energy must be provided (i) to minimize the existence of gradients of properties inside the cultures, of both culture conditions and nutrients, and (ii) to improve the light regime to which the cells are exposed inside the reactor. Energy required to recirculate the culture in any photobioreactor can be calculated using Bernouilli's equation, which is an energy balance into the system (Eq. 6.11). In the case of tubular photobioreactors, the utilization of this equation is quite clear, whereas in raceway reactors, Manning's equation has been traditionally used. However, it has already been demonstrated that Bernoulli's equation is also suitable for estimating the energy consumption for liquid circulation in raceway reactors (Mendoza et al., 2013a). According to this equation, the energy required to circulate a liquid between two points (1–2) is a function of the difference of pressure between both points (P_1, P_2), velocity in both points (v_1, v_2), height of both points (z_1, z_2), and pressure drop into the system. For tubular photobioreactors, this equation can be applied considering the surface of the bubble column and end of the loop as positions 1 and 2, respectively. The difference of pressure is then null, the velocity at the surface of the bubble column being negligible; only the kinetic energy corresponding to liquid velocity at the end of the loop remains relevant, and no difference of height exists between positions 1 and 2. The only remaining factor is then the pressure drop into the loop (Eq. 6.12). The pressure drop into the system can be calculated as a function of the friction coefficient (C_f), length of the tube (L), velocity of the liquid (v), and diameter of the tube (D) (Eq. 6.13). The friction coefficient can be 0calculated using the Blaussius equation (Eq. 6.14), such as a function of the Reynolds number (Eq. 6.15) (Molina et al., 2001). It is mathematically demonstrated that the contribution of the kinetic energy is much lower than the contribution of pressure drop into the tubes, then it can be disregarded (Molina Grima et al., 1999).

$$\frac{P_2 - P_1}{\rho} + \left(\frac{v_2^2}{2\alpha_2} - \frac{v_1^2}{2\alpha_1}\right) + g(z_2 - z_1) + \Sigma F = W \tag{6.11}$$

$$\left(\frac{v_2^2}{2\alpha_2}\right) + \Sigma F = W \tag{6.12}$$

$$\Sigma F = 4C_f \frac{Lv^2}{2D} \tag{6.13}$$

$$C_f = 0.0791\,\mathrm{Re}^{0.25} \tag{6.14}$$

$$\mathrm{Re} = \frac{v\rho D}{\mu} \tag{6.15}$$

A similar analysis can be performed in the case of raceway reactors; in this case the selected points 1 and 2 are the inlet and outlet of the paddlewheel. In the case of raceway reactors, equal than for tubular ones, the pressure at the inlet and outlet is the atmospheric pressure, also the height of water being the same at the inlet and outlet; in addition the liquid velocities in both points are equal, thus the only factor remaining is the pressure drop into the system (Eq. 6.16). Thus, the energy consumption in raceway reactors can be calculated using the same Eq. (6.13) as before for tubular photobioreactors. The equivalent diameter is defined as two times the hydraulic radius, calculated as the transversal section to the wet perimeter of the section, both being a function of width of the channel and depth of the culture (Eq. 6.17).

$$\Sigma F = W \tag{6.16}$$

$$D_h = 2r_h = 2\frac{wh}{w + h + h} \tag{6.17}$$

Fig. 6.5 summarizes the energy consumption in both tubular and raceway reactors as a function of liquid velocity and characteristic dimension of each type or reactor, the tube diameter in the case of tubular photobioreactors, and the width of the channel in the case of raceway reactors (because the water depth is normally fixed at 0.2 m in this type of photobioreactor). As the increase of liquid velocity has a large impact into the energy consumption, whatever the reactor type, it is recommended to reduce the liquid velocity as much as possible. However, liquid velocity must be kept above a minimum value to avoid the sedimentation of the cells and to minimize the existence of gradients along the reactor; in the case of tubular reactors this ranges from 0.4 to 0.8 m/s, whereas in raceway reactors, liquid velocities is usually fixed at 0.2 m/s (Acién et al., 2017). Concerning the effect of characteristic dimension of each type of reactor, results show that the energy consumption largely reduces when increasing the tube diameter in tubular photobioreactors, also reducing in raceway reactors when increasing the width of the channel. In any case, the energy consumption into the raceway reactor is much lower than for tubular photobioreactors, in general less

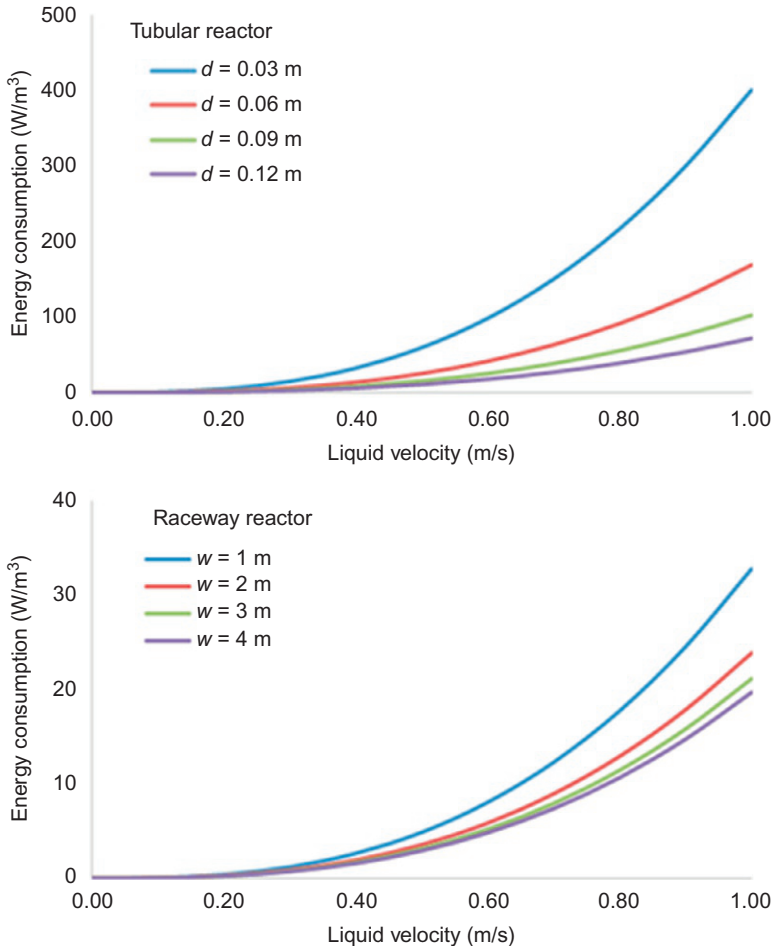

Fig. 6.5

Influence of liquid velocity on energy consumption of tubular and raceway reactors as a function of tube diameter and wide of the channel, respectively.

than 10 times lower for equivalent conditions. Thus, in tubular photobioreactor the energy consumption range is 100–400 W/m^3 (Molina et al., 2001), whereas for raceway reactors the energy consumption can be lower than 10 W/m^3 (Mendoza et al., 2013a). This is a critical factor when deciding on which type of reactor to use, especially for low-cost applications for which only open raceway reactors are suitable due to their lower energy consumption.

Fluid-dynamics also influence the light regime to which the cells are exposed inside the reactor, but to determine the real light regime taking place in each case, specific studies using computational fluid-dynamic studies must be performed. Thus, a general pattern cannot be defined, and each case must be analyzed individually.

6.2.2.3 Mass transfer

The mass transfer capacity of any bioreactor is related to the capacity of the system to transport any component to the cells. Whatever compound dissolved into the liquid phase is easily accessible for the cells because they are suspended in the same liquid and the liquid/culture is well mixed. However, transporting components between gas and liquid phase is more difficult. In the case of microalgae photobioreactors, the two major components to be exchanged between the liquid and gas phases are CO_2 and O_2. To provide CO_2 to microalgae cultures, it is possible to inject pure CO_2, mixtures of CO_2 with air, or flue gases containing CO_2. To remove oxygen from microalgae cultures, it is possible to supply air; in addition, the injection of any CO_2-rich gas removes oxygen from the culture. The challenge is to provide CO_2 and to remove O_2 at an efficient enough rate to satisfy the requirements of microalgae cells, at minimum cost. CO_2/O_2 requirements can be easily estimated on the basis of expected biomass productivity. Thus, from elemental analysis of the biomass it was concluded that up to 1.8 gCO_2/gbiomass must be supplied, whereas up to 1.33 gO_2/gbiomass must be removed. Considering an overall biomass productivity of 30 gbiomass/m^2·day, and taking into account that biomass production occurs mainly in the central hours of the solar period (approximately 6 h), the requirement of CO_2 supply is up to 9.2 gCO_2/m^2 h, whereas the requirement of O_2 desorption is up to 6.7 gO_2/m^2 h.

Considering oxygen as an example, it can be summarized that the mass transfer capsacity in any system is a function of three parameters: (i) the mass transfer coefficient (K_La_L); (ii) the driving force as the difference between the concentration of the component into the liquid phase ($[O_2]$) and that in equilibrium with the gas phase ($[O_2^*]$); and (iii) the volume of the system (V) (Eq. 6.18). The dissolved oxygen concentration in equilibrium with any gas can be calculated using Henry's law (Eq. 6.19), as a function of Henry's constant for the component (H_{O_2}), the total pressure (P), and the partial pressure of the component into the gas phase. In the case of CO_2, the same equations can be applied, such as Eqs. (6.20) and (6.21). It is important to note that although two different mass transfer coefficients for O_2 and CO_2 are defined, they are quite similar, thus $K_La_{L,\,O_2} = 0.91 \cdot K_La_{L,\,CO_2}$, both being mainly a function of mixing/contact between gas and liquid phase.

$$NO_2 = K_La_{L,O_2}\left([O_2] - \left[O_2^*\right]\right)V \tag{6.18}$$

$$\left[O_2^*\right] = H_{O_2} \cdot P \cdot y_{O_2} \tag{6.19}$$

$$NCO_2 = K_La_{L,CO_2}\left([CO_2] - \left[CO_2^*\right]\right)V \tag{6.20}$$

$$\left[CO_2^*\right] = H_{CO_2} \cdot P \cdot y_{CO_2} \tag{6.21}$$

The value of Henry's coefficient for O_2 (4.3×10^{-3} mol/L·atm) is much lower than for CO_2 (3.2×10^{-2} mol/L·atm), then the driving force for O_2 removal being much lower than for CO_2 absorption, it largely influencing the overall mass transfer phenomena. As shown in Fig. 6.6A, the concentration of dissolved oxygen in equilibrium with air is 8.7 mg/L, whereas the maximum achievable concentration at atmospheric pressure in equilibrium with pure oxygen is

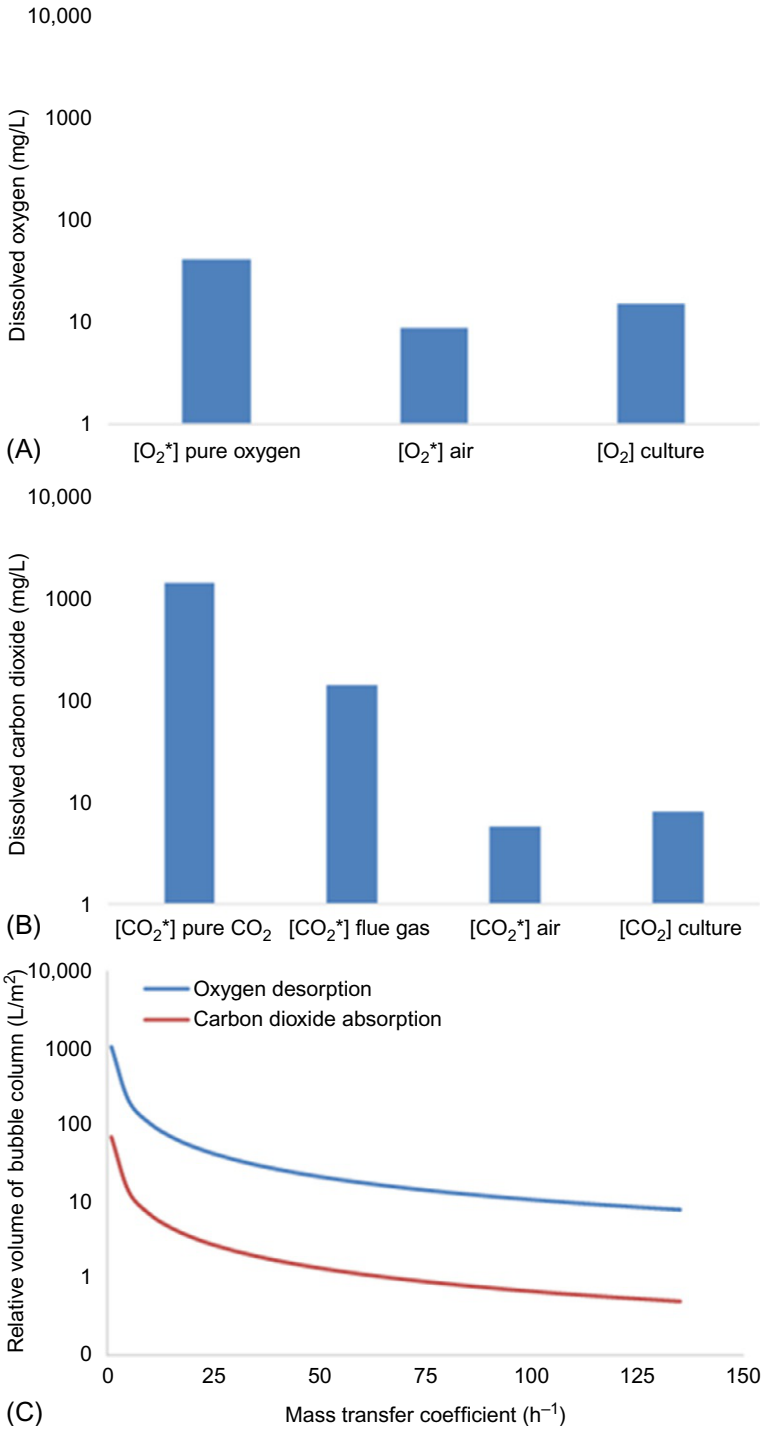

Fig. 6.6

Variation of dissolved oxygen (A) and carbon dioxide (B) concentration into the culture as a function of the gas in equilibrium with the liquid phase and that expected into microalgae cultures. (C) Variation of relative volume of reactor requested to achieve the required oxygen removal and carbon dioxide absorption capacities as a function of mass transfer coefficient.

41.6 mg/L. It has previously been indicated that if dissolved oxygen concentration into the culture is greater than 15 mg/L the photosynthesis rate strongly reduces, and the maximal driving force for oxygen desorption in real cultures is then lower than 10 mg/L. This means that to be able to remove the oxygen produced by photosynthesis in a large reactor, the capacity of mass transfer must be extremely high; thus the mass transfer coefficient and the volume of the mass transfer unit must be large. In the case of CO_2, the values are quite different. Thus, the concentration of CO_2 in equilibrium with pure CO_2 is up to 1400 mg/L; this value reduces to 140 mg/L if a flue gas containing 10% of CO_2 is supplied (Fig. 6.6B). The concentration of CO_2 into the culture is a function of the total inorganic carbon and equilibrium constant for the carbonate/bicarbonate buffer existing in any culture medium. Considering a total inorganic carbon concentration of 0.1 g/L and pH 8, the concentration of CO_2 into the culture is 8 mg/L. This means that the driving force for CO_2 absorption is up to 1400 mg/L when using pure CO_2 and up to 132 mg/L if using flue gases. It is important to note that in the case of equilibrium with air, the concentration of CO_2 is up to 5.6 mg/L, which is similar to the CO_2 concentration into the culture of 8.0 mg/L; CO_2 into the air is so low that including if the air is excellently supplied to the microalgae cultures, using excellent mass transfer units, the net amount of CO_2 provided to the culture is anyway too low, then limiting the performance of microalgae cells. In this sense, when using pure CO_2 or flue gases, the driving force for CO_2 absorption are two and one order of magnitude higher that in the case of oxygen, respectively, indicating that the absorption of CO_2 into the culture is much easier, and the required mass transfer coefficient and volume of the mass transfer unit can be smaller than in the case of O_2 desorption. Because the same system is usually used for both O_2 removal and CO_2 absorption, the limiting factor for designing this section of the reactor must be the O_2 desorption phenomenon.

Fig. 6.6C shows that for a reactor with an oxygen production rate of 6.7 gO_2/m^2 h and a CO_2 consumption rate of 9.2 gCO_2/m^2 h, the relative volume of mass transfer unit to satisfy these requirements reduces when increasing the mass transfer coefficient into the bubble column, which is much larger for O_2 desorption than for CO_2 absorption. Data shows that for adequate O_2 removal, the relative volume of the bubble column must be larger than 100 L/m^2 if the mass transfer coefficient is lower than 10 h^{-1}, reducing to 10 L/m^2 only if mass transfer coefficients higher than 100 h^{-1} are achieved into the mass transfer unit. For example, for a raceway reactor of 0.2 m water depth and 5 m^2, it means that the volume of the mass transfer unit reduces from 500 to 50 m^3 if the mass transfer coefficient into the mass transfer unit increases from 10 to 100 h^{-1}. Thus, careful consideration of the mass transfer phenomenon is critical for the adequate design and management of any microalgae photobioreactor.

6.2.2.4 Heat transfer

Heat transfer is the capacity of removing/supplying heat to any system to control its temperature. In the case of biological processes, the energy dissipated during the growth is negligible; therefore, most of the biological processes the requirements of heat exchange are

low. However, photobioreactors are exposed to sunlight, thus absorbing large amounts of energy by radiation. In addition, they are exposed to ambient temperatures, which also influence the temperature of the culture. To design any heat exchanger system adequately, it is necessary to determine the inlet and outlet heat flows, or at least the most relevant of them.

Considering all the possible mechanisms related to heat exchange in a photobioreactor, the overall heat balance is defined by Eq. 6.22. The heat transfer by radiation from the sun ($Q_{radiation}$) is a function of impinging global solar radiation on the surface of the reactor (G), the absorptivity of the culture (a), and the overall surface exposed to the solar radiation ($A_{radiation}$) (Eq. 6.23). The heat transfer by convection ($Q_{convection}$) corresponds to the heat exchanged with the air by this mechanism, which is a function of the convective heat transfer coefficient ($h_{convection}$), the surface in contact with the air ($A_{ambient}$), and the difference of temperature of the ambient ($T_{ambient}$) to that of the culture ($T_{culture}$) (Eq. 6.24). Conduction takes place if the reactor is in contact with soil, as in raceway reactors. In this case, heat transfer by conduction ($Q_{conduction}$) is a function of the conductivity of the material used to build the reactor ($k_{conduction}$), the surface of the reactor in contact with the soil (A_{soil}), the temperature of the soil (T_{soil}), and the thickness of the material used to build the reactor ($e_{conduction}$), in addition to the temperature of the culture ($T_{culture}$) (Eq. 6.25). The two last terms correspond to the evaporation and radiative phenomena. Heat transfer by evaporation ($Q_{evaporation}$) corresponds to the removal of heat by water evaporation from the reactor. Although this can be calculated by measuring the net amount of water evaporated and taking into account the heat removed by each mass unit of water evaporated, it can also be estimated as a function of a heat transfer by evaporation coefficient ($h_{evaporation}$), the surface of the reactor on which evaporation take place ($A_{evaporation}$), the vapor pressure of water in equilibrium with air at the ambient temperature ($PV_{ambient}$), and that at the real vapor pressure of water at the temperature of the culture ($PV_{culture}$) (Eq. 6.26). It is important to note that the heat transfer coefficient by evaporation ($h_{evaporation}$) is a function of environmental conditions such as wind. Finally, the heat lost by radiative dissipation ($Q_{radiative}$) is highly relevant at night, when the temperature of the sky is lower. This heat loss is a function of the Stefan-Boltzman constant (σ), the surface exposed to the sky ($A_{radiative}$), the emissivity of the culture (ε), the temperature of the sky (T_{sky}), and that of the culture ($T_{culture}$) (Eq. 6.27).

$$Q_{accumulated} = Q_{radiation} + Q_{convection} + Q_{conduction} + Q_{evaporation} + Q_{radiative} \tag{6.22}$$

$$Q_{radiation} = G \cdot a \cdot A_{radiation} \tag{6.23}$$

$$Q_{convection} = h_{convection} \cdot A_{ambient} \cdot (T_{ambient} - T_{culture}) \tag{6.24}$$

$$Q_{conduction} = k_{conduction} \cdot A_{soil} \cdot (T_{soil} - T_{culture}) / e_{conduction} \tag{6.25}$$

$$Q_{evaporation} = h_{evaporation} \cdot A_{evaporation} \cdot (PV_{ambient} - PV_{culture}) \tag{6.26}$$

$$Q_{radiative} = \sigma \cdot A_{radiative} \cdot \varepsilon \cdot \left({T_{sky}}^4 - (T_{culture} + 273)^4 \right) \tag{6.27}$$

The challenge of any photobioreactor design is to optimize the temperature of the culture, both during the daylight period and throughout the year. Because to control the temperature of large reactors requires enormous amounts of energy, most of the large scale reactors only have systems capable to avoid overheating of the cultures that by sure provoke the death of the cells. To minimize the amount of energy required for that, the temperature in the location where the reactor is installed must be as close as possible to that required by the selected microalga strain. Next, the total energy required to control the temperature is calculated from the heat balance of the system. In any photobioreactor, the absorption of heat by radiation is important; absorptivity of the culture is close to 1, whereas the global radiation and surface exposed to sunlight are easily known. Heat transfer by convention is always low whatever the photobioreactor type because the convection coefficient ($h_{convection}$) for natural convection is lower than 20 kcal/h m^2°C; in addition, the difference of temperature between ambient and culture is also low, typically no greater than 10°C. In the case of heat transfer by conduction, the same criteria can be applied, thus the conductivity of the materials used to build the reactors is low, lower than 0.4 kcal/h m °C; they are mainly plastics with a large thickness, typically up to 0.5 cm. Additionally, the temperature of the soil is quite stable over time, being close to ambient temperature. Heat exchange by evaporation is relevant in open reactors because the surface in contact with the air is large, and large volumes of water are evaporated, up to 10 L/m^2·day. This is not the case for closed photobioreactors, where evaporation losses are minimal. Radiative heat exchange is only relevant during the night, whatever the reactor, contributing to reduce the temperature during this period. Fig. 6.7 shows the heat balance for a raceway reactor located in temperate climates. Typically, the heat accumulated is positive because the inlet by radiation is

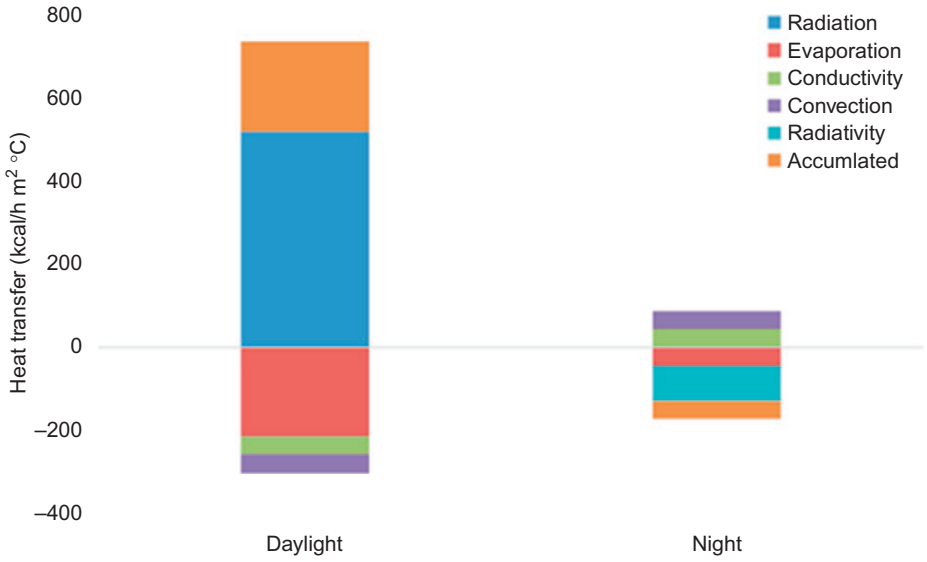

Fig. 6.7
Most relevant heat transfer phenomena taking place in outdoor microalgae cultures performed in raceway reactors during both daylight and night periods.

higher than losses by convection, conductivity, and evaporation, whereas during the night the accumulation is negative because losses by radiative and evaporation are higher than inlets by convection and conductivity. In this example, to prevent the overheating of the culture during the daylight period up to 215 kcal/h m^2 must be removed, whereas during the night, to avoid excessive reduction of temperature, up to 43 kcal/h m^2 must be supplied. This mean that for an industrial raceway reactor of 5 m^2, up to 1.8×10^6 kcal/h can be required to avoid overheating during daylight period. This energy is approximately double that chemically stored in the biomass.

6.3 Photobioreactor types

6.3.1 Raceway reactors

Raceway ponds are currently the most extended reactors for the production of microalgae worldwide; more than 90% of global production is performed using these reactors, due mainly to its low cost (Fig. 6.8). The cost investment for these systems ranges from 0.13 to 0.37 M€/ha at 100 ha scale (Norsker et al., 2011; Chisti, 2012). Facilities producing microalgae in raceway reactors are located worldwide, such as the United States, Thailand, China, Chile, and Israel; the biomass produced is mainly used for human consumption. In raceway ponds the biomass concentration remains below to 1.0 g L^{-1} due to the high water depth; this facilitates the contamination of the cultures by other microorganisms and increases the risk of failures, both strongly reducing the overall productivity of these systems. In order to minimize these problems, only robust and fast-growing strains, tolerant to extreme conditions, are produced in these reactors, such as *Spirulina*, *Chlorella*, and *Dunaliella*. Although biomass productivities

Fig. 6.8
Image of 500 m^2 raceway reactor located at Estacion Experimental Las Palmerillas from Fundación Cajamar (Almería, Spain).

up to 40 g m^{-2} day^{-1} (equivalent to 150 t ha^{-2} $year^{-1}$) have been reported (Lundquist et al., 2010), on average, much lower productivities are usually claimed, in the range of 9–15 g m^{-2} day^{-1} when producing *Tetraselmis suecica* or *Nannochloropsis* sp. (Chiaramonti et al., 2013), 13 g m^{-2} day^{-1} when producing *Chlorella* (Hase et al., 2000), or 21 g m^{-2} day^{-1} when producing *Spirulina* (Vonshak and Guy, 1992). The reliability of raceway reactors also depends on additional factors such as rainfall and storms, the presence of dust and pollutants, the presence of biological contaminants such as insects or fungi, etc.

Raceway ponds were firstly proposed by Oswald for wastewater treatment (Oswald and Golueke, 1968), although after that they were also utilized to produce microalgae biomass in clean water (Weissman and Goebel, 1987). The design of this type of photobioreactor has been recently updated, in particular to improve the fluid-dynamics and mass transfer capacity of these reactors, for both oxygen desorption and CO_2 absorption (Sompech et al., 2012; Chiaramonti et al., 2013; Mendoza et al., 2013a; de Godos et al., 2014).

Concerning geometry, the overall design of any raceway reactor usually consists of two channels along the culture which are recirculated by a paddlewheel. This provides the energy to overpass the pressure drop into the system, especially into the bends. Raceway reactors are built on compacted soil covered by polymers, although the utilization of only compacted soil has also been proposed (Craggs et al., 2012). Although some designs including up to four channels have been proposed, they are not recommended due to the larger pressure drop caused by the additional bends. The length-to-width ratio of this type of reactor ranges from 10 to 20, and the utilization of lower length/wide ratios is preferable to reduce the pressure drop into the system, especially at large scale. Due to the limited capacity of the paddlewheel to provide energy, raceway reactors up to 5 m^2 are constructed at industrial scale; larger reactors are not generally feasible because of the large pressure drop into the channels and bends. Industrial facilities are then composed of multiple units of this industrial size standard unit. The water depth into the channel ranges from 0.2 to 0.4 m, low water depth being recommendable to increase the light availability and then the biomass concentration inside the culture. Opposite, high water depth is more recommendable when using the raceway reactor to treat wastewater because of the larger volume of the reactor and then the larger wastewater treatment capacity for the same hydraulic residence time.

Regarding fluid-dynamics, the liquid velocity in this type of photobioreactor ranges from 0.2 to 0.3 m/s; larger velocities are not used in order to minimize the energy consumption, whereas lower velocities favor the sedimentation of the biomass and existence of death zones in the reactor. It is important to note that usually liquid velocity is assumed to be fixed at 0.2 m/s to avoid sedimentation of microalgae cells. However, the settling velocity of microalgae is 10^{-7} m/s, and thus requires more than 250 h to get down in a 0.2 m water depth culture. Otherwise, the liquid velocity significantly affects the recirculation time; at industrial scale up to 30 min are required, which largely affects the capacity of the reactor to adjust the culture conditions to the optimal ones. To minimize energy consumption, the design of channels and

accessories, such as bends, sumps, or deflectors, must be carefully planned. It has been reported that bends can provide larger pressure drops than channels in this type of reactor, and to minimize this effect, two configurations of bends are proposed: those with additional baffles and those with island type bends (Sompech et al., 2012; Mendoza et al., 2013a). To provide the energy required for the circulation of the culture along the reactor, the utilization of a paddlewheel is generally preferred. This consists of a wheel with 10–12 paddles, with a total diameter of four times the water depth, rotating at velocities from 10 to 20 rpm. The precision into the hydraulic closure of the paddlewheel with the walls of the reactor largely determines its performance. In general, performances are low, from 10% to 60% as a function of liquid velocity and pond design (Weissman and Goebel, 1987; Lundquist et al, 2010), which is not particularly high; however, considering the low energy required and the low cost and simplicity of this impulsion system, it is considered sufficient. Although alternative impulsion systems such as centrifugal pumps or propellers can be used, they have never been used at large scale (Chiaramonti et al., 2013). Only recently FCC Aqualia patented the LEAR (low energy algae reactor) using a turbine for the circulation of the culture, claiming an energy consumption up to 50% of that achieved with a conventional paddlewheel system; however, its cost is much higher than that of the paddlewheel.

Concerning the influence of fluid-dynamics on the light regime to which the cells are exposed in this type of reactor, it has recently been reported that due to the large water depth and low vertical movement of cells at the circulation conditions prevailing in this type of photobioreactor, no possibility of light integration exists (Fernández-Del Olmo et al., 2017; Barceló-Villalobos et al., 2019a). Thus, at steady state no more than 25% of the total volume of the culture has enough irradiance to allow the cells to perform photosynthesis, the remaining 75% of the culture being in dark (Fig. 6.9). The vertical movement of the cells on this reactor is so low that an average vertical velocity of 2×10^{-4} m/s is estimated, which means that to move from dark to light zones, the cells requires up to 15 min, frequencies of light exposition up to 1×10^{-4} Hz being determined (Fig. 6.9) (Fernández-Del Olmo et al., 2017; Barceló-Villalobos et al., 2019a). Due to the low frequency of light exposition, the cells are adapted to the light that they receive at each moment in each position of the rector, on this way the overall performance being lower than if adaptation to average irradiance would take place.

Concerning mass transfer, raceway reactors have usually been considered adequate for oxygen removal due to the large surface of culture exposed to air on these reactors, whereas to optimize the absorption of CO_2, the utilization of diffuser providing small bubbles is recommended. However, it has been determined that due to the low mixing between culture and atmosphere along the channels, the mass transfer capacity on this section is very poor, with mass transfer coefficients up to $0.7\,h^{-1}$ being determined (Mendoza et al., 2013b). This value of mass transfer coefficient is similar to those reported, of 0.5–1.1 h^{-1}, for circulation of liquids without intense mixing between air-liquid phases (Weissman and Goebel, 1987; Camacho-Rubio et al., 1999). This means that raceway reactors also have problems of dissolved oxygen accumulation along the channel, such as those typically reported for closed

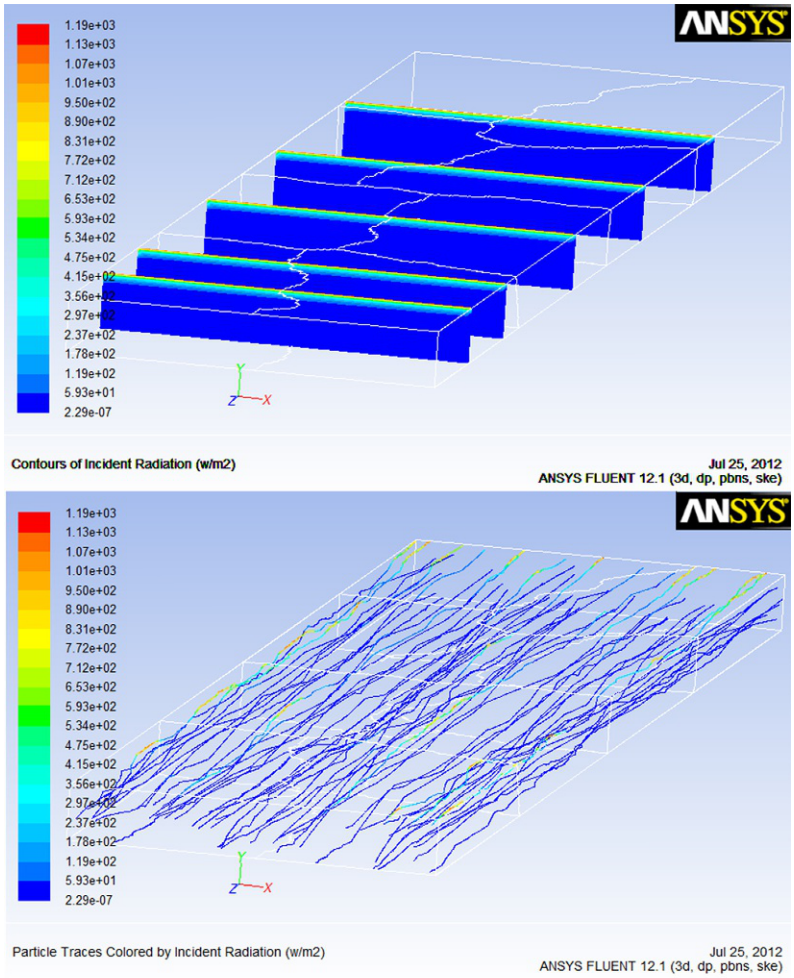

Fig. 6.9

Simulations about light availability and light regimen to which the cells are exposed in a 100 m^2 raceway reactor, located at Estacion Experimental Las Palmerillas from Fundación Cajamar (Almería, Spain). Upper part shows the light profile into the reactor, whereas the lower part shows the light received by the cells according to its movement along the reactor.

photobioreactors. Thus, values up to 450 %Sat. have been reported in raceway reactors producing *Chlorella* (Weissman and Goebel, 1987), whereas for those producing *Spirulina*, dissolved oxygen concentration values up to 375 %Sat. have been achieved (Jiménez et al., 2003). Extreme values of 500 %Sat. have been reported in raceway reactors, the performance of the cultures deteriorating and causing the cultures to die (Marquez et al., 1995; Singh et al., 1995; Vonshak, 1997). To solve this problem, the design/installation of adequate sumps is essential, with values of mass transfer coefficient up to 160 h^{-1} being reported on these systems (Mendoza et al., 2013b). Mass transfer coefficients from

50 to 350 h^{-1} have been reported for other aeration devices, which suggests that the gas-liquid mass transfer into the sump can be increased (Carvalho et al., 2006). Moreover, due to the variation of photosynthesis rate as a function of the daily variation of culture conditions into the reactor, the air flow rate supplied to the sump can be regulated to optimize the performance of the sump and of the entire raceway reactor by adjusting it to the actual demands of the system (Barceló-Villalobos et al., 2018).

Regarding CO_2 absorption, it is critical to supply it, otherwise the productivity of the cultures will be limited to 5 g/m^2·day. Injecting CO_2-rich gases is the most frequent method to supply CO_2 and to control the pH of the cultures. Because the driving force for CO_2 absorption is much higher than for O_2 desorption, the same volumetric mass transfer coefficient allows the required CO_2 to be supplied more easily than for the O_2 be removed. Thus, the same sump can be used for both oxygen removal and CO_2 supply. In any case, the challenge is to maximize the CO_2 absorption efficiency. If CO_2 is injected into the channel (0.2 m water depth), the efficiency of CO_2 absorption is very low, with up to 80%–90% of the CO_2 injected being loss to the atmosphere (Weissman and Goebel, 1987; Richmond et al., 1990; Richmond, 2003), whereas when CO_2 is injected into the sump, the efficiency of CO_2 absorption increases, and less than 5% of CO_2 is lost to the atmosphere (de Godos et al., 2014). The application of advanced control strategies allows the efficiency of CO_2 utilization to be improved; the performance of the cultures is also enhanced by reducing the variations of pH to which the cells are exposed inside the cultures, ultimately improving the overall performance of the system (Pawlowski et al., 2015, 2016).

Regarding temperature, the biomass productivity in raceway reactors is largely influenced by the temperature prevailing into the location where the reactor is installed, because in this type of reactor it is not possible to control it at large scale. Temperatures of 20–30°C are optimal for most microalgae, but the temperatures of cultures used in raceway reactors when exposed to high solar radiation can increase up to 40–50°C. The most relevant mechanism reducing the temperature of raceway pods is the evaporation of water, as this is heavily influenced by the humidity and temperature of the air. If the temperature of the culture is excessive and damages the performance of the cells, the only possibility is to increase the water depth to enlarge the thermal inertia of the system. However, doing this means that the water depth also increases, thus reducing the light availability to which the cells are exposed inside the culture, and ultimately the overall productivity.

6.3.2 Tubular reactors

Tubular photobioreactors are currently the recommended technology to produce high-quality biomass for high-value applications, especially when involving the production of sensible strains not tolerating their production in raceway ponds (Fig. 6.10) (Tredici et al., 2010; Torzillo and Zittelli, 2015). The cost investment for tubular photobioreactors is approximately

Fig. 6.10
Image of tubular photobioreactors of 3.0 m^3 located at IFAPA research center (Almería, Spain).

0.51 M€/ha at 100 ha scale, which is more than two times that for raceway ponds (Norsker et al., 2011). Facilities producing microalgae in tubular photobioreactors were installed only recently, and are now located worldwide, such as in Germany, France, Portugal, Spain, the United States, Israel, and China. In tubular photobioreactors the biomass concentration range was 1.0–3.0 g/L due to the lower water depth (tube diameter), which ranges from 0.03 to 0.12 m. Because the reactors are closed and the biomass concentration is higher, the prevalence of problems such as contamination and risk of failure reduces in comparison with raceway reactors. This allows these reactors to be used to produce sensible strains such as T-ISO, *Phaeodactylum*, *Nannochloropsis*, *Tetraselmis*, *Porphyridium*, and *Haematococcus*. In tubular photobioreactors, biomass productivities of 8–18 g/m^2·day have been reported for the production of T-ISO (van Bergeijk et al., 2010; Ippoliti et al., 2016a), whereas for the production of *Phaeodactylum*, values up to 18 g/m^2·day have been identified (Silva Benavides et al., 2013), and for the production of *Scenedesmus*, productivity values ranging from 20 to 50 g/m^2·day have been reported (Acién et al., 2012). These figures do not differ significantly from those provided for raceway reactors, thus contradicting the general assumption that tubular photobioreactors are more productive than raceway reactors. This assumption is generally related to the better control of culture conditions that is possible when using tubular photobioreactors than using raceways. However, including using closed tubular photobioreactors, it is not possible to adjust the culture conditions completely to those required by the cells, due to the large variation of solar radiation and temperature outdoors. Data already published demonstrated that including in tubular photobioreactors the culture conditions cannot be completely fitted to the optimal ones for the microalgae cells, then the overall performance of the cells reduces to one-third of the maximal one due to deviation of culture conditions from

the optimal ones (Ippoliti et al., 2016a). In spite of this fact, the major advantage of tubular photobioreactors is the reliability of producing biomass in these systems, by operating at higher biomass concentrations, increasing the control of culture parameters. Although reduction of contamination risk has also been claimed for tubular reactors, recent evidence demonstrated that this is only partially true (Forehead and O'Kelly, 2013; Carney et al., 2014). Thus, recently a number of important parasites have been identified in microalgae mass culture systems; the number and prevalence of these parasites increase when increasing the size and number of commercial facilities (Hoffman et al., 2008; Carney et al., 2014).

Tubular photobioreactors were first proposed to maximize the productivity of *Chlorella* cultures (Little, 1953), although since then, they have been utilized to produce other strains and to increase the quality of the biomass produced. The design of this type of photobioreactor has been updated to maximize the light utilization efficiency, mass transfer capacity especially for oxygen desorption, and control of temperature (de Vree et al., 2015; Torzillo et al., 2015; Brindley et al., 2016; Fernández et al., 2017).

Concerning geometry, the overall design of tubular photobioreactors consists of a solar collector made with tubes along which the microalgae culture is recirculated to a bubble column or tank, using a centrifugal pump that provides the energy required to overpass the pressure drop into the solar loop (Fig. 6.11). The major factors determining the geometry of the reactor are: (1) the overall distribution/configuration of the tubes into the solar collector; (2) the tube diameter; and (3) the length of the tubes. Different configurations of the solar collector have been proposed such as serpentine/manifold, vertical/horizontal, and helical/flat, which determine the net amount of solar radiation actually intercepted and available on the surface of the reactor (Robinson et al., 1988; Richmond et al., 1993; Tredici and Zittelli, 1998; Fernández-Sevilla et al., 2010; Tredici et al., 2010). Small tube diameters, lower than 0.1 m, are required to maximize the performance of tubular photobioreactors; however, reducing the tube diameters too much heavily increases the energy consumption, thus minimum values of 0.03 m are recommended (Molina et al., 2001). The major factor limiting the length of the solar collector is the accumulation of dissolved oxygen, thus maximal lengths of 100 and 400 m are recommended for manifold and serpentine configurations, respectively. Due to the existence of limit values for both tube diameter and length of the tubular loop, the overall volume of single tubular photobioreactors is also limited to values up to 20 m^3 per unit; industrial facilities consist of repeated units of tubular photobioreactors up to maximum surfaces of 10 ha.

Regarding fluid-dynamics, the liquid velocity in tubular photobioreactors ranges from 0.4 to 1.0 m/s. This higher liquid velocity, with respect to that found in raceway reactors, is related to the higher accumulation of dissolved oxygen taking place in this type of reactor, and it is necessary to recirculate the culture thought the bubble column to minimize this problem. Thus, in tubular photobioreactors the circulation time typically ranges from 3 to 10 min. The high liquid velocity increases the energy consumption on these systems up to 100–400 W/m^3, which is much higher than in raceway reactors. The lower water depth and higher liquid velocity found in tubular photobioreactors compared to raceway reactors allows the cells to move more

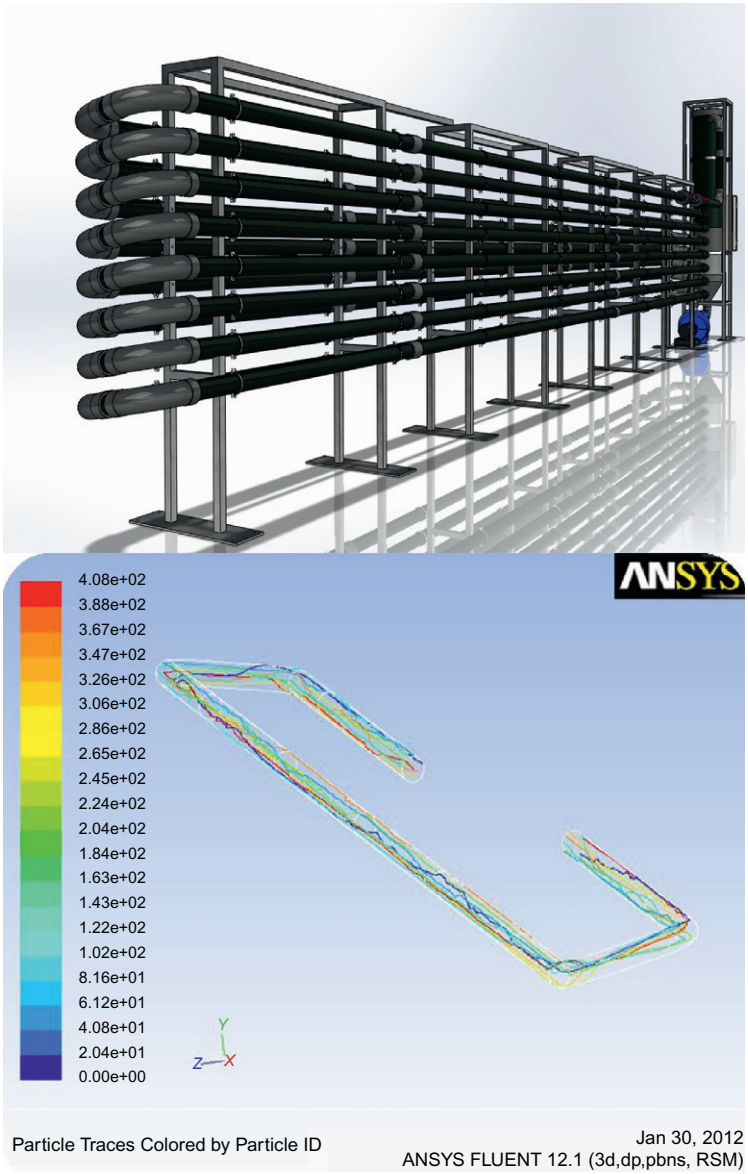

Fig. 6.11

Scheme and light regime to which the cells are exposed in a 3.0 m^3 tubular photobioreactor located at IFAPA research center (Almería, Spain). Upper part shows the scheme of tubular reactor, whereas lower part shows the light regime at which the cells are exposed to in the same tubular photobioreactor.

quickly from the inner dark zones to the outer illuminated zones on the tubes, thus allowing an increase in the light frequency to which the cells are exposed in this type of system (Fig. 6.11) (Brindley et al., 2016; Fernández-Del Olmo et al., 2017). This fact allows the cells to achieve higher light integration than in raceway reactors, which ultimately increases biomass productivity (Brindley et al., 2016; Fernández-Del Olmo et al., 2017). However, an excess of

turbulence in tubular photobioreactors, and especially when using centrifugal pumps, can reduce the performance of the cultures (Alías et al., 2004), and the utilization of adequate impulsion systems at the optimal liquid velocity is a challenge.

Concerning mass transfer, in tubular photobioreactors air and CO_2 supply are usually separated. The former is supplied to the bubble column/tank, which is optimized to maximize the oxygen desorption capacity, whereas CO_2 is added into the loop to maximize the absorption of CO_2 absorption and the CO_2 utilization efficiency. Typically, the air supply at the bubble column is approximately 0.1 v/v/min, whereas the CO_2 supply at the loop is about 0.001 v/v/min (Camacho-Rubio et al., 1999; Fernández et al., 2017). In these conditions, the dissolved oxygen concentration remains below 250 %Sat., while CO_2 utilization efficiencies up to 95% can be achieved (Camacho-Rubio et al., 1999; Fernández et al., 2017). Recently, the application of advanced control strategies has been demonstrated to improve both the control of pH and the removal of oxygen, maximizing the efficiency of the system, and ultimately biomass productivity, while simultaneously reducing the biomass production cost (Fernández et al., 2010; Pawlowski et al., 2014).

Concerning temperature, in tubular photobioreactors it is possible to use heat exchanger systems to control the temperature of the cultures, especially to avoid overheating in outdoor conditions, because the size of these systems is much lower than of raceway reactors. Two systems are typically used: water spraying and internal heat exchangers (Richmond et al., 1993; Ippoliti et al., 2016b). The utilization of water-spray systems is suitable for locations with low humidity, and in general the utilization of heat exchangers is recommended. By recirculating water from large reservoirs, it is possible to keep the temperature of the culture close to the ambient temperature, thus a maximal of 10°C upper than the ambient temperature being measured.

6.3.3 Other designs

Other designs of photobioreactors, apart from raceway and tubular photobioreactors, have been proposed, although they are not used at large scale.

6.3.3.1 Flat panels

Flat panel reactors consist of two parallel panels between which the microalgal suspension is aerated (Carvalho et al., 2006) (Fig. 6.12). The height of the panel is normally lower than 1.8 m, while its length can be in theory unlimited, although designs below 20 m have been recommended. The key factor in the design of this type of reactor is the width of the panel; narrow panels are preferred, ranging from 0.03 to 0.10 m, in order to maximize the average irradiance inside the culture and the biomass productivity, with values greater than 2 g/L day being reported (Tredici et al., 1991). Because the size of a single unit is in the range of 1–3 m^3, industrial facilities are also built by installing a large number of these reactors, the maximum size of facilities using this technology already published is 1 ha (Tredici et al., 2015).

Fig. 6.12
Image of a flat panel reactor located at IBVF (Sevilla, Spain) and thin-layer reactor located at IFAPA research center (Almeria, Spain).

The advantages of flat panels include the possibility to control the culture conditions because they are almost closed photobioreactors, thus preventing contamination problems, and optimizing the irradiance on the reactor surface by distributing the solar radiation on a large surface per land unit, which is named the "light dilution effect" (Tredici et al., 2015).

Flat plate photobioreactors can be set up vertically or tilted to optimize solar energy capture. These reactors can be oriented toward the sun, permitting better efficiency in terms of energy absorbed from incidental sunlight (Carvalho et al., 2006). Panel orientation has a large effect on productivity, and at higher latitudes the difference between north-south and east-west orientation can be up to 50% (Slegers et al., 2011). A large diversity of microalgae has been produced in flat panel reactors, such as *Botryococcus braunii* and *Nannochloropsis*, with productivities ranging at 5–35 $g/m^2 \cdot day$ (Tredici et al., 2015; Bazaes et al., 2012; Ruiz et al., 2013). Higher biomass productivities are reported when reducing the width of the panel (Hu et al., 1996; Zou and Richmond, 1999). However, thin photobioreactors are by far more

expensive to construct, more difficult to clean, and more readily subjected to light inhibition and temperature fluctuation (Wang et al., 2012).

Engineering aspects of this type of reactors have been studied only recently (Sierra et al., 2008; Jacobi et al., 2010; Massart et al., 2014; Chen et al., 2016). In these reactors, mixing and mass transfer are simultaneously provided by bubbling air. In this case, the aeration rate must be reduced to minimize the energy consumption of the system, with values below 0.1 v/v/min being recommended. In these conditions, the flat plate behaves as a mixed-tank system (Jacobi et al., 2010). The power consumption by aeration on these systems (P_G/V_L) is a function of the density of the liquid (ρ_L), the gravitational acceleration (g), and the superficial gas velocity in the aerated zone (U_G) (Eq. 6.28), whereas the volumetric gas-liquid mass transfer coefficient potentially increases with the power supply (Eq. 6.29) (Sierra et al., 2008).

$$\frac{P_G}{V_L} = \rho_L g U_G \tag{6.28}$$

$$K_L a_L = 2.39 \times 10^{-4} \left(\frac{P_G}{V_L}\right) \tag{6.29}$$

According to these equations, the energy consumption in flat panel reactors ranges from 20 to 200 W/m^3, intermediate between that determined for raceway and tubular reactors (Sierra et al., 2008). Moreover, values of mass transfer coefficient up to 25 h^{-1} are achieved in these systems, with the entire volume of the reactor being suitable for mass transfer, so the mass transfer capacity on these reactors exceeds that required by the microalgae cells to control the pH/CO_2 and dissolved oxygen concentrations adequately (Sierra et al., 2008). Flat panel photobioreactors can be cooled by spraying water at the surface of the reactor (Tredici et al., 1991; Griffiths, 2013), although they can also be controlled by using internal heat exchangers (Sierra et al., 2008). Major problems related to this type of photobioreactor involve the existence of biofouling phenomena, biomass attaching to the wall of the reactor, then reducing the light availability inside the culture, and finally the culture collapsing. Biofouling and outdoor contamination risks could be overcome by using disposable materials to build the reactors, also bringing a substantial cost reduction. Thus, the "Green Wall Reactor" was patented (Rodolfi et al., 2009). The reactor design consists of a flexible transparent plastic bag contained between two rigid frames confining a relatively thin vertical panel.

6.3.4 Thin-layer reactors

Thin-layer reactors were developed at the Institute of Microbiology in Třeboň, Czech Republic (Šetlík et al., 1970) (Fig. 6.12). The design of these reactors is analogous to tubular photobioreactors because it can be also divided into three major parts: (i) the surface or loop where photosynthesis is performed; (ii) the retention tank (degasser) where the culture is managed; and (iii) the pump used to recirculate the culture from the tank to the surface.

The surface is slightly tilted (0.1%–1.0%) to facilitate the flow of the culture by gravity at velocities ranging from 0.1 to 1.0 m/s. The major advantage of this type of reactor is being able to minimize the water depth, including below 1.0 cm, thus maximizing the light availability to which the cells are exposed, and therefore biomass productivity. A crucial advantage of thin-layer reactors is the efficient mixing, inducing fast light-dark (L/D) cycling of cells in a "short" light-path. In thin-layer reactors, the cells are exposed to light every few milliseconds, including at liquid velocities of 0.5 m/s and high biomass concentrations up to 20 g/L (Masojídek et al., 2011). Similarly to tubular photobioreactors, the length of the illuminated surface is determined by the maximal dissolved oxygen concentration admissible (<200 %Sat.). Because in these reactors the light availability is much higher than in tubular photobioreactors, the oxygen production rate is also higher, thus the total length of the surface must be lower than the tube length in tubular photobioreactors; a maximal length lower than 100 m is recommended. In thin-layer reactors of 80 m length, the dissolved oxygen concentration along the channel increases so much due to photosynthesis, that values up to 250 %Sat. are measured at the end of the channel, this fact greatly reducing the performance of microalgae cells (Barceló-Villalobos et al., 2019b). High dissolved oxygen concentrations in this type of photobioreactor have already been reported (Morales-Amaral et al., 2015).

Power consumption in thin-layer reactors is related to the different height between the tank and the initial of the surface, being equal to 9.8 J/kg of culture recirculated and meter of height difference. Typical values reported ranged at 1–10 W/m^2, equivalent to 40–400 W/m^3, thus being larger than for raceway reactors but lower than those corresponding to tubular photobioreactors. The volume of the tank from which the culture is recirculated must be enough to store the culture during the night period but also to accumulate the water coming from storms and rainfall if taking place. This tank must be also designed to absorb CO_2 efficiently when provided for pH control and carbon supply. In general, the design of this type of reactors has still not been optimized, thus large problems of inadequate pH control, low efficiency CO_2 transfer, and including excess of temperature have been observed (Barceló-Villalobos et al., 2019b). Despite these drawbacks, the biomass productivity already reported in this type of reactor is one of the highest when compared with the other types, and may still be increased twofold if the reactor is adequately designed/operated (Barceló-Villalobos et al., 2019b). It is important to mention that in this type of reactor, just as in raceway reactors, the major mechanism contributing to the cooling of the culture is water evaporation, which makes it very difficult to implement temperature control strategies.

Concerning the production capacity of thin-layer reactors, it has been reported that using this type of photobioreactor high biomass concentrations, up to 40–50 g/L, are achieved, which also enable high biomass productivities to be achieved, as much as 55 g/m^2 day in summer, even in temperate climate zones (Masojídek and Prášil, 2010). Values up to 42 $g/m^2 \cdot$day have been reported when producing *Scenedesmus* in this type of reactor and using centrate as a nutrient source (Morales-Amaral et al., 2015). It has previously been reported that during summer time

in Trebon (Czech Republic), the average productivity achieved in this type of reactor was in the range of 23–38 g/m^2 day with *Chlorella* sp., using a 6–7 mm culture depth (Doucha and Lívanský, 2006; Doucha et al., 2009). In spite of this high productivity, only some pilot scale units have been operated: up to 100 m^2 areal surface in Trebon (Czech Republic) and Almeria (Spain), up to 900 m^2 in Rupite (Bulgaria), and up to 1.5 m^2 at Pataias (Portugal). The reason that this technology has still not been widely used is because its design and operation are not yet completely optimized.

6.4 Future trends

Future trends in the field of photobioreactors for the production of microalgae can be summarized in three major aspects: (i) development of technologies improving the performance of the current ones; (ii) scale-up of the actual production systems; and (iii) industrialization of microalgae production. New technologies allowing optimization of the control of culture conditions and for them to be fitted to the requirements of the microalgae cells are required, also allowing the biomass production cost to be reduced. The scale-up of the production processes is related to the definition of scale-up criteria and development of suitable machinery for the robust operation at larger scales. In this case, the implementation of scale-up techniques and machinery from other industries such as wastewater treatment is recommended. The need for control of contamination and robustness of the cultures is vital for this task, especially when using open systems. On the other hand, the successful operation of closed reactors still requires a solution to the problem of biofouling that periodically collapse the cultures. Finally, a cost-competitive industrialization requires the methods and facilities for microalgae production to conform to industrial standards, including continuous operation, control strategies, accomplishment of good manufacture practices, etc. Although microalgae production is increasing worldwide, only advances in these areas will allow a relevant scale to be achieved and enable a valuable industry based on microalgae to be established.

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CHAPTER 7

Wastewater treatment based in microalgae

Alfredo de Jesús Martínez-Roldán[a,b], Rosa Olivia Cañizares-Villanueva[c]

[a]CONACYT-TecNM/IT Durango, Departamento de Ingenierías Química y Bioquímica, Maestría en Sistemas Ambientales, TecNM/IT Durango, Durango, Mexico [b]Maestría en Sistemas Ambientales, TecNM/IT Durango, Durango, Mexico [c]Laboratorio de Biotecnología de Microalgas, Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de Mexico, Mexico

Chapter outline

7.1 Introduction 165
7.2 Wastewater treatment 166
7.3 Microalgae for WW treatment 168
7.3.1 Removal of nitrogen and phosphorus 169
7.3.2 Heavy metal removal 172
7.3.3 Elimination of colorants 176
7.3.4 Elimination of emerging pollutants 177
7.4 Conclusion 178
References 179

7.1 Introduction

Life on Earth is dependent on water, which is a vital part of its composition and environment. Even humans are dependent on the utility of water to survive (Pohorille and Pratt, 2012; Pohorille and Andew, 2018). The availability of hydric resources depends on many factors, such as population growth and production of commodities and services; all these factors cause a gradual diminution of the volume available to be used (Lenzen et al., 2013). In addition to its vital usage, water loses a portion of its quality when used, becoming wastewater (WW). The FAO defines WW as "water that loses its characteristics before its employment." Some authors have estimated the volume of generated WW as higher than 4000 km^3 $year^{-1}$ (Schwarzenbach et al., 2006).

The principal problem caused by WW is not its generation itself, but the fact that a huge portion of WW is normally discharged without treatment. This can negatively impact aquatic ecosystems, particularly primary producers (aquatic plants, algae, and cyanobacteria).

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Moreover, it is important to highlight the huge diversity of contaminants and their very diverse effects. Although nitrogen (N) and phosphorus (P) primarily have a physiological impact on nutrient fluxes, other heavy metals tend to accumulate persistently inside the cell, and this constitutes its access to the trophic webs. Once in the trophic levels, a biomagnification phenomenon can occur and toxic concentrations reach higher levels (Kelly et al., 2007; Rajendran et al., 2003).

In environmental sciences, an aquatic pollutant is described as a substance that does not pertain to the ecosystem and can damage its members or misbalance the relation between the members of the ecosystem; it can be an organic or inorganic substance or even a biological particle (Cañizares-Villanueva et al., 2013; Laws, 2017). The global flux of pollutants in the world can reach millions of tons, and a huge portion ends up primarily in rivers, lagoons, and lakes. Specifically, N and P contamination are caused by the excessive fertilization of different kind of crops, pollution with heavy metals is caused by the industrial sector, and colorants are normally released by the textile industry. Hence, it is important to include the "classical pollutants" and so-called "emerging contaminants." Emerging contaminants are becoming important because they are very diverse, present in almost all WW, and many of them affect human and animal health (Geissen et al., 2015; Schwarzenbach et al., 2006).

7.2 Wastewater treatment

WW treatment includes the removal of different classes of components in the residual previous to its discharge into natural water bodies, such as river, lakes, or lagoons (Metcalf and Eddy, Inc., et al., 2002). The removal of the polluting substances can be achieved by physical, chemical, or biological strategies and is dependent on the complexity of WW. Actually, the diversity of contaminants is so vast that normally a combination of different process are employed in wastewater treatment plants (WWTPs) (Geissen et al., 2015; Stoddard et al., 2003).

Since 1970, a huge number of WWTPs have been installed in many countries worldwide. These WWTPs perform a succession of different processes with diverse objectives and are normally classified as preliminary, primary, secondary, and tertiary treatment; recently a quaternary process has also been included. This quaternary stage is included for the elimination of specific pollutants that cannot be eliminated by the aerobic and anaerobic processes normally used in WWTPs (Gupta et al., 2016; Stoddard et al., 2003; van Puijenbroek et al., 2019).

The preliminary stage normally eliminates materials of huge size such as branches, gravels, garbage, or even fats and oils. This stage is useful to promote the good operation of the subsequent stages of the treatment (Tchobanoglous et al., 2003). In the primary treatment, sedimentation of suspended particles, such as sands or small gravels (with diameters of

<0.5 mm), occurs. For this, normally, screens with different pore diameters (if the size is too large) or sedimentation tanks with a laminar flow regime for sands and gravels (Comas et al., 2004; Metcalf and Eddy, Inc., et al., 2002) are used.

After sedimentation/separation comes the secondary treatment with the objective of eliminating organic matter in the liquid (regardless of whether it is suspended or soluble), and normally this is possible by the use of microorganisms (Comas et al., 2004; Metcalf and Eddy, Inc., et al., 2002). Organic matter can be removed from WW by different strategies, and the selection of the processes depends on the characteristics of WW. Some of the parameters that are important when a technology is to be selected are organic matter content, pH, temperature, flux, N and P concentration, and N speciation. However, a more commonly employed strategy is activated-sludge. This strategy eliminates organic matter by aerobic organisms but at a faster rate because of the recirculation of biomass from the secondary sedimentation process to the bioreactor. If the organic matter concentration is too high, it is convenient to employ an anaerobic processes instead of the activated-sludge process (Cañizares-Villanueva et al., 1994; Comas et al., 2004; Metcalf and Eddy, Inc., et al., 2002; van Puijenbroek et al., 2019).

The tertiary treatment consists of the removal of biomass from WW normally by sedimentation or another strategy that permits the efficient separation of water and sludge. The quaternary stage has recently been added with the objective to remove specific pollutants that are normally not eliminated in previous phases. This process normally includes the elimination of recalcitrant substances, heavy metals, emerging pollutants, or even N and P (Franco Martínez et al., 2017; Metcalf and Eddy, Inc., et al., 2002; Olguin, 2003).

The complexity of the treatment process depends on two main factors: (1) its provenance, depending on the type of water use, and the type and concentration of the pollutants; and (2) later uses, because certain employment of the treated water (such as irrigation) does not require complete removal of the pollutants (Molinos-Senante et al., 2015; Muga and Mihelcic, 2008).

If WW has high levels of organic matter, it is subjected to anaerobic digestion, and normally the effluent has high amounts of N and P because this treated water promotes the eutrophication of the receptor water bodies; this can also occur in aerobic processes if its operation is suboptimal (Dodds and Smith, 2016; van Beusekom, 2018). Owing to the risk of eutrophication, the quaternary stage is performed, and if the objective is the removal of N and P, microalgae can be used because they are very efficient in the elimination of N and P. Other advantages of the use of microalgae are the increment of oxygen concentration in the effluent and the consequent reduction in negative impacts on the receptor water bodies (Martínez-Roldán and Cañizares-Villanueva, 2019; Martínez-Roldán and Ibarra-Berumen, 2019; Olguin, 2003).

7.3 Microalgae for WW treatment

The potential of microalgae for WW treatment was proposed in 1950. In 1957, the efficient removal of N and P and the beneficial role of microalgae in the stabilization ponds were proved (Oswald and Golueke, 1960). In the United States, Oswald et al. (1957) developed different design criteria for the construction of oxidation ponds, and described the interaction between microalgae and aerobic bacteria in the water column. In this relationship between bacteria and microalgae, microalgae produce O_2, whereas the bacteria utilize the O_2 produced for the mineralization of the resting organic matter while liberating CO_2 by a respiration process, which is used by microalgae for their photosynthetic process (Craggs et al., 2013; Gonçalves et al., 2017; Oswald et al., 1957; Wang et al., 2017).

Previously, microalgae were normally used in the facultative process. This process is performed in an unmixed and low-depth lagoon. Nevertheless, if its operation is suboptimal, an anaerobic portion can appear at the bottom, causing a loss in efficiency and the generation of bad odor (Almasi et al., 2015; Wang et al., 2017).

In the 1960s, Professor Oswald and his research group at the University of California, Berkeley, developed a WW treatment process with higher efficiencies compared with that by facultative lagoons; this process was called the high-rate algal pond (HRAP). The HRAP consisted of a shallow lagoon (<50 cm) mixed by a paddle wheel. The diminution in the depth and addition of mixing increased the efficiency of biomass production dramatically and, consequently, the rates of N and P removal were higher (Craggs et al., 2013; Oswald et al., 1957; Oswald and Golueke, 1960). In the following years, many HRAPs were installed in the United States, particularly in small communities (Craggs et al., 2013; Park and Craggs, 2010).

Nevertheless, the composition and level of pollution of WW changed drastically. Many substances that normally did not reach measurable values and were considered as trace are present in alarming concentrations today. Moreover, many new pollutants have been found, of which many are recalcitrant and cannot be eliminated by classical processes; therefore, the development of new processes using different microorganisms, such as microalgae or even fungi, is necessary (Escapa et al., 2015; Geissen et al., 2015; Gonçalves et al., 2017; Matamoros et al., 2015).

The current literature proves the capability of microalgal cultures for the elimination of very diverse pollutants, and some of the most important pollutants are as follows:

- N and P: These contaminants are the most important because they are discharged into water bodies in huge quantities, causing a huge imbalance in aquatic environments. N normally exists in inorganic forms such as nitrate of ammonium, whereas orthophosphate is the most common speciation of P. The removal of these substances in WWTPs is normally not very efficient, and their concentrations in the effluents are sufficient to cause eutrophication (Abdel-Raouf et al., 2012; Franco Martínez et al., 2017; Olguin, 2003; Perez et al., 2015).

- Heavy metals: Contamination by heavy metals is vital due to the fact that many economies in the world are practicing extraction and refinement of different metals. This increases the volume of material-contaminated growth every year. The main problem of pollution by heavy metals is the fact that they accumulate in the tissue and many of them are toxic or even carcinogenic (Das et al., 2014; Hengstler et al., 2003; Mulligan et al., 2001). Some of the most important heavy metals are zinc, copper, lead (Perales-Vela et al., 2007), mercury (Blue et al., 2008), nickel, chromium (Peña-Castro et al., 2004; Jácome-Pilco et al., 2009), and cadmium (Pellón et al., 2008; Cañizares-Villanueva, 2000; Cañizares-Villanueva et al., 2017; Jácome-Pilco et al., 2009; Peña-Castro et al., 2004; Perales-Vela et al., 2006, 2007).
- Colorants: These kind of compounds are normally produced by the textile industry as well as by the cosmetics and food industries (El-Sheekh et al., 2009). A majority of the colorants include aromatic rings in their molecular structure difficulting its removal and degradation. Nevertheless, in some cases it is possible to perform biotransformation, but some molecules are transformed into species that result in more toxic compounds than the original compounds. This is very common in the derivatives of aniline (Chung, 2016). The presence of colorants in aquatic ecosystems is toxic to fish and inhibits the photosynthetic processes in aquatic plants and microalgae, causing imbalances in the flux of nutrients (El-Sheekh et al., 2009).
- Emerging pollutants: These are a group of substances that were not considered previously because their concentrations in WW were very low and in some cases undetectable. However, in the past few years, some authors have demonstrated their adverse effects even at low concentrations. Moreover, some of the pollutants are today present in higher concentrations and are now considered in toxicological studies (Gavrilescu et al., 2015; Geissen et al., 2015). Emerging pollutants are molecules produced in industries such as pharmaceutical (used for human beings and livestock) and chemicals, and include compounds such as antibiotics, antipyretics, hormones, antifungal, plasticizers, and surfactants (Bolong et al., 2009; Gavrilescu et al., 2015). Another problem is the fact that currently there are no regulations about their concentration in treated water discharges because their presence reached quantifiable values only recently (Bolong et al., 2009). The problem is critical because these pollutants cannot be eliminated by classic processes of WW treatment, such as activated-sludge, anaerobic digestion, or oxidation ponds, and they are discharged into water bodies (Bolong et al., 2009; Bueno et al., 2012).

7.3.1 Removal of nitrogen and phosphorus

The use of microalgae for the elimination of N and P has been practiced for almost 70 years (Oswald et al., 1957; Oswald and Golueke, 1960). However, the study of metabolic processes and their relation with the efficiency of nutrient elimination is currently valid. This is because

the sources of WW that contain quantities of N and P are varied and their elimination in the processes for the removal of organic matter is not very efficient. Moreover, the effluents of WWTPs normally contain important amounts of these nutrients (Abdel-Raouf et al., 2012; Franco Martínez et al., 2017; Olguin, 2003).

The main sources of N and P are agriculture, the food industry, cattle raising, and municipal drainage (Grizzetti et al., 2013; Leip et al., 2015). Analyzing the input of domestic WW is important because, regardless of the process employed for its treatment (facultative lagoons, activated-sludge, or anaerobic digestion), important amounts of both pollutants are present as a consequence of organic matter degradation, and its removal is thus necessary by the addition of an extra stage in the treatment train (Cooper et al., 1994).

For the removal for N and P, it is important to highlight the use of microalgal cultures because this system affords many advantages compared to the processes using bacteria (Cañizares-Villanueva et al., 1994, 1995; Cañizares et al., 1994; Franco Martínez et al., 2017; González et al., 1997; Martínez-Roldán and Cañizares-Villanueva, 2015, 2017; Martínez-Roldán and Ibarra-Berumen, 2019; Peñaranda et al., 2012). Some of the advantages are as follows:

1. High removal efficiencies: Both contaminants can be completely removed from WW (Franco Martínez et al., 2017; Martínez-Roldán, 2008) because these are essential components of the biomass and are required for very diverse physiological processes, such as protein and nucleic acid production, energy transfer inside the cell, and cellular division. In the case of P, it is consumed in an accelerated manner irrespective of the requirement and tends to be accumulated in the cytosol as inclusions such as polyphosphate grains (Jácome-Pilco et al., 2009; Peña-Castro et al., 2004; Perales-Vela et al., 2006, 2007). N can reach volumetric consumption rates of close to $40\,mg\,N\,L^{-1}\,d^{-1}$ (Franco Martínez et al., 2017; Klok et al., 2013; Martínez-Roldán, 2008), and for P, the rate can be even higher than $4\,mg\,P\,L^{-1}\,d^{-1}$ (Franco Martínez et al., 2017; González et al., 1997; Martínez-Roldán, 2008).
2. Lower operation costs: The elimination of N and P by microalgae does not require an organic carbon source (Cai et al., 2013b).
3. No sludge generation: This is important in comparison with physicochemical processes, such as coagulation or flocculation, wherein high amounts of precipitates can be produced (Aguilar et al., 2002; Xie et al., 2005). In the process with microalgae, the biomass produced can be used for different purposes (Martínez-Roldán and Ibarra-Berumen, 2019; Rawat et al., 2011; Zhu, 2015).
4. Obtaining high-value products: If the process of N and P elimination is developed in a biorefinery concept, it is possible to produce high-value products such as biomass, fatty acids, biodiesel, unicellular protein, pigments, and polyunsaturated fatty acids, and these products can favor financial feasibility (Abdel-Raouf et al., 2012; Borowitzka, 2013; Martínez-Roldán and Ibarra-Berumen, 2019; Rawat et al., 2011).

Many studies have demonstrated the capability of different microalgal species (monoalgal or axenic culture) for the removal of N and P from culturing media, synthetic effluents, or even real WW. Nevertheless, the experiments were usually performed in laboratory conditions (Franco Martínez et al., 2017; Gonçalves et al., 2017; Jácome-Pilco et al., 2009; Martínez-Roldán, 2008; Peña-Castro et al., 2004). However, in the field, the use of lagoons, open ponds, or pivots is frequent, and in these kinds of reactors the risk of contamination is very high. This obligates the use of microalgae with high growth rate or specific growing conditions to reduce the probability of contamination (Cai et al., 2013a, b; Quijano et al., 2017; Torzillo et al., 2010).

Domestic effluent normally has a content of organic matter (N and P) that does not inhibit the growth of microalgae; therefore, treatment using this kind of microorganism is very efficient (Franco Martínez et al., 2016, 2017). Many species are proposed for the treatment of WW, and Table 7.1 shows some examples. Good results are attributed to ammonia (NH_4^+) level and also

Table 7.1: WW treatment by microalgae.

Effluent	Specie	Removal/efficiencies	Reference
Municipal	Microbial consortia (algae/bacteria) isolated from WWTP	DQO and N > 85%, P > 65%	Su et al. (2011)
Secondary piggery effluent	*Phormidium* sp.	100% for N and urea, P ~ 30%	Cañizares-Villanueva et al. (1995)
Aeriated piggery effluent	*Spirulina maxima* immobilized in K-carrageenan	70% for N-NH_3, and 50% for P	Cañizares et al. (1994)
Anaerobically treated piggery WW diluted at 25%	*Phormidium* sp.	90% for P and N-NO_3, and 50% for N-NH_3	Cañizares-Villanueva et al. (1994)
Piggery WW	*Spirulina maxima*	40% for N-NH_3, and 90% for P	Cañizares-Villanueva and Domínguez (1993)
Textile industry WW	*Chlorella vulgaris*	10% for the color and 30% for COD, N-NH_3, and P	Lim et al. (2010)
Anaerobically treated piggery WW	*Chlorella* sp.	83% for N, and 75% for P	Wang et al. (2010)
Agribusiness WW	*Chlorella vulgaris*	95% for N, and 55% for P	González et al. (1997)
Municipal WW	*S. obliquus*	~100% for N and P	Ruiz-Marin et al. (2010)
Municipal WW	*C. vulgaris*	~100% for N and P	Franco Martínez et al. (2017)
Municipal WW	Mixed microalgae and bacteria culture	~100% for N and P	Delgadillo-Mirquez et al. (2016)
Anaerobically digested wastewater	*Scenedesmus dimorphus*, *Scenedesmus quadricauda*, *Chlorella sorokiniana*, *Chlorella vulgaris ESP-6*	70% for N-NH_3, and P-PO_4	Chen et al. (2018)
Anaerobic digestion effluent generated in biogas production	*Chlorella vulgaris*	85% for N and P	Xie et al. (2018)

because organic matter does not reach inhibitory values (Cai et al., 2013b; Franco Martínez et al., 2016; Martínez-Roldán, 2008). Higher volumes of water contaminated with N and P are produced in cattle raising, and this contaminated water is normally discharged into rivers (Cai et al., 2013b; Gonçalves et al., 2017). Liquid effluents of the cattle raising industry are very difficult to treat owing to their concentrations of organic matter, suspended solids, or even the presence of drugs, such as antibiotics or hormones (Chen et al., 2017; Cheng et al., 2018). Moreover, the amounts of N and P cause problems because N is primarily present as ammonia and can reach values from 60 to 1900 $mg\,L^{-1}$ (Cai et al., 2013a, b), whereas P can reach concentrations from 10 to 1200 $mg\,L^{-1}$, primarily as orthophosphate (Cai et al., 2013a, b; Cañizares-Villanueva et al., 1993, 1995). The toxicity of this type of WW for microalgal cultures is very high because the predominant species of N is ammonium, but if the pH reaches values greater than 8.5, the predominant species is ammonia, and this molecule is very toxic for microalgae because it can cross the cell membrane and unbalance the amount of ions inside the cell (Cai et al., 2013b; Cañizares-Villanueva et al., 1993, 1995; Franco Martínez et al., 2016, 2017).

Nevertheless, the toxicity of the ammonia-ammonium system can be circumvented by different strategies; the simplest ones are culturing strategy and immobilization.

1. The objectives of immobilization are as follows: firstly to create a barrier between the microorganisms and the residual/pollutant, which causes the concentration of the contaminant that the microorganism "sees" to be lower than the real one because it depends of the diffusion inside the support, and secondly to simplify biomass separation from the treated effluent (de-Bashan and Bashan, 2010). Different matrices have been assayed, leading to very diverse results, highlighting the employment of matrices such as κ-carrageenan (Cañizares et al., 1994), alginate (Cañizares-Villanueva et al., 2001), polyurethane foam, silica gel, and agar (de-Bashan and Bashan, 2010).
2. Culturing strategy: Different operation modes can be used in the culture, such as cyclic batch, feed-batch, or even continuous culture (Martínez-Roldán, 2008; Park et al., 2011). The objective of these methods is to generate a dilution of the WW to favor the diminution of pollutant concentration and, consequently, its toxicity (Cañizares-Villanueva et al., 1993; Martínez-Roldán, 2008).

Although satisfactory results have been obtained by the employment of these strategies, a bioprocess must be designed according to the specifications and characteristics of the WW to be treated.

7.3.2 Heavy metal removal

Another environmental problem that is becoming more important with each passing year is the pollution of aquatic environments with heavy metals because these metals are recalcitrant, persistent, and highly toxic, and also because some of them exhibit

Table 7.2: Effect of heavy metals on the human body.

Heavy metal	Toxic effect
Arsenic	Skin irritation, cancer, and vascular disease
Cadmium	Kidney damage and cancer
Chromium	Headache, diarrhea, nausea, and cancer
Copper	Insomnia, hepatic damage, and Wilson's disease
Nickel	Skin irritation, asthma, cough, and cancer
Zinc	Depression and neurological damage
Lead	Brain damage in fetus and kidney, circulatory, and nervous damage
Mercury	Rheumatoid arthritis, damages in the circulatory and nervous systems, and kidney damage

Modified from Kurniawan, T.A., Chan, G.Y., Lo, W.H., Babel, S., 2006. Comparisons of low-cost adsorbents for treating wastewaters laden with heavy metals. Sci. Total Environ. 366, 409–426; Barakat, M.A., 2011. New trends in removing heavy metals from industrial wastewater. Arab. J. Chem. 4, 361–377.

carcinogenicity (Fu and Wang, 2011). Furthermore, for humans, some metals have a specific effect on some systems or organs. Table 7.2 describes the effects of the most common metals. The dynamics of metals in the environment are very different compared with those of other pollutants because they cannot be degraded and microorganisms can only modify their oxidation state (Das, 2014; Muñoz and Guieysse, 2006). The impossibility of elimination leads to a tendency to accumulate in the tissue of organisms and generates a biomagnification phenomenon in the food chain, culminating in huge values in the higher and superior levels of the food chain (Kelly et al., 2007).

Pollution with heavy metals can be caused by different industries or can even be present in domestic effluent (from drainage pipes). Nevertheless, higher volumes and concentrations are generated by plating of metals, mining, tanning, and battery and pesticide production (Das, 2014; Fu and Wang, 2011). Traditionally, heavy metals were removed from WW by chemical processes such as precipitation, coagulation, or electrocoagulation, but these processes produce huge amounts of sludge with a high metal concentration, and must therefore be disposed of as dangerous waste (Das et al., 2014). Another disadvantage of the chemical processes is the fact that its efficiency depends on the metal concentration, which is very low when the concentration of the metal is below 1–2 ppm, and at these concentrations many heavy metals are still toxic to aquatic environments (Barakat, 2011).

Industries that produce effluents polluted with heavy metals normally discharge high volumes, making the treatment of effluents difficult; thus, favoring the development of new technologies and using biomass is an option owing to low obtaining costs and efficiency in the removal of heavy metals reaching values close to 100% (Cañizares-Villanueva et al., 2017; Dwivedi, 2012). Furthermore, it is important to highlight the fact that, in some cases, the process is reversible and recuperation of the metals is very simple by the modification of operational conditions such as pH (Fu and Wang, 2011). Some of the materials employed, such as sorbents for the removal of heavy metals, are metallic slags, clays, sand, zeolites, activated carbon, and biomass from different sources (Barakat, 2011; Naja and Volesky, 2011).

Biomass is an effective sorbent; it is renewable and cheap, and can be obtained from plants or agro-industrial residues, seeds, fruit peels, or even biomass from fungi, bacteria, or microalgae (Cañizares-Villanueva et al., 2017; Naja and Volesky, 2011; Wang and Chen, 2009).

The employment of microalgal biomass as a sorbent for the removal of metallic ions from WW is becoming important due to the following advantages:

- It is easy to obtain and low cost (Monteiro et al., 2011, 2012).
- It has a high specific surface area compared with other sorbents (Cañizares-Villanueva et al., 2017; Monteiro et al., 2012; Mudhoo et al., 2012).
- The surface contains many well-distributed active sites that easily trap the metallic ions (Monteiro et al., 2011, 2012).
- The active sites of the cell wall composed of functional groups, such as hydroxyl, thiol, carboxyl, and carbonyl, can be activated very easily by the modification of pH (Cañizares-Villanueva, 2000; Cañizares-Villanueva et al., 2017).
- The yield of metal removed by unit of biomass is very high (Cañizares-Villanueva, 2000; Monteiro et al., 2011, 2012).

Nevertheless, one of the advantages that sorbent presents is the fast income of metallic ions into the food chain as the biomass rapidly integrates the metals (Monteiro et al., 2011, 2012). The use of microalgal biomass for the removal of heavy metals is important when the concentration is low ($<10\,mg\,L^{-1}$), because at these concentrations the physicochemical processes are either inactive or not so efficient (Cañizares-Villanueva et al., 2017; Monteiro et al., 2011, 2012). Biomass can be used in two strategies:

- Active biomass: In this case, the amount of metal that can be eliminated is higher than the amount removed with inactive biomass. The process occurs in two different stages: the first process is rapid (from 4 to 40 min) wherein the metallic ions are eliminated by an interaction between the positive charges of the metals and the negative charge of the functional groups in the cell wall (carboxyl, thiol, hydroxyl, etc.). In the second process, the metal is incorporated to the cell and undergoes gradual bioaccumulation, and this stage can take hours or even days (Cañizares-Villanueva, 2000; Cañizares-Villanueva et al., 2017). In the case of using active biomass, many metabolic strategies can normally be developed to reduce the toxic effect of the metals; these strategies include the production of phytochelatins, metallothioneins, or even confinement of the metals inside some organelles, such as the vacuole and nucleus (Perales-Vela et al., 2006, 2007).
- Inactive biomass: In this process, the biomass is not viable because it is inactivated by lyophilization, autoclaved, and dried. Because of its inactivation, the biomass is adsorbent and its action is via the functional groups present in the cell wall that interact with the metallic ions. The main advantage of using inactive biomass is the fact that it does not require nutrients or controlling culturing conditions. The recuperation for the adsorbed

metal is very easy and washing with an acidic pH is sufficient to remove the ions in the biomass (Cañizares-Villanueva, 2000; Cañizares-Villanueva et al., 2017; Monteiro et al., 2011, 2012).

The bioadsorption process (by interactions between charges) is fast and only a few minutes are required. In active biomass, the metal removed by this process can be close to 80% of the total, and the rest is eliminated by bioabsorption. Bioabsorption can take hours or even days, and the removal is close to 20% (Cañizares-Villanueva, 2000; Monteiro et al., 2011, 2012).

The main advantage of the employment of microalgae to remove heavy metals is the fact that some species exhibit a special affinity for specific metals (Table 7.3). This permits the development of specific processes to remove specific metals such as gold and silver and rare elements such as cesium, platinum, palladium, or uranium (Das, 2010; Varbanov et al., 2017).

Table 7.3: Removal of heavy metals by microalgal biomass or cultures (de-Bashan and Bashan, 2010; Cañizares-Villanueva et al., 2017; Suresh Kumar et al., 2015).

Metal	Genus/microorganisms	Condition	Metal removed
Au	*Chlorella*	Immobilized in alginate	~90%
	Tetradesmus	01 g L^{-1} of biomass and 5	100%[a]
Cd	*Chlorella*	Immobilized	66% 57/7.7 mg g^{-1}
	Scenedesmus	Immobilized	73% 69/7–574 mg g^{-1}
	Spirulina	Immobilized on silica gel	12–45 mg g^{-1}
	Porphyridium	Dead cells	7.5 mg g^{-1}
	Chaetoceros	Free live cells	1055.3 mg g^{-1}
Co	*Oscillatoria*	Dead cells	15.3 mg g^{-1}
	Spirogyra	Dead cells	12.8 mg g^{-1}
	Spirulina	Dead cells	0.01 mg g^{-1}
Cr	*Scenedesmus*	Immobilized	31%–36%
	Chlorella	Immobilized	34%–48%/14–41 mg g^{-1}
	Anacytis	Continuous culture	65%–85%
	Spirulina	Live and dead cells	304 and 167 mg g^{-1}
	Chlamydomonas	Dead cells	18–25.6 mg g^{-1}
Cs	*Chlorella*	Immobilized	~70%
Cu	*Chlorella*	Live cells	2–220 mg g^{-1}
	Anabaena	Live cells	12.6 mg g^{-1}
	Ceratium	Live and dead cells	2.3 y 5.7 mg g^{-1}
	Chlorella	Dead cells	23–108 mg g^{-1}
	Scenedesmus	Dead cells	2.8 y 20 mg g^{-1}
Hg	*Calothrix*	Dead cells	19 mg g^{-1}
	Chamydomonas	Dead cells	72.2 mg g^{-1}
	Chlorella	Dead cells	16–18
	Scenedesmus	Dead cells	9–20 mg g^{-1}
Ni	*Chlorella*	Live cells	0.64–59.3 mg g^{-1}
	Chlorella	Dead cells	12.1–42 mg g^{-1}
	Spirulina	Live cells	1378 mg g^{-1}

Continued

Table 7.3: Removal of heavy metals by microalgal biomass or cultures—cont'd

Metal	Genus/microorganisms	Condition	Metal removed
Pb	*Arthrospira platensis*	Dead cells	102.6 mg g^{-1}
	Chlamydomonas reinhardtii	Dead cells	96.3 mg g^{-1}
	Chlorella vulgaris	Dead cells	17.1–127 mg g^{-1}
	Scenedesmus acutus	Dead cells	90 mg g^{-1}
U	*Chlorella regularis*	Live and dead cell suspension	14.3 y 28.3 mg g^{-1}
Zn	*Chlorella*	Immobilized	78%–85%
	Scenedesmus	Immobilized	84%–91%
	Chlorella vulgaris	Dead cells	6.4–43.4 mg g^{-1}
	Planothidium lanceolatum	Live cells	118.6 mg g^{-1}
	Scenedesmus	Dead cells	5–22.3 mg g^{-1}
	Scenedesmus subspicatus	Live cells	72.1 mg g^{-1}

[a](Shen and Chirwa, 2018).

7.3.3 Elimination of colorants

The first artificial colorant was developed by William Henry Perkin in 1856, and since then, the diversity and quantity of colorants used in the textile industries have grown exponentially. An actual estimation of the number of different pigmenting molecules used to dye different fibers with different colors is close to 10,000 (Saratale et al., 2011).

The impact of the textile industries on the environment is huge, primarily because it is one of the most WW-generating industries. In addition, the colorants have different effects on the ecosystems such as: (1) diminution of penetration of light into the water bodies because the colorants act as a type of filter that diminishes the amount of light that enters the water; (2) reduction in oxygen concentration, caused by the diminution of light available for the photosynthetic processes performed by aquatic plants and microalgae; and (3) effect on biodiversity because some molecules of metabolites produced by biotransformation of the colorants can cause severe toxic effects and, in extreme conditions, can be fatal for the flora and fauna in the ecosystem (Bafana et al., 2011; Saratale et al., 2011).

Similar to the processes for other types of pollutants, there is a very diverse group of chemical and physiochemical processes to eliminate these kinds of substances. Some of the processes include the use of hydrogen peroxide, UV radiation, flocculation, or electrocoagulation, but these technologies only transfer the colorants from the liquid to the sludge produced or break the molecules to generate new molecules that can be more toxic than the original ones (Saratale et al., 2011; Singh and Arora, 2011). Biotechnological processes are presented as an alternative and have been proved by using (Joshi et al., 2008) fungi, yeast (Jadhav et al., 2007), and, more recently, microalgae and cyanobacteria (Jadhav et al., 2007; Joshi et al., 2008; Roy et al., 2018; Saratale et al., 2011). Table 7.4 shows some genera of microalgae used for the removal of colorants.

Table 7.4: Colorant removal by microalgae and cyanobacteria.

Microorganism	Colorant	Removal (%)	Time	Reference
Chlorella vulgaris	Reactive Black 5 Dye, 800 ppm	52.4	24 h	Aksu and Tezer (2005)
Cosmarium sp.	Malachite green, 10 ppm	74	3.5 h	Daneshvar et al. (2007)
Chlorella vulgaris	Congo red, 10 ppm	77	96 h	Hernández-Zamora et al. (2014)
Nostoc linckia	Reactive red 198, 100 ppm	93	–	Mona et al. (2011)
Spirogyra sp.	Café Directo 1, 15 ppm	40	300 h	Mohan et al. (2008)

A major portion of colorant removal is due to the interaction of the molecule with the reactive sites of the cell wall. This is an advantage because it permits the elimination of the colorant in a rapid manner, similar to that with metallic ions (da Rosa et al., 2018; Hernández-Zamora et al., 2014; Saratale et al., 2011). Nevertheless, this is also a disadvantage because the majority of the active sites in the cell wall are negative, so it is useful only for colorants with a positive effective charge. Although it has been recently proved that the activity of enzymes (such as peroxidase and azoreductase) can be involved in the degradation of colorants (El-Sheekh et al., 2009), these enzymes can only transform and mineralize the pollutants. This is important for molecules that can produce metabolites more toxic than the original compound, such as aniline and its derivatives (Saratale et al., 2011).

Regardless of the advantages of employing microalgae to eliminate colorants, it is important to highlight the fact that a concentration of 200 ppm or more can be toxic for these microorganisms; therefore, its use in highly contaminated WW can be possible only in the quaternary phase when the concentration is lower than that present in the original WW (Hernández-Zamora et al., 2014; Roy et al., 2018; Saratale et al., 2011).

7.3.4 Elimination of emerging pollutants

Emerging pollutants are a group of compounds that are normally eliminated in the different stages of a classical WWTP. However, it has been recently demonstrated that their concentration reaches alarming concentrations and tends to accumulate because currently there are no regulations on its discharge, and for some molecules no standard quantification method is available (Gavrilescu et al., 2015). Emerging pollutants include: industrial additives such as repellents, antifoaming and antiadhesion agents, polishers, and flame retarding; drugs such as antibiotics, analgesics, antiinflammatories, and hormones; pesticides (Bolong et al., 2009; Bueno et al., 2012; Kantiani et al., 2010); and other molecules (Küster and Adler, 2014; Kantiani et al., 2010).

The effects of these kinds of molecules are important because: (1) there is no regulation about a specific limit of concentration; (2) many of these molecules are generally distributed as drugs;

Table 7.5: Removal of emerging pollutants using microalgae.

Genus/Species	Substrance	Removal	Reference
Chlorella sorokiniana	Diclofenac	~30%	Escapa et al. (2016)
Chlorella vulgaris	Diclofenac	~22%	
Scenedesmus obliquus	Diclofenac	~80%	
Desmodesmus subspicatus	17α-Ethynylestradiol	68%	Maes et al. (2014)
Nannochloris	Ibuprofen	40%	Bai and Acharya (2017)
	Trimethoprim	10%	
	Ciprofloxacin	100%	
	Carbamazepine	20%	
	Triclosan	100%	
Chlorella pyrenoidosa	Cefradine	76%	Chen et al. (2015)
Chlamydomonas reinhardtii	Estradiol	100%	Hom-Diaz et al. (2015)
	17α-Ethynylestradiol	100%	
Navicula	Ibuprofen	60%	Ding et al. (2017)
Chlorella vulgaris	Levofloxacin	10%–92%	Xiong et al. (2017)

(3) their nonpunctual generation; (4) the majority are not removed in the WWTP; and (5) they affect the primary producers of the aquatic ecosystems (aquatic plants and microalgae) as well as superior organisms, such as fish (Bueno et al., 2012; Escapa et al., 2015; Geissen et al., 2015).

The removal of emerging contaminants is normally done by physicochemical processes such as photo-oxidation, ozonization, or oxidation via metallic catalysis, but currently it is performed only at a small scale (demonstrative basis) (Gomes et al., 2017; Keen et al., 2014). Information on the use of microalgae for the removal of this kind of pollutant is limited and normally focuses on the effect of photosynthesis, respiration, and other physiological processes, such as electronic transport (Chen and Guo, 2012; Hernández-Zamora et al., 2014). However, there are novel studies on the removal of emerging pollutants using microalgae, and part of this research is listed in Table 7.5. Owing to the growing importance of the effects of these contaminants on microalgae metabolism, it is very probable that the number of studies will increase exponentially.

7.4 Conclusion

The necessity of reusing WW has stimulated the development of different processes with the objective of removing specific contaminants. However, currently, WW characteristics and composition have caused the addition of extra stages to ensure good quality of the effluent. The inclusion of a quaternary treatment permits the employment of microalgae to improve the quality of the effluent, this because the removal of some of the remaining pollutants in the treated WW. The addition of a microalgae-based process permits the elimination of

contaminants, increases the oxygen concentration, and reduces the impact of the discharge of the water on natural water bodies.

Recently, the biorefinery concept was proposed for financial feasibility. In this concept, it is possible to obtain many products from the biomass, and its commercialization enables increased economic benefits. Moreover, it is possible to couple a WW treatment process with microalgae for the elimination of gaseous pollutants in exhaust gases from industrial chimneys. Some of the possible products include biomass (for animal feed), pigments, carbohydrates, and polysaccharides.

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CHAPTER 8

Carbon dioxide capture and utilization using microalgae

Patricia Ruiz-Ruiz, Adrián Estrada, Marcia Morales

Processes and Technology Department and Doctoral/Master Program in Natural Sciences and Engineering, Metropolitan Autonomous University Campus Cuajimalpa, Mexico City, Mexico

Chapter outline

8.1 Introduction 185
8.2 Photosynthesis and carbon dioxide fixation by microalgae 186
8.2.1 The light reactions of photosynthesis 186
8.2.2 CO_2 fixation: The Calvin-Benson cycle and the CO_2 concentrating mechanism 187
8.3 Cultivation systems for CO_2 capture using microalgae 189
8.3.1 CO_2 sources and physicochemical properties 189
8.3.2 Strains 190
8.3.3 Physicochemical aspects involved in CO_2 capture by microalgae 191
8.3.4 Culture systems for CO_2 capture with microalgae 192
8.3.5 Carbon balances for evaluation of microalgae-based CO_2 capture systems 194
8.4 Strategies for CO_2 capture improvement 197
8.4.1 Genetic engineering and metabolic modifications for the improvement of CO_2 capture 197
8.4.2 Photobioreactors: Enhancing design for CO_2 capture improvement 199
8.5 Remarks and conclusion 201
Acknowledgments 202
References 202

8.1 Introduction

Atmospheric CO_2 concentrations have reached levels unprecedented in at least 800,000 years, with a rise from 280 ppm since the Industrial Revolution to 414 ppm today. If the current trend continues, a further increase of 60% by 2100 is expected (Ciais et al., 2013; Kumar et al., 2018; CO_2 Earth, 2019). This situation has led to a 0.87°C rise in mean annual global temperatures (Hoegh-Guldberg et al., 2018). Depending on the actions taken to reduce CO_2 and other greenhouse gas emissions, the atmospheric CO_2 concentrations will probably reach between 550 and 1000 ppm by the end of the century (Dusenge et al., 2019), leading to further global mean temperature increases of 1–3.7°C (Ciais et al., 2013). Despite the Paris Agreement in

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2015 and the strong drive toward reducing CO_2 emissions by member countries, the British Petroleum (BP) Statistical Review of World Energy (British Petroleum, 2018) reports that CO_2 emissions increased from 29,714 million tons in 2009 to 33,444 million tons in 2017. The adverse environmental effects of the CO_2 rise include: increase in the frequency and duration of heat waves; warming of ocean and atmosphere; rise in sea level; and melting of Greenland and Antarctic ice sheets (House et al., 2011; Meinshausen et al., 2009). The abovementioned phenomena can cause harmful health effects for humans (Mistry et al., 2019).

The Intergovernmental Panel on Climate Change (IPCC) estimates that to limit global warming to 1.5°C above preindustrial levels, anthropogenic CO_2 emissions must be reduced by approximately 45% from 2010 levels by 2030 and reach net zero around 2050 (Zhou et al., 2018), otherwise the scenario is not optimistic for humanity.

In order to reduce the adverse effects of CO_2 emissions, it is necessary to develop sustainable and effective technologies for CO_2 capture and utilization. One approach is biological CO_2 capture by photosynthetic organisms present in terrestrial as well as aquatic environments. The use of microalgae can contribute to managing atmospheric carbon levels. Furthermore, the focus has shifted to carbon capture and utilization rather than just sequestration (Seth and Wangikar, 2015). These microorganisms use CO_2 to biosynthesize, accumulate, or secrete a wide range of primary and secondary metabolites, many of which are valuable substances with potential applications in the food, pharmaceutical, environmental, and cosmetic industries (Morales et al., 2015).

8.2 Photosynthesis and carbon dioxide fixation by microalgae

Photosynthesis provides, directly or indirectly, the energy supply and carbon input to all life-forms on Earth for their metabolism and growth. Through this process, microalgae convert light energy and inorganic compounds into organic matter. Oxygenic photosynthesis can be divided into two subprocesses: the light reactions, which comprise all steps needed to provide energy (ATP) and reducing equivalents (NADPH), and the dark reactions, where photosynthetic organisms consume ATP and NADPH to transform inorganic carbon into carbohydrates via the Calvin-Benson cycle (Wobbe and Remacle, 2014).

8.2.1 The light reactions of photosynthesis

The main role of the light reactions is to provide the biochemical reductant (NADPH) and the chemical energy (ATP) for the assimilation of inorganic carbon. The photosynthetic light reactions occur in the thylakoid membranes, which contain the major complexes carrying out the electron transport pathways to produce NADPH, ATP, oxygen, and protons (Wobbe and Remacle, 2014). Light energy is captured by the two pigment-protein complexes Photosystem II (PSII) and Photosystem I (PSI), which contain pigments bound to specific apoproteins and operate in series connected by a chain of electron carriers (Raven and Beardall, 2016).

Light-harvesting and energy transfer to the reaction centers are carried out by the light-harvesting complexes and can be defined as: (1) hydrophilic phycobiliproteins, which are found in cyanobacteria and red algae; and (2) hydrophobic pigment-protein complexes, such as light-harvesting complexes I and II (LHCI and LHCII), which contain chlorophyll *a*, chlorophyll *b*, and carotenoids, and are found in green algae and higher plants (Masojídek et al., 2013; Raven and Beardall, 2016). In all oxygenic photosynthetic organisms (except the cyanobacterium *Acaryochloris* and its relatives, which use chlorophyll *d*), the reaction centers use the red absorption peak of chlorophyll *a* at 680 nm in PSII and at 700 nm in PSI (Raven and Beardall, 2016).

Upon illumination, two electrons are extracted from water (releasing O_2) and transferred through a chain of electron carriers to produce one molecule of NADPH. Electrons resulting from the water splitting in PSII reduce $NADP^+$ as the terminal acceptor. To connect PSII with the final acceptor, plastoquinone (PQ), Cyt b_6f complex, plastocyanin (PC), PSI, and ferredoxin (Fd) are employed. The oxidation of PC and subsequent reduction of Fd comprise a light-driven reaction catalyzed by PSI, and the last step of NADPH formation requires the enzyme ferredoxin-$NADP^+$ reductase (FNR). The flow of protons from the lumen to the stroma through the ATP synthase leads to the generation of ATP from ADP and P_i (Mitchell and Moyle, 1967). The minimum photon cost of photosynthetic electron transport (photophosphorylation) is nine photons to oxidize two water molecules, reduction of two $NADP^+$, and the phosphorylation of three ADP (Raven and Beardall, 2016). The global reaction is expressed as follows:

$$2H_2O_{(\text{lumen})} + 2NADP^+{}_{(\text{stroma})} + 3ADP_{(\text{stroma})} + 3\text{phosphate}_{(\text{stroma})} + 9\,\text{photons}\,(400-700\,\text{nm}) \rightarrow O_{2(\text{lumen})} + 2NADPH_{(\text{stroma})} + 2H^+{}_{(\text{stroma})} + 3ATP_{(\text{stroma})} + 3H_2O_{(\text{stroma})}$$

Cyclic photosynthetic electron transport also exists in addition to the linear mode, which contributes to forming the proton gradient but does not result in NADPH accumulation. In cyclic electron transport, electrons are recycled from either reduced Fd or NADPH to PQ, and subsequently to the Cyt b_6f complex (Wobbe and Remacle, 2014).

8.2.2 CO_2 fixation: The Calvin-Benson cycle and the CO_2 concentrating mechanism

Fixation of CO_2 happens in the dark reactions using the NADPH and ATP produced in the light reactions of photosynthesis. The conversion of CO_2 to carbohydrates (or other compounds) occurs in four distinct phases in the Calvin-Benson cycle (Masojídek et al., 2013):

1. *Carboxylation phase*. CO_2 is added to the 5-carbon sugar, ribulose bisphosphate (Ribulose-bis-P), to form two molecules of phosphoglycerate (glycerate-P). This reaction is catalyzed by the enzyme ribulose bisphosphate carboxylase/oxygenase (RuBisCO).
2. *Reduction phase*. In order to convert phosphoglycerate to 3-carbon products (triose-P) the energy must be added in the form of ATP and NADPH in two steps: phosphorylation of phosphoglycerate to form diphosphoglycerate and ADP, and secondly, reduction of

diphosphoglycerate (glycerate-bis-P) to phosphoglyceraldehyde (glyceraldehyde-P) by NADPH.

3. *Regeneration phase*. Ribulose phosphate (ribulose-P) is regenerated for further CO_2 fixation in a series of reactions combining 3-, 4-, 5-, 6-, and 7-carbon sugar phosphates. Generation of 5-carbon sugars from 6-carbon and 3-carbon sugars is accomplished by the action of the transketolases and aldolases.
4. *Production phase*. Primary end products of photosynthesis are carbohydrates, but fatty acids, amino acids, and organic acids are also synthesized in photosynthetic CO_2 fixation. Several end products can be formed under different conditions, e.g., light intensities, CO_2 and O_2 concentrations, and nutrition.

Two molecules of NADPH and three molecules of ATP are required to fix one molecule of CO_2. The global reaction can be expressed as follows:

$$2\mathrm{NADPH}_{(\mathrm{stroma})} + 2\mathrm{H}^{+}{}_{(\mathrm{stroma})} + 3\mathrm{ATP}_{(\mathrm{stroma})} + 3\mathrm{H_2O}_{(\mathrm{stroma})} + 1\mathrm{CO_2}_{(\mathrm{stroma})} \rightarrow 1(\mathrm{CH_2O})_{(\mathrm{stroma})} + 1\mathrm{H_2O}_{(\mathrm{stroma})} + 2\mathrm{NADP}^{+} + 3\mathrm{ADP}_{(\mathrm{stroma})} + 3\mathrm{phosphate}_{(\mathrm{stroma})}$$

All cyanobacteria and eukaryotic microalgae depend on RuBisCO and the Calvin-Benson cycle for net assimilation of inorganic carbon. RuBisCO is thus the main carboxylating enzyme and probably the most abundant enzyme on the planet, involved in more than 99% of primary production (Beardall and Raven, 2016). Despite its central role, RuBisCO's efficiency surprisingly struggles, as the maximal rate of RuBisCO is an order of magnitude lower than the average of central metabolism enzymes. The low specificity toward CO_2 substantially reduces its effective carboxylation efficiency (Kubis and Bar-Even, 2019).

A major productivity limitation for many phototrophs is photorespiration, an energetically expensive process in which the undesirable oxygenase activity of RuBisCO leads to a net loss of fixed carbon and metabolic energy (Ort et al., 2015). In this competing process, RuBisCO acts as an oxygenase, catalyzing the reaction of O_2 with ribulose bisphosphate to form phosphoglycolate. After dephosphorylation, glycolate is converted, in several steps, to serine, ammonia, and CO_2 (Masojídek et al., 2013). This phenomenon occurs at elevated temperatures and high O_2:CO_2 ratios (Kliphuis et al., 2011). Thus, photorespiration depends on the relative concentrations of oxygen and CO_2, where a high O_2:CO_2 ratio stimulates this process, whereas a low O_2:CO_2 ratio favors carboxylation. RuBisCO has low affinity to CO_2, its K_m (half saturation) being approximately equal to the level of CO_2 in air. Therefore, under high irradiance, high O_2 level, and reduced CO_2, the reaction equilibrium is shifted toward photorespiration. For optimal yields in microalgal mass cultures, it is necessary to minimize the effects of photorespiration. This might be achieved by an effective stripping of O_2 and by CO_2 enrichment. For this reason, microalgal mass cultures are typically grown at a much higher CO_2:O_2 ratio than that found in air (Masojídek et al., 2013).

Nature has evolved several strategies to suppress oxygenation by sequestering RuBisCO into compartments in which CO_2 is concentrated (Ort et al., 2015). Many microalgae are equipped with CO_2 concentrating mechanisms (CCMs) that reduce photorespiratory losses by pumping bicarbonate into the cell and converting it back to CO_2 in a subcellular structure called a pyrenoid (carboxysome in cyanobacteria) where it is dehydrated to CO_2 by carbonic anhydrase (CA). It is thought that both carboxysomes and pyrenoids present a diffusion barrier for CO_2 and O_2, keeping the CO_2 in and O_2 out, and thus enhancing carboxylation and suppressing oxygenation (Mangan et al., 2016). CMMs are found in almost all cyanobacteria and microalgae, with only some exceptions (Beardall and Raven, 2016). Inorganic carbon (HCO_3^- or CO_2) utilization by cells occurs directly by active transport and cation exchange, as well as indirectly through action of CA that catalyzes the conversion of HCO_3^- to CO_2 and OH^-. Three types of CAs have been reported: the periplasmic carbonic anhydrase (pCA), the cytosolic carbonic anhydrase (cyCA), and the chloroplast carbonic anhydrase (chCA). The function of pCA is to balance the CO_2 and HCO_3^- and continuously supply CO_2 for cells. The cyCA may accelerate the transport of CO_2 and HCO_3^- through the plasma membrane to chloroplasts. The chCA is considered a key CA in CCM (Zhao and Su, 2014) as the inorganic carbon transport system located on the chloroplast envelope delivers HCO_3^- to the stroma (Badger and Price, 1994). The uncatalyzed conversion rate of HCO_3^- into CO_2 is 10,000 times slower than the rates of CO_2 fixation by RuBisCO (Badger and Price, 1994). Hence, the ability of CAs to catalytically convert HCO_3^- into CO_2 is critical in microalgal CCM (Morales et al., 2015). The CMMs represent an effective strategy for carbon acquisition that allows microalgae to survive and proliferate when the CO_2 concentration limits photosynthesis (Wang et al., 2015).

8.3 Cultivation systems for CO_2 capture using microalgae

CO_2 capture using microalgae involves a wide range of simultaneous processes. The course starts with the transfer of CO_2 in a gaseous stream to the culture medium where microalgae grow, until CO_2 is fixed in the form of biomass. One of the main concerns associated with optimizing the carbon fixation efficiency is finding strains that favor CO_2 capture and a suitable cultivation system, considering configuration and operating conditions such as light availability, temperature, pH, and mixing.

8.3.1 CO_2 sources and physicochemical properties

The main industrial sources for CO_2 emissions include: coal (40%), oil (35%), gas (20%), cement (4%), and gas flaring (0.7%) (Bekun et al., 2019; British Petroleum, 2018). Exhaust gases from these industries could contain up to 15% CO_2, providing a CO_2-rich source for

microalgal cultivation, because the atmosphere contains only 0.03%–0.06% of CO_2, and it cannot sustain the CO_2 requirements for intensive biomass production (Morales et al., 2015).

While terrestrial plants generally utilize CO_2 from the atmosphere, after dissolution in cellular water, microalgae acquire inorganic carbon from a liquid phase and the amount of dissolved CO_2 from the gas into a liquid phase depends on the composition of the gas and liquid phases, besides operational conditions (pressure and temperature) (Acién et al., 2016). Henry's law constant for CO_2, H_{CO2}, is usually considered as constant, a value of 3.4×10^{-2} mol/L atm being reported for pure water at standard conditions (25°C, or 298 K, and 1 atm, 0.1 MPa). Consequently, under standard conditions, the solubility of CO_2 in water varies from 1.36×10^{-5} mol/L in equilibrium with air to 3.40×10^{-2} mol/L when in equilibrium with pure CO_2. Nonetheless, CO_2 solubility also varies with temperature and pressure; thus, solubility of CO_2 in equilibrium with air goes from 1.97×10^{-5} to 1.13×10^{-5} mol/L for temperatures ranging from 10°C to 30°C that are usually found in microalgal cultures (Acién et al., 2016).

Dissolved inorganic carbon (DIC) is present in the aqueous nutrient medium in the form of dissolved CO_2, carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) under dynamic ionization equilibrium described by the following equations (Morales et al., 2015):

1. CO_2 dissolves in water (slow reaction)	$CO_2(g) \leftrightarrows CO_2(aq)$
2. $CO_2(aq)$ reacts with water to form carbonic acid (slow reaction)	$CO_2(aq) + H_2O \leftrightarrows H_2CO_3$
3. Carbonic acid reacts with water to form bicarbonate and H_3O^+ ions (fast reaction)	$H_2CO_3 + H_2O \leftrightarrows HCO_3^- + H_3O^+$
4. Bicarbonate finally reacts with water to form carbonate and H_3O^+ (fast reaction)	$HCO_3^- + H_2O \leftrightarrows CO_3^{2-} + H_3O^+$

In addition, in an aqueous solution, the diffusion rate of CO_2 is much slower (by a factor of 10^4) than in air, and where much of the available DIC is present (except in some inland waters) as the charged anion HCO_3^- rather than as CO_2 (Beardall and Raven, 2016). The above is particularly represented in seawater where CO_2 concentrations at air equilibrium are in the order of 10–15 μM, but HCO_3^- is found at around 2 mM. The other component of DIC, carbonate ion, is only found in significant concentrations at high pH and is not used as a source of carbon in photosynthesis. CO_2 availability is frequently a major limiting factor in algal mass cultures (Beardall and Raven, 2016).

8.3.2 Strains

The screening of adequate microalgae strains for CO_2 capture is one of the main concerns regarding the improvement of CO_2 capture processes (Vuppaladadiyam et al., 2018). Regularly, the highest biofixation rates in microalgae take place when gas streams with a

concentration of CO_2 between 2% and 5% are supplied to the cultivation system at temperatures between 16°C and 30°C (Morales et al., 2015); unfortunately, flue gases usually have higher concentrations of CO_2 and are emitted at higher temperatures, some reaching 120°C. Due to the abovementioned reasons, the main characteristics sought in these strains are: tolerance to high CO_2 concentrations (10%–15% CO_2); high temperatures (Bhatia et al., 2019); high CO_2 fixation rates; and the ability to grow in the presence of toxic compounds such as NO_x, SO_x, and H_2S (Cheah et al., 2015; Yen et al., 2015). It has been found that species typically from the genders *Chlorella* (Kao et al., 2014; Marín et al., 2018), *Scenedesmus* (Kandimalla et al., 2016; Sun et al., 2016), *Chlorococcum* (Ota et al., 2015), and *Nannochloropsis* (Cheng et al., 2018b; Meier et al., 2015) have successfully captured CO_2 present in effluents with similar characteristics to those emitted from industrial activities.

8.3.3 Physicochemical aspects involved in CO_2 capture by microalgae

8.3.3.1 Light and nutrients

Microalgal cultures require the proper amount of light and nutrients to ensure cell proliferation. In autotrophic cultures, light is the energy source and is one determinant factor for photosynthetic activity (Morales et al., 2015), while nutrient supply is a limiting aspect to regulate key metabolic processes related to both CO_2 fixation and biomass synthesis (Morales et al., 2018). The growth of microalgae in autotrophic cultures may be hindered if the self-shading phenomenon becomes present due to high cellular concentrations (Yen et al., 2019). On the other hand, cellular damage may occur if the amount of light is too much for the photosynthetic apparatus to harvest (Morales et al., 2018) due to the oxidative stress responses that take place in the cell. Studies have shown that not only light intensity but also the wavelength of the light emitted to microalgal cultures play a key role in controlling biosynthetic processes (Morales et al., 2018; Sirisuk et al., 2018).

The elemental composition of microalgal biomass is fundamental to design a culture medium (Morales et al., 2018). The main elements that constitute algal biomass are carbon, nitrogen, phosphorus, magnesium, and sulfur; considering this, Chisti (2007) reported a general molecular formula for microalgal biomass: $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$. The major microalgal biomass structural components are carbon and nitrogen, accounting for approximately 60% of its molecular composition, where nitrogen plays a key role in photosynthesis and carbon fixation, given its influence in chlorophyll and protein biosynthesis (Toledo-Cervantes et al., 2013).

8.3.3.2 Temperature

Temperature is a fundamental parameter to enhance both CO_2 availability and cell growth. However, while at relatively high temperatures (>25°C) enzymatic and chemical reactions are favored, an inverse effect is observed on the solubility of CO_2 (Morales et al., 2018). The reduction of CO_2 solubility in liquid media as a function of temperature is described by Henry's

law (Acién et al., 2016). In the range of 10–30°C, Henry's constant for the transfer of CO_2 present in air to water is reduced by 42%. Despite this, high temperature operation is a typical condition when mitigating CO_2 from gas streams such as flue gases, which promotes carbon fixation reactions in microalgae cells (Morales et al., 2015).

8.3.3.3 pH

Another important physicochemical parameter that affects CO_2 solubilization is pH. The value for this parameter determines the relative abundance of CO_2, HCO_3^-, and CO_3^{2-} in relation to the total inorganic carbon concentration (Acién et al., 2016). Microalgae cultures usually present neutral or alkaline pH values (7–10) (Morales et al., 2018). Acidification of microalgae cultures is mainly associated to the formation of H_2CO_3 when supplying CO_2 to the culture while alkalization is related to the release of hydroxyl ions, generally due to the assimilation of NO_3^- and HCO_3^-. Alkaline conditions are usually preferred to cultivate microalgae due to the positive effect on CO_2 solubilization, and this could prevent culture contamination.

8.3.3.4 Mixing

Efforts to enhance the transfer of CO_2 to the liquid medium have also been focused on improving mixing in culture systems. Mixing efficiency is highly dependent on the type of reactor and mixing system used in a microalgae culture. Supplying gas streams in the form of bubbles is usually the most effective way to transfer CO_2 to the liquid medium so that it can later be delivered to microalgae, compared to superficial mass transfer; these factors play an active role in maintaining adequate growth conditions for microalgae within a culture system. Particularly, mass transfer phenomena are highly dependent on mixing, temperature, and pH present in the culture medium.

8.3.4 Culture systems for CO_2 capture with microalgae

Generally, photobioreactors (PBRs) can be divided into open and closed systems (Fig. 8.1). In open systems, the culture broth has direct contact with its surroundings, contrary to the closed systems, where the culture medium is separated from their surroundings by a translucid plastic or glass layer.

The typical configurations for CO_2 capture systems are the same as those used for microalgae cultures. Tubular PBRs are commonly used for CO_2 capture due to the relatively good scalability and low risk of contamination (Verma and Srivastava, 2018; Yen et al., 2019). This type of system can be divided into horizontal tubular reactors (HTRs) and bubble columns (BCs). Both systems generally perform well when used for the production of high added value products since the culture broth is not in direct contact with the environment (Acién et al., 2016). The main advantages of using HTRs for biomass production and CO_2 capture are the large surface area exposed to light and the relatively low losses of CO_2 (Morales et al., 2015)

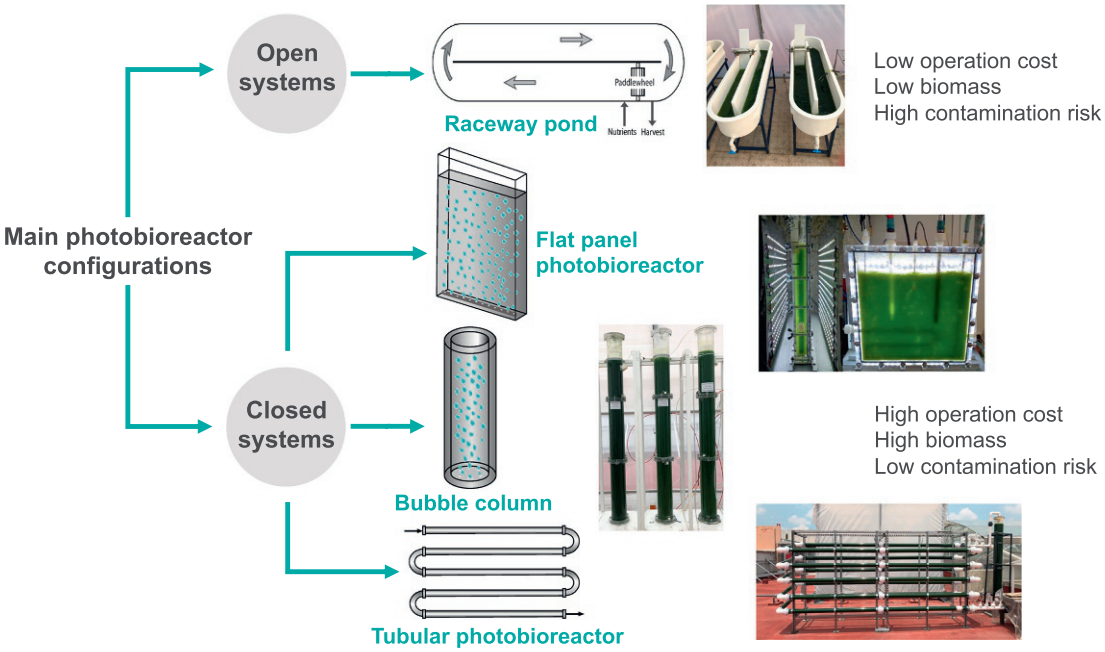

Fig. 8.1
Main photobioreactor configurations for microalgal cultures.

due to desorption; this is commonly reflected in high biomass productivities and carbon fixation efficiencies (Babcock et al., 2016; Moraes et al., 2019). However, an important disadvantage related to microalgal processes that involve the use of HTRs is the accumulation of oxygen in the culture medium. This is of particular importance because it can cause damage in the photosynthetic apparatus of the microalga, which in turn can lead to a decrease in biomass production and CO_2 uptake (Morales et al., 2018).

On the other hand, BCs have the advantage of high mass transfer and good mixing, which has also made them adequate systems for biomass production and CO_2 sequestration (Kargupta et al., 2015; de Morais et al., 2015). A major disadvantage of BCs is the small illumination area, which may induce the self-shading phenomenon at high biomass concentrations, leading to a decrease in growth rate.

Another common configuration used for CO_2 capture with microalgae is flat-plate PBRs. These systems are used for similar purposes to tubular PBRs; however, an important advantage over HTRs and BCs is the short light path and high illumination area (Morales et al., 2015). This enables microalgae to achieve high photosynthetic efficiencies and thus increased CO_2 fixation rates (Koller et al., 2017; Xia et al., 2018). An important drawback when operating flat-plate reactors lies in the low mixing and high shear stress that microalgae experience.

Open ponds have also been implemented for CO_2 capture processes due to their low costs and relatively simple operation (Morales et al., 2015; Verma and Srivastava, 2018). Although presenting lower biomass productivities, algae cultures in raceway ponds have proven useful in capturing CO_2 and producing biomass (Cheng et al., 2018b). Nonetheless, losses to the atmosphere of supplied CO_2 ranging from 80% to 90% (Putt et al., 2011), as well as an elevated risk of contamination, are still major problems associated with raceway ponds operation to be addressed in order to integrate this configuration further into CO_2 capture systems (Acién et al., 2016; Verma and Srivastava, 2018).

Operating open systems is simpler and usually less expensive than closed systems; however, the risk of contamination from airborne microbes is significantly higher. Mixing in these systems is also less efficient. Microalgal cultures in closed systems can achieve higher biomass concentrations and are easier to control than in open systems, but have a significant downside regarding their operation costs (mixing, artificial lighting, etc.) (Morales et al., 2015).

8.3.5 Carbon balances for evaluation of microalgae-based CO_2 capture systems

Common calculations to determine the performance of a CO_2 capture systems are the removal efficiency (RE) and elimination capacity (EC); these parameters are defined as follows:

$$\mathrm{RE} = (100)\left(\frac{X_{\mathrm{CO_2,in}} - X_{\mathrm{CO_2,out}}}{X_{\mathrm{CO_2,in}}}\right) \quad \mathrm{EC} = \frac{((F_{\mathrm{in}})(X_{\mathrm{CO_2,in}}) - (F_{\mathrm{out}})(X_{\mathrm{CO_2,out}}))}{V}$$

where F_{in} and F_{out} are the gas flows at the inlet and exit of the system, $X_{\mathrm{CO_2,\ in}}$ and $X_{\mathrm{CO_2,\ out}}$ are the fractions of CO_2 in F_{in} and F_{out}, respectively, and V is the volume of the culture broth.

However, they do not provide full information about the fate of the carbon supplied to the system. This carbon could be fixed as biomass, dissolved in the culture medium or desorbed from the system (Fig. 8.2).

The carbon balance for a microalgae-based CO_2 capture system operated in batch mode with continuous supply of CO_2 without a source of organic carbon can be established as follows:

$$\mathrm{C_{(g),in}} = \mathrm{C_{(l)}} + \mathrm{C_{(X)}} + \mathrm{C_{(g),out}}$$

where $\mathrm{C_{(g),in}}$ is the carbon found in the gas stream supplied to the system, $\mathrm{C_{(l)}}$ is the dissolved carbon retained in the culture broth, $\mathrm{C_{(X)}}$ is the carbon found in microalgal biomass, and $\mathrm{C_{(g),out}}$ is the carbon present in the gas stream at the exit of the culture system.

8.3.5.1 Gaseous carbon

To estimate the fraction of carbon that does not remain in the system, either dissolved in the culture medium or in the form of biomass, it is necessary to measure the concentration of carbon species in the gas stream at the outlet of the system. The most common techniques used to measure CO_2 in a gas stream are gas chromatography (GC), infrared sensors (IRs), and titration

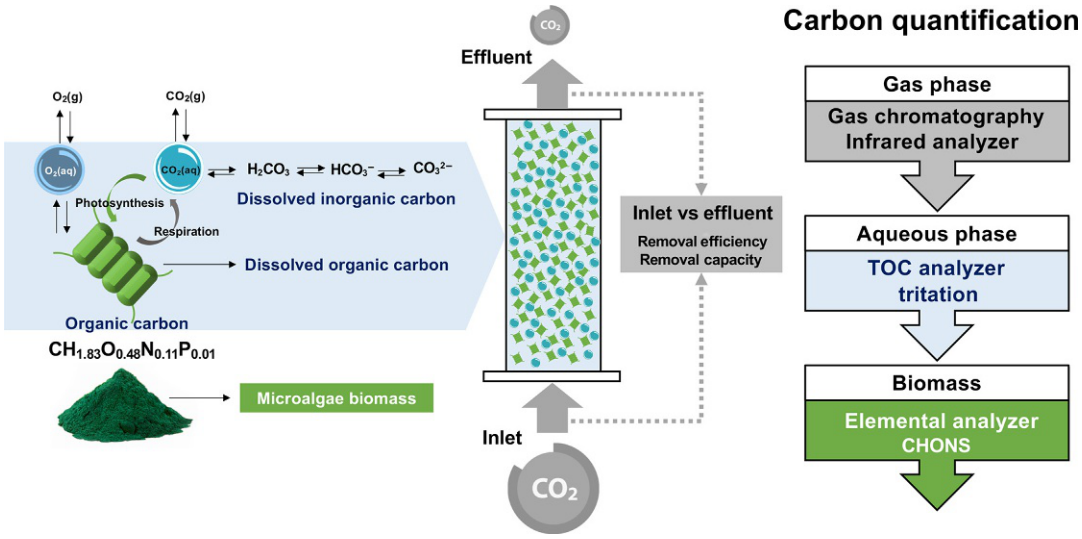

Fig. 8.2
Carbon fate and quantification techniques for microalgal systems.

of the dissolved CO_2 present in effluent capture in an alkaline bath (Pfeiffer et al., 2011). Although chromatography is the most precise method, IR detectors are commonly used in portable CO_2 measuring devices (Gibson and MacGregor, 2013).

Using the analytical techniques previously mentioned, the mass of carbon entering and exiting the system can be calculated as follows:

$$C_{(g),in} = (t)(F_{in})(X_{CO_2,in})\left(\frac{PM_{CO_2}}{RT_{in}}\right)\left(\frac{M_C}{M_{CO_2}}\right) \text{ and}$$

$$C_{(g),out} = (t)(F_{out})(X_{CO_2,out})\left(\frac{PM_{CO_2}}{RT_{out}}\right)\left(\frac{M_C}{M_{CO_2}}\right)$$

where t is time at which CO_2 is fed, T_{in} and T_{out} are the temperatures of the inlet and outlet gas streams, respectively, R is the ideal gas constant, P is the atmospheric pressure, M_{CO_2} is the molecular weight of CO_2, and M_C is the molecular weight of carbon. When both $C_{(g),in}$ and $C_{(g),out}$ are calculated, the fraction of carbon in the gas phase (C_{gas}) can be obtained using the following equation:

$$C_{gas} = \left(\frac{C_{(g),out}}{C_{(g),in}}\right) = \frac{(F_{out})(X_{CO_2,out})(T_{in})}{(F_{in})(X_{CO_2,in})(T_{out})}$$

8.3.5.2 Dissolved carbon

Carbon can also remain dissolved in the liquid, depending on the physicochemical conditions in which the capture system is operated (Morales et al., 2018). In order to measure the amount of

carbon dissolved in the system, it is usually necessary to obtain a sample of culture medium without biomass and use analytical techniques such as titration or high temperature catalytic oxidation. The latter is a more precise measurement technique due to its sensitivity and its capacity in some equipment to distinguish between organic and inorganic dissolved carbon.

The carbon retained in the culture medium ($C_{(l)}$) can be calculated by multiplying the difference between the dissolved carbon concentration ($D_{(l)}$) after a certain time and the initial value ($D_{(l),0}$) when CO_2 supply started:

$$C_{(l)} = \left(D_{(l)} - D_{(l),0}\right)(V)$$

With this information, the fraction of carbon dissolved in the culture medium (C_{Liquid}) can be obtained with the following equation:

$$C_{Liquid} = \left(\frac{C_{(l)}}{C_{(g),in}}\right) = \frac{\left(D_{(l)} - D_{(l),0}\right)(V)}{(t)(F_{in})(X_{CO_2,in})\left(\frac{PM_{CO_2}}{RT_{in}}\right)\left(\frac{M_C}{M_{CO_2}}\right)}$$

8.3.5.3 Carbon in biomass

In order to calculate the amount of carbon fixed from the gas stream into biomass, it is necessary to know the biomass concentration ($X_{biomass}$) in the cultivation system and the fraction of carbon in the biomass. This is not the case when the culture medium contains relatively high amounts of carbonate or bicarbonate (Guo et al., 2019; Könst et al., 2017), nor when an organic source of carbon is also present in the medium, such as carbohydrates or organic acids—for example, during wastewater treatment coupled to CO_2 removal (Zhu et al., 2013).

The most common methods to obtain $X_{biomass}$ are directly, by measuring the dry weight of biomass in a given sample volume (DW), or with the optical density of the culture broth at a given wavelength (OD). The second method is used when rapid measurements are required for a given experimental setting or volume restrictions, while DW is more trustworthy (Harris and Kell, 1985). It is also important to obtain the composition of the biomass generated during mitigation of CO_2. Elemental analysis is usually carried out for the determination of total carbon, hydrogen, oxygen, nitrogen, and sulfur (C, H, O, N, S).

Taking into account the concentration of biomass at the beginning of the culture ($X_{biomass,0}$) and the one measured at a certain time t ($X_{biomass}$) as well as the volume of the culture broth (V), the carbon present in the system as biomass ($C_{(X)}$) is given by the following expression:

$$C_{(X)} = \left((X_{biomass})\left(\frac{M_{carbon}}{M_{biomass}}\right)_t - (X_{biomass,0})\left(\frac{M_{carbon}}{M_{biomass}}\right)_0\right)(V)$$

where $(M_{carbon}/M_{biomass})_t$ is the fraction of carbon present in the microalgal biomass at a specific sampling time and $(M_{carbon}/M_{biomass})_0$ is the fraction of carbon in the microalgal

biomass at the beginning of CO_2 supply to the system. This parameter can change significantly over time if microalgal cells are exposed to environmental stress or fluctuating temperature, light intensity, etc. To calculate the fraction of carbon present in the biomass suspended in the culture broth, the following formula can be used:

$$C_{biomass} = \left(\frac{C_{(X)}}{C_{(g),in}}\right) = \frac{\left((X_{biomass})\left(\frac{M_{carbon}}{M_{biomass}}\right)_t - (X_{biomass,0})\left(\frac{M_{carbon}}{M_{biomass}}\right)_0\right)(V)}{(t)(F_{in})(X_{CO_2,in})\left(\frac{PM_{CO_2}}{RT_{in}}\right)\left(\frac{M_C}{M_{CO_2}}\right)}$$

8.4 Strategies for CO_2 capture improvement

Two global strategies to improve CO_2 capture by microalgae are being extensively studied: genetic modification of microalgae and design of photobioreactors (Fig. 8.3).

8.4.1 Genetic engineering and metabolic modifications for the improvement of CO_2 capture

Several strategies have been adopted to enhance the overall carbon fixation rate of the microalgae cells (Gomaa et al., 2016). One of the main approaches has been modification of RuBisCO in order to improve its selectivity and velocity. However, limited success has been

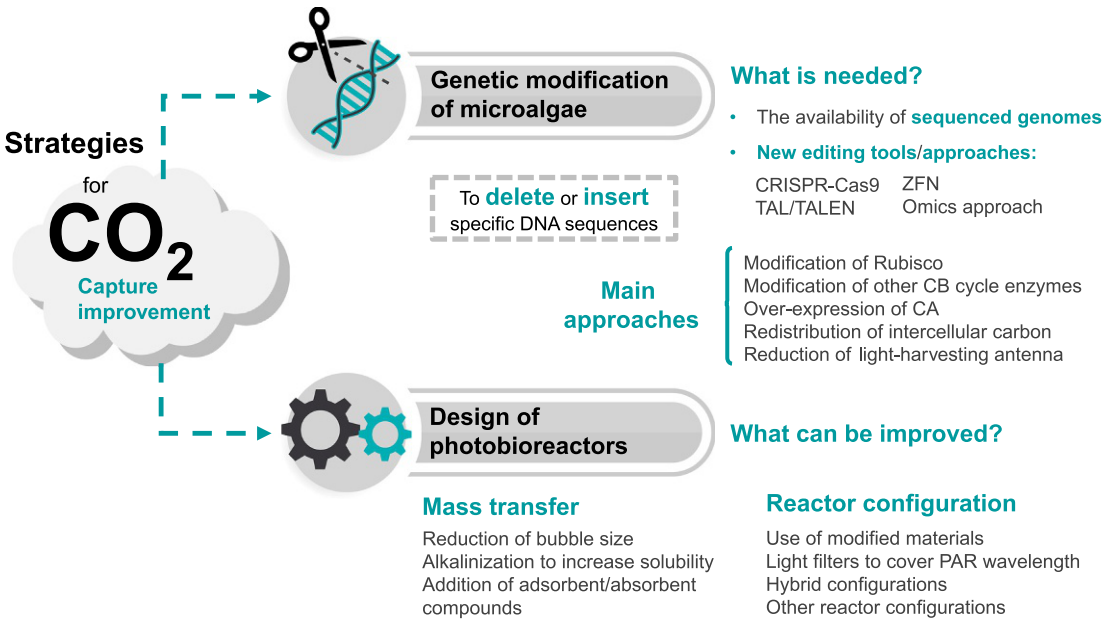

Fig. 8.3
Global strategies for CO_2 capture improvement.

reported, as RuBisCO's selectivity and velocity could not be enhanced simultaneously (Zhu et al., 2010). Thus, RuBisCO may be improved by considering two approaches: the catalytic rates or the relative affinity toward CO_2. Successful improvements of RuBisCO's activity have been reported, reaching increases of up to four times its activity (Atsumi et al., 2009; Iwaki et al., 2006; Liu et al., 2010) by overexpressing genes encoding for RuBisCO's subunits or through the overexpression of some natural variants of this enzyme. Furthermore, co-overexpression of RuBisCO and CA to a certain ratio as well as increasing the activity/amount of bicarbonate transporter have demonstrated an increase in growth and biomass productivity (Chen et al., 2012; Kamennaya et al., 2015). Overall, genetic modification of RuBisCO to increase catalytic velocity is preferable to improving selectivity, since the selectivity problem could be overcome by bioreactor design with a high concentration of CO_2 (Ng et al., 2017). On the other hand, RuBisCO is not the only enzyme to enhance carbon fixation. Other enzymes of the Calvin-Benson cycle have also been engineered for enhancing carbon fixation, such as sedoheptulose-1,7-bisphosphatase, transketolase, aldolase, and so on. Details of each case are presented in Ng et al. (2017), and progress on improvement of carbon fixation through metabolic engineering and redistribution of intercellular carbon flux is reviewed by Zhou et al. (2016).

Abiotic factors also affect the efficiency of carbon fixation. For example, excess of light induces photoinhibition, which results in inefficient utilization of light and decreased photosynthetic efficiency. Several genetic modifications of microalgae have been proposed that can potentially enhance photosynthetic efficiency. A number of recent reviews are available that discuss molecular approaches toward improvement of photosynthetic efficiency: Blankenship and Chen (2013), Ort et al. (2015), Seth and Wangikar (2015), and Wobbe et al. (2016). An approach that is being extensively studied is the concept of the truncated light-harvesting antenna (TLA), in which a reduction of the antenna size in microalgae allows a greater light penetration and prevents over-absorption of photons by individual cells, enabling deeper sunlight penetration into the culture, and affording an opportunity for more cells to be productive, in effect raising photosynthetic productivity of the culture as a whole (Kirst and Melis, 2014). Several studies have reported that shrinking antenna size allows more light transmission and higher absorptive capacity of light, resulting in higher biomass productivity (Beckmann et al., 2009; Masuda et al., 2003; Mussgnug et al., 2007; Stefano et al., 2014).

Genetic engineering technologies provide the potential to exploit microalgae as light-driven biotechnology platforms for CO_2 capture and the synthesis of a wide range of products (Spicer and Purton, 2016). However, they require the availability of sequenced genomes and genetic tools such as transformation techniques, constitutive/inducible promoters, and established methods for the knock-down/out of individual genes (Wobbe and Remacle, 2014). Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated protein 9 (CRISPR-Cas9) and Transcription Activator-Like (TAL) Effector Nucleases (TALEN), which

are the new genome-editing tools, as well as Zinc-Finger Nucleases (ZFN), have been used in gene modification (Shin et al., 2016; Spicer and Purton, 2016). These tools can be used to create mutations within native genes to suppress (knockout) or insert DNA sequences into specific sites within the genome (knock-in). Desired microalgae phenotypes without gene modification could be also achieved by gene-interfering tools, such as CRISPR-dCas9, micro RNA (miRNA), and silence RNA (siRNA), to repress or activate the gene expression (Huang et al., 2016; Yao et al., 2016). The omics approach is likewise an integrative technology for microalgal research in sustainable development (Banerjee et al., 2016). Advances in this area are being made at an ever-increasing pace, and it is likely that all approaches will be successfully developed and applied within microalgae for the production of natural bioproducts as well as new functionalities (Spicer and Purton, 2016).

8.4.2 Photobioreactors: Enhancing design for CO_2 capture improvement

8.4.2.1 Increasing mass transfer and reactor configurations

8.4.2.1.1 Manipulation of bubble dynamics

Bubble size is often a fundamental parameter when optimizing CO_2 mass transfer to the culture medium. It directly affects the area/volume quotient of gas supplied to the reactor: when the individual size of bubbles is smaller, the value of this quotient is higher, hence, mass transfer to the culture medium is enhanced. Ying et al. (2013) increased CO_2 transfer to a liquid medium by 30% using a microbubble generation system. A similar study performed with microbubbles showed that by reducing bubble size, the mass transfer coefficient of CO_2 to the culture media and the final biomass concentration were enhanced by 80.9% and 30%, respectively (Cheng et al., 2019). By optimizing the gas diffuser geometry and the gas-liquid velocities in a raceway pond numerically, it was possible to reduce bubble size by 50.7%, and when validating the results obtained, this led to a higher growth rate and biomass production (26.6%, 50.7%) (Cheng et al., 2018a).

Another strategy involving the manipulation of bubbling supplied to the culture systems is increasing the gas-liquid contact time. Xia et al. (2018) studied the effect of an internal arc trough in the transfer of CO_2 to the culture medium. In biomass production and CO_2 fixation rate, gas-liquid contact time was increased from 0.448 to 256 s, enhancing biomass concentration and CO_2 fixation rate by 20.9% and 26%, respectively. Other modifications include the addition of baffles to raceway ponds with continuous supply of CO_2, which have increased gas residence time by 27%, leading to a rise in biomass concentration of 29% (Cheng et al., 2016). The rational design of gas spargers also contributed to modify the behavior of bubbles and enhance mass transfer, achieving a biomass concentration 83.44% higher than that found with the use of a commercial microbubbles aerator (Huang et al., 2017).

8.4.2.1.2 Modifying the liquid medium: Alkalinization and addition of adsorbent/absorbent materials

pH influences the physicochemical equilibrium between the concentration of CO_2, HCO_3^-, and CO_3^{2-}, and alkalinization of the culture medium has been investigated as a possible strategy to optimize the solubilization of gaseous CO_2 in the liquid medium where microalgae grow. Duarte-Santos et al. (2016) supplied CO_2 on demand into a raceway pond by maintaining the pH of the culture at a value of 8, allowing for a higher fraction of the injected CO_2 to remain in the liquid medium or to be fixated by microalgae cells.

On the other hand, the use of two-stage processes based in alkaline solutions has also been studied. Könst et al. (2017) used a carbonate-based absorption liquid to facilitate CO_2 capture in the form of HCO_3^-. Guo et al. (2019) added Na_2CO_3 to improve the absorption of CO_2; the addition of this compound led to an increase of 500% in the growth rate in the culture system as well as an overexpression of cellular components related to CO_2 fixation and photosynthesis.

The addition of adsorbent/absorbent materials to the culture has also been studied to enhance CO_2 capture and fixation. da Rosa et al. (2018) added monoethanolamine (MEA) to a microalgal culture with CO_2 supply and increased dissolved inorganic carbon (DIC) and biomass productivity significantly. Another study performed by Rosa et al. (2019) showed that the fed-batch addition of MEA and CO_2 heightened DIC by 22% as well as the CO_2 fixation rate (CR) in comparison with the sole injection of CO_2. Cardias et al. (2018) increased CR by 61% with the addition of diethanolamine (DEA) and potassium carbonate (K_2CO_3); similarly, da Silva Vaz et al. (2019) increased CR by 58% with the addition of iron oxide polymeric nanofibers to the culture broth.

8.4.2.1.3 Built-in materials for photobioreactors

Modified materials to be used for improvements in light-harvesting processes in microalgae need to present high quantum efficiency and be able to absorb in a wide wavelength range, covering the part of the spectrum where low light-harvesting efficiency is found in microalgae cells, as well as presenting low-cost and long-term photostability (Delavari Amrei et al., 2014).

Delavari Amrei et al. (2014) defined an integrated wavelength-shifting strategy for enhancement of microalgal growth rate by coating the photobioreactors with a mixture of acrylic thermoplastic and different proportion of fluorescent dye photobioreactors. They found that *Chlorella* biomass productivity was enhanced up to 74% compared to the PBR configuration without the dye. Mohsenpour and Willoughby (2013) used a light filter to expose a culture of *Chlorella vulgaris* to light of different wavelengths, which resulted in increases of 20.3% and 14.4% when using red and green filters, respectively.

8.4.2.1.4 Hybrid photobioreactors

Hybrid systems combine the characteristics of two or more system configurations to enhance their benefits and overcome the difficulties. Bahr et al. (2014) used a combination of an absorption column with a high rate algal pond for biogas upgrading. The CO_2 is transferred to the liquid phase in the bubble column where photosynthetic activity is practically negligible, and carbon fixation occurs in the open pond where the light exposure is favored due to the high surface area. Moreover, Liu et al. (2019) combined an open pond and a bubble column, enhancing the final biomass concentration by 16% compared to the one obtained only with an open pond. Likewise Deprá et al. (2019) combined a nontransparent bubble column with an illumination platform composed of open tubular structures, reaching an CO_2 conversion and maximum specific growth rate of 45.32 kg_{CO2}/m^3/day and 0.96/day, respectively.

8.4.2.1.5 Other bioreactor configurations

An important concern regarding traditional microalgae cultivation systems is the need for large amounts of water for their operation (Berner et al., 2015). A solution to this is cultivating microalgae in a solid base medium such as biofilms. Biofilms can be defined as aggregates of microorganisms in which cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhering to each other and/or to a surface (Vert et al., 2012). This reactor configuration may enhance CO_2 transfer to microalgae cells due to the absence of liquid resistance to gas solubilization. Blanken et al. (2017) modeled biomass growth and CO_2 utilization efficiency, concluding that concentrated CO_2 streams under plug flow conditions could be treated efficiently by connecting photobioreactor units in series, fed with a flue gas stream.

Other innovative alternatives are the offshore reactors with the possibility of nutrient, heat, and light supply from the environment surrounding the sea (Harris et al., 2013), which would reduce the energy-associated costs of a microalgal process. A theoretical study showed that offshore cultures supplied with CO_2 present in flue gases may be able to reduce the annual CO_2 emissions by half (Smith et al., 2018). The use of a magnetic field to stimulate microalgae metabolism has also been studied in the field of CO_2 capture. Deamici et al. (2019) used magnetic field exposure to increase biomass productivity and CO_2 fixation rate, which increased by 34% and 50%, respectively.

8.5 Remarks and conclusion

CO_2 biofixation by microalgae can contribute significantly to the reduction in the greenhouse effect. This chapter provided an overview of basic issues on CO_2 capture by microalgae and some challenges and recent advances that the scientific community is developing to establish innovative approaches that include biological modifications through molecular and genetic tools and technological challenges to optimize CO_2 capture processes.

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CHAPTER 9

Dewatering and drying of algal cultures

Jaison Jeevanandam[a], **Michael Kobina Danquah**[b]

[a]*Department of Chemical Engineering, Curtin University, Miri, Sarawak, Malaysia* [b]*Chemical Engineering Department, University of Tennessee, Chattanooga, TN, United States*

Chapter outline

9.1 Introduction 207
9.2 Historic timeline of algal biomass production 208
9.3 Conventional dewatering methods of algae processing 209
9.3.1 Flocculation 209
9.3.2 Centrifugation 210
9.3.3 Filtration 211
9.3.4 Gravity sedimentation 211
9.3.5 Flotation 212
9.3.6 Electrophoresis methods 213
9.4 Conventional drying methods for algae processing 213
9.4.1 Solar drying 213
9.4.2 Convective drying 214
9.4.3 Spray-drying 214
9.4.4 Freeze-drying 215
9.4.5 Other drying methods 215
9.5 Latest trends in dewatering and drying of algae for commercialization 216
9.6 Future perspective 217
9.7 Conclusion 218
References 218

9.1 Introduction

Algae have been extensively explored for the production of useful bioactive compounds and biofuels (Michalak and Chojnacka, 2015). It is noteworthy that algal species such as *Nostoc*, *Spirulina*, and *Aphanizomenon* have been used as food for more than 1000 years. These edible algal species contain a wide variety of proteins, carbohydrates, and lipids, which make them highly nutritious (Spolaore et al., 2006). They are highly beneficial in elevating human and animal nutrition and are used as pet and farm animal feeds, due to their exclusive nutritional value (Cuellar-Bermudez et al., 2015). Moreover, algal extracts are useful in

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cosmetics (Mourelle et al., 2017), aquaculture (Tredici et al., 2009), wastewater treatment (Escudero-Oñate and Ferrando-Climent, 2019), and carbon capture applications (Schipper et al., 2019). In addition, algae also possess high value molecules such as fatty acids, pigments, and stable biochemical isotopes, which enhances their commercial value (Chew et al., 2017). Thus, commercially viable culturing and harvesting processes for algae have gained much attention among researchers to enhance the yield of value-added products and to meet the growing demand for algal products.

Algal culturing starts with the inoculation of desired algal species into the culture medium, which contains the required nutrients for their growth. Antibiotics are also added to avoid the growth of bacterial contaminants in the culture media. The culture media with the inoculum is maintained at ambient conditions for growth, and algae can be harvested after cultivation (Sing et al., 2014). The processing of algae for the desired applications is commenced after harvesting to avoid loss of algal compounds due to lack of nutrients (Khanra et al., 2018). The presence of water in the in the culture can affect the characteristics and concentration of the extracts as well as the efficacy of the extraction technique. Thus, dewatering and drying of algal cultures for biomass production and extraction are important. Centrifugation, flocculation, filtration, gravity sedimentation, flotation, and electrophoresis techniques are the conventional dewatering methods widely used (Pragya et al., 2013). Similarly, solar, convective, spray-, and freeze-drying are the conventional methods used to dry algal cultures (Del Campo et al., 2007). Each method has specific pros and cons, requiring proper operational optimization and management to achieve improved dewatering and drying outcomes. This chapter provides an overview of conventional dewatering and drying processes, and their merits and demerits for large-scale processing of algal cultures. Additionally, recent advances in dewatering and drying methods for commercial applications are discussed.

9.2 Historic timeline of algal biomass production

As shown in Fig. 9.1, the historic timeline of algal biomass production dates back to the 1st century AD, and in the 1950s, the commercial value of algae was highly recognized, resulting in escalating interest in large-scale cultivation of algal culture (Spolaore et al., 2006). Large-scale production of algal biomass requires continuous dewatering and drying steps to increase the yield of the extract (Moheimani et al., 2015). The dewatering and drying technologies used for algae harvesting and biomass production are dependent on the species and the specificity of desired applications (Rizwan et al., 2018). The identification of drawbacks in the conventional dewatering and drying processes and development of improved technologies started in early 2000 to support commercial-scale production of algal products.

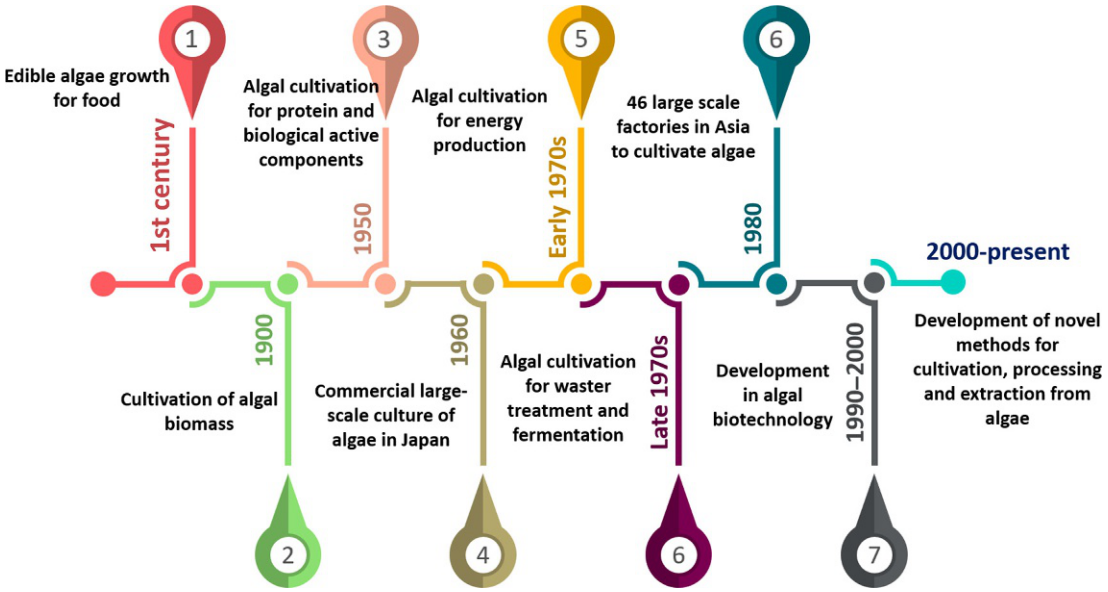

Fig. 9.1
Timeline of developments in commercial algal biomass production.

9.3 Conventional dewatering methods of algae processing

The high cost of harvesting and dewatering algal cultures hinders the developmental of algal products such as biofuels as an alternative to conventional fuels. There are several dewatering methods such as centrifugation, flocculation, filtration, gravity sedimentation, flotation, and electrophoresis that are currently used for algal culture (Barros et al., 2015; Fig. 9.2).

9.3.1 Flocculation

Flocculation is usually considered as a primary harvesting method to increase the particle size of algae cells before dewatering and drying (Rastogi et al., 2018). Flocculation increases the rate of agglomeration of algal cells via the use of flocculants to increase their size (called floc) and facilitate the dewatering process (Ummalyma et al., 2016). These flocs either settle to the bottom by gravity sedimentation or are aerated and move to the surface to form a cream (Chatsungnoen and Chisti, 2016b). Cationic polymers, cationic flocculants, and metal salts such as alum, aluminum sulfate, ferric chloride, and ferric sulfate are used to initiate coagulation, flocculation, and aggregation of algal cells (Wan et al., 2015). The ionic charge of the metal ions used for algal flocculation is a significant factor that determines the flocculation efficiency (Lavrinovičs and Juhna, 2017). Generally, alum is considered as a highly effective metal salt that facilitates algal flocculation; however, it is not

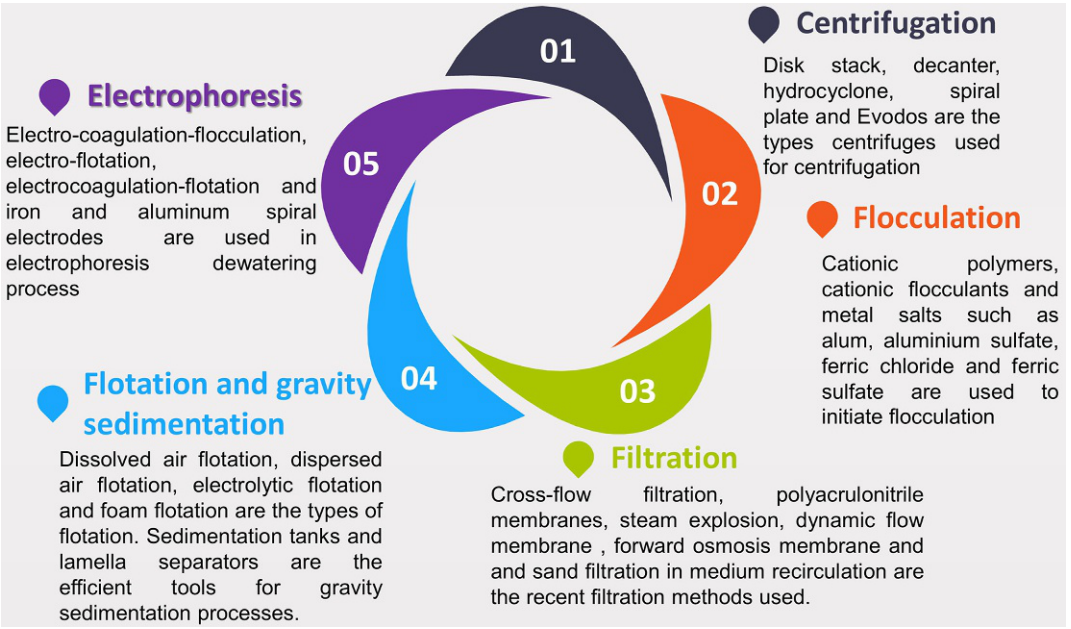

Fig. 9.2
Conventional dewatering methods for algal cultures.

advisable for use in food and medicinal applications as it may lead to toxicity via heavy metal accumulation (Ahmad et al., 2011; Lee et al., 2015). Advanced flocculants such as polyferric sulfates, prepolymerized metal salts, nonanionic polymer Magnafloc LT-25, ultrasound, pH modification, electrolytes, synthetic polymers, natural polymers including cationic starch, *Moringa oleifera* seed flour, and chitosan are used as novel algal flocculation agents (Wan et al., 2015). Control of carbon dioxide using pH-responsive cellulose nanocrystals (Eyley et al., 2015), reversible flocculation using magnesium hydroxide (Vandamme et al., 2015a), auto-flocculation (Parr, 2018), alkaline-electrolyzed water (Lee et al., 2018), and ballast agent-mediated flocculation (Gorin et al., 2015) are also used as pretreatments to initiate algae dewatering.

9.3.2 Centrifugation

The separation of solids from liquids using centrifugal forces based on the particle size of the solid and the density of the liquid is called centrifugation. Centrifugation is classified under secondary dewatering processes as the algal culture is transformed into a slurry form at the primary stage (e.g., flocculation). Thus, high energy is required at this stage, compared to primary processes, and this increases the cost involved in the dewatering process (Amaro et al., 2011). Different types of centrifugation systems are useful for the dewatering of various algal types depending on their size and cellular intactness (Fasaei et al., 2018).

Disk stack, decanter, hydrocyclone, spiral plate, and Evodos centrifugation systems offer different degrees of separation suitable for specific algal culture characteristics (Sharma et al., 2013). Naghdi and Schenk (2016) utilized dissolved air flotation and centrifugation to recover algal biomass and extract oil. In this study, *C. muelleri* culture was initially centrifuged to separate algae and water, and later to fracture algal cells to free up lipids. Centrifugation resulted in a concentration of more than 60% algal debris, compared to flotation, with 40% algal debris (Naghdi and Schenk, 2016). Centrifugation possesses major drawbacks such as requirement of high energy, maintenance, operating costs, and scale-up challenges (Han et al., 2015; Schenk et al., 2008). In addition, high-speed centrifugation can disrupt algal cells and cause premature leaching of intracellular contents, making them more effective under low speed conditions, and this affects the dewatering efficiency (Xu et al., 2015).

9.3.3 Filtration

Filtration uses a permeable medium to separate a suspension where the liquid passes through the medium and the solids are retained. A pressure-drop applied across the medium via gravity, vacuum, or centrifuging, is essential to force the liquid through the filtering medium. Surface and depth (or deep bed filters) are the two major types of filtration, where a thin film is used as a filtering medium to deposit solids, and where the solids are deposited within the filter medium, respectively (Uduman et al., 2010). Other filtration methods, such as tangential flow filtration (TFF), have been developed to overcome the challenges of conventional filtration methods. In the TFF method, the suspension flows tangentially along the membrane and the retentate is recirculated to concentrate the solid content and minimize fouling. This method can be highly beneficial for large-scale algal dewatering and recovery operations (Björnmalm et al., 2015). In recent times, cross-flow filtration (Giménez et al., 2018), polyacrulonitrile membrane (Marbelia et al., 2016), dynamic flow membrane (Lorente et al., 2017), forward osmosis membrane (Kim et al., 2015), and sand filtration in medium recirculation (Fret et al., 2016) have been investigated for use in algal dewatering. Filtration methods also possess certain disadvantages such as high operating and capital cost, frequent membrane replacement processes, and slower separation rates compared to centrifugation (Sharma et al., 2013). However, ultra, micro, and nanofiltration methods are considered to be simpler and cost-effective compared to centrifugation for large-scale algal dewatering (Lopresto et al., 2017).

9.3.4 Gravity sedimentation

Generally, coagulation and flocculation steps are followed by gravity sedimentation, which separates the algal suspension into clear liquid and concentrated slurry. Sedimentation tanks and lamella separators with recovery efficiencies of 1.6% and 3%, respectively, are widely used

for the dewatering of algae larger than 70 μm in size via natural gravity mediated sedimentation (Richardson, 2018). Chatsungnoen and Chisti (2016a,b) have developed a continuous flow process for dewatering algal slurries, where flocculation mediated sedimentation is used to concentrate *Nannochloropsis salina* effectively. The flocculation-mediated sedimentation uses aluminum sulfate as an inexpensive and safe flocculant to recover algal biomass suspension with 0.5 g/L of initial dry biomass concentration (Chatsungnoen and Chisti, 2016a). Golzarijalal et al. (2018) evaluated the algal sheer-induced flocculation and sedimentation dewatering process using a coupled computational fluid dynamics (CFD) population balance approach. The results showed that variations in the algal shear rate affect sedimentation rate, flocculation behavior, and recovery efficiency. Even though gravity sedimentation is usually considered to be a simple and inexpensive method, algal concentration achieved is lower without flocculation, and this reduces its reliability (Barros et al., 2015). Similarly, the concentration of algae obtained from lamella separators is low, and this can be improved by adding thickening agents such as polyelectrolyte (Pant and Adholeya, 2007) and vibration lamellar thickeners (Zhou et al., 2012).

9.3.5 Flotation

In this method, the suspension of solid-liquid algae culture is interacted with gas or air, where the solid particles surface is attached with gaseous molecules and the particles are carried to the liquid surface and float (Garg et al., 2012). The size and surface charge of the algae are the significant factors that determine the efficiency of the flotation process. The particle size of the algal cells has to be below 500 μm and the surface charge facilitates stability. Stable particles result in enhanced particle-air contact (Laamanen et al., 2016). Dissolved air flotation, dispersed air flotation (Ndikubwimana et al., 2016), electrolytic flotation (Garg et al., 2012), and foam fractionation (Coward et al., 2013) are the conventional flotation methods that are employed for algal dewatering processes.

Recently, Shi et al. (2017) studied the synergistic effect of electro-flocculation and flotation for *Chlorella vulgaris* culture dewatering using aluminum electrolysis. The result revealed that increase in ammonium present in the algae growth medium elevated the recovery of algae (Shi et al., 2017) via electrostatic interactions. Likewise, Garg et al. (2015) demonstrated that the marine algae *Tetraselmis* sp. M8 can be separated from an aqueous medium via froth flotation using various surfactants, namely, *N*-dodecylpropane-1,3-diamine hydrochloride (DN2), dodecyl amine hydrochloride (DAH), sodium dodecyl sulfate (SDS), and dodecyl pyridinium chloride (DPC), at different pH conditions. The results showed that the DPC at 15 ppm helped the dewatering process, leading to a 23-fold increase in concentration and 99% recovery of marine microalgae (Garg et al., 2015). Gemini surfactant (Huang et al., 2019), liquid biphasic flotation (Phong et al., 2017), flocculant-free electrolytic system (Luo et al., 2017), and ozone flotation (Oliveira et al., 2018) are other advancements in flotation methods for algae dewatering.

9.3.6 Electrophoresis methods

The addition of chemicals for algae dewatering increases the concentration of total dissolved solids in water and may result in water pollution and environmental toxicity (Mishra et al., 2004; Verma et al., 2012). Hence, electrophoresis methods are employed for coagulation, flotation, and flocculation to separate water and algal cells via electric field application (Vandamme et al., 2011). In recent times, dewatering of algae has been performed via the combination of different methods such as electro-coagulation-flocculation in batch mode (Fayad et al., 2017), and electrocoagulation-flotation (Golzary et al., 2015). Likewise, electro-flotation is performed using alternating current (Florentino et al., 2019), iron and aluminum spiral electrodes (Baierle et al., 2015), stainless steel electrodes (Ghernaout et al., 2015), and corrosive electrodes (Hung, 2017) to enhance the dewatering efficiency. In addition, biocathode coupled electro-coagulation cell (Dong et al., 2017), photovoltaic electro-flocculation-flotation (Rahmani et al., 2017), and sand-enhanced electro-flocculation (Xiong et al., 2015) have been employed to improve the dewatering performance of flocculation. It is noteworthy that the process of electrophoresis-assisted dewatering has to be optimized for each algal species depending on their size and surface charge. Electrophoretic dewatering of algae requires further improvement and extensive research before being released for full-scale commercial application.

9.4 Conventional drying methods for algae processing

Dewatering processes separate water from algal cells, and drying is used to remove water completely to generate a dry biomass. The drying process is another crucial step that determines the yield of value-added products from the algal culture. Increasing the temperature of dewatered algal slurry by heating it via various sources such as solar and convection are the conventional drying methods used in large-scale production of algal biomass. Spray-drying, freeze-drying, roller/drum drying, fluidized bed drying, and infrared light-mediated drying methods are used to address the drawbacks of conventional drying methods and to enhance the yield of value-added products from algal biomass (Chen et al., 2015; Fig. 9.3).

9.4.1 Solar drying

Drying of algal cultures in direct sunlight in an open area is called solar drying, and is considered the simplest and cheapest drying method. However, this method requires a large drying surface and longer duration for drying completion (Brennan and Owende, 2010). Open and closed are the two different types of solar drying processes. Open drying results in slow drying of algal cultures due to decreased insolation. Open solar drying may be beneficial in tropical regions, where solar energy is abundant (Doucha et al., 2005). Closed solar drying requires a temperature of 35–60°C for about 3–5 h of drying to generate a final moisture content below 10% (Ho et al., 2019). Overheating is a major challenge in this method

Fig. 9.3
Conventional drying methods for commercial algal biomass production.

as it can disrupt the algal cells, denature value-added products, and reduce yield (Show et al., 2015b). In recent times, solar dryers have been used extensively for the drying of algae to avoid overheating and to enhance drying efficiency (Elberis et al., 2016).

9.4.2 Convective drying

Algal dehydration is another name for convective drying. This method uses convective hot air drying such as oven drying or microwave drying (Desmorieux and Decaen, 2005; Suganya et al., 2016). It is reported that algal species can withstand a specific threshold temperature below which they can be dried to avoid denaturing and disruption of cells (Guldhe et al., 2014). Thus, it is important to optimize the drying conditions on a small scale before applying to a large scale (Villagracia et al., 2016). Draft oven (Oliveira et al., 2010), convective tunnel drying (Simioni et al., 2019), continuous conveyer belt dryer (Hosseinizand et al., 2017), and convective tray drying (Larrosa et al., 2018) are being used for drying algal biomass. Convective drying can also lead to overheating of the algal biomass if the temperature is not properly controlled.

9.4.3 Spray-drying

Spray-drying is highly recommended for drying high-value products from algae. It possesses the ability to generate algal biomass with high yield and quality. Further, this method can also retain more nutrients, especially proteins, compared to convective drying (Chaiklahan et al., 2018). Recently, a spray-drying method was used to obtain high-quality chlorophyll *a* and total carotenoid from *Isochrysis galbana* and *Nannochloropsis oculate*

biomass. These high-value products are used as coloring agents in food products. The spray-drying process affected the color and textural properties of the products (Palabiyik et al., 2018). The steps involved in spray-drying of algae are liquid atomization, gas or droplet mixing, and drying of liquid droplets. The atomized water droplets are sprayed downward and hot gases are passed into the vertical column. The drying process is completed within a few seconds, the dried algae or algal products are collected from the bottom, and the waste gas stream is exhausted via a cyclonic dust separator of the tower (Show et al., 2015a). Spray-drying is less susceptible to lipolysis upon algal storage, and allows carotenoids to oxidize readily, compared to freeze-drying methods (Ryckebosch et al., 2011). Thus, it is essential to characterize the nutrients that are lost or retained before spray-drying is used on a large scale.

9.4.4 Freeze-drying

Freeze-drying can preserve the biomolecular contents of the algal biomass without disrupting their cell wall. Generally, the algae or algal products are frozen for 24 h at −70°C and later subjected to freeze-drying for 5 days (Wong and Cheung, 2001). Freeze-drying helps in retaining the nutritional quality of algae compared to oven drying, and it is suitable for applications relating to functional foods (Bennamoun et al., 2015). In another study, the *Sargassum muticum* algae culture was freeze-dried to avoid degradation of thermolabile compounds. The freeze-dried algae biomass was used to extract phenolics, fatty acids, and fucoxanthin via the supercritical CO_2 extraction method (Conde et al., 2015). Recently, freeze-dried *Chlorella vulgaris* and *Nannochloropsis oculate* biomass were used for selective extraction of neutral lipids. The results showed that freeze-drying plays a significant role in the extraction process in generating neutral lipids along with phospholipids and glycolipids (Obeid et al., 2018). Freeze-drying was also used to retain eicosapentaenoic acid (EPA) from the biomass of *Nannochloropsis salina*. The results showed that the drying time and freezing temperature have a profound effect on the oxidation of carotenoids, EPA, and tocopherols after 56 days of storage (Safafar et al., 2017).

9.4.5 Other drying methods

In addition to these drying methods, there are some further novel and hybrid methods that are used to dry and obtain high-value products from algae. For example, roller/drum (Fasaei et al., 2018; Nicolò et al., 2017), fluidized-bed (Azadi et al., 2015), rotary, flash, incinerator, vacuum-shelf drying, cross-flow air drying (Show et al., 2015b), swirl flash (Ljubic et al., 2019), and infrared drying (Ghnimi et al., 2019) are some novel methods that are used to dry algal biomass. Each method has specific advantages and disadvantages. Thus, it is highly recommended to optimize and select the best drying method for a specific algal application. Moreover, it is important to monitor the effect on algal disruption to avoid the loss of high-value products.

9.5 Latest trends in dewatering and drying of algae for commercialization

The recent advancements in nanotechnology have made it possible to use nanosized particles and materials in most of the commercial algal processes to enhance dewatering and drying. Nanoparticles are widely used in dewatering, as the large surface area of the nanosized particles binds with algal cells and removes water molecules from them. The binding of nanoparticles with algae is facilitated via electrostatic force of attraction, which increases their aggregation capacity and enhances separation. Several magnetic nanoparticles are used for magnetophoretic separation of algae and water molecules in dewatering processes. Recently, surface functionalized iron oxide nanoparticles were used to enhance the separation of *Chlorella* and water molecules via centrifugation (Toh et al., 2014). Nanoparticles are also used in the flocculation of algal culture. Magnetite nanoparticles easily attach to the microalgal cells via surface functionalization and electrostatic attractions, and this elevates the viscosity and density to facilitate formation of flocs and sedimentation. The magnetic nanoparticles can be removed from the microalgal biomass via lateral centrifugal and magnetic sedimentation (Matsuda et al., 2016). Magnetic nanoparticles grafted with amino-rich dendrimer (Wang et al., 2016), cationic surfactant-decorated iron oxide nanoparticles (Seo et al., 2016), chitosan/magnetic nanoparticle composites (Lee et al., 2013), and highly charged cellulose-based nanocrystals (Vandamme et al., 2015b) are the recent nanosized flocculants that are used as dewatering agent in algal dewatering.

In filtration-based dewatering processes, nanofiltration using a filter membrane with nanosized pores is widely used to remove water from algae cultures (Cicci et al., 2013). In the case of dewatering, nano-filter-based membranes are designed to filter algal cells and are permeable to water molecules (Lopresto et al., 2017). Crosslinked polyvinylidene fluoride (PVDF) (Van Goethem et al., 2019), nano titanium dioxide-modified hollow fiber membrane (Hu et al., 2015), coal-based carbon membrane (Tao et al., 2017), and nylon 6, 6 nanofiber (Azizo et al., 2017) are recently fabricated membranes with nanosized pores for algal culture dewatering. Similar to flocculation, nanoparticles are used in gravity sedimentation processes for algal dewatering. Recently, silica nanoparticles were used to form micro-clusters to facilitate gravity sedimentation of green algae and dewater the algal cells (Tan et al., 2018). Moreover, organo-building blocks of nanoclays (Farooq et al., 2013), needle-shaped hydroxyapatite nanoparticles (MubarakAli, 2019), and magnetic nanoparticles modified microalgal cells (Safarik et al., 2016) are some of the nanomaterial-based agents used to enhance gravity sedimentation process.

Similar to flocculation and sedimentation, flotation based on magnetic nanoparticles is also used in the dewatering process of microalgae (Seo et al., 2017). BS-12 assisted silica nanoparticles for novel flotation column (Hu et al., 2018), iron oxide nanoparticle incorporated poly vinyl chloride (PVC) nanocomposites (Liu et al., 2019), and surface layered polymeric microspheres for buoy-bead flotation are some of the recent nanomaterial-based flotation

methods targeted toward enhanced algal biomass. In electrophoretic dewatering of algae cultures, nanoparticles such as nano-orifice (Zhao and Li, 2017), silver nanoparticles (Mozhayeva and Engelhard, 2017), titanium dioxide (Morelli et al., 2018), and nanoscale thermoplastic columns (Weerakoon-Ratnayake et al., 2016) are being investigated to enhance dewatering performance. However, although nanoparticles can improve algal dewatering processes, their toxicity and the cost of production remain challenges to commercial application (Toh et al., 2016; Table 9.1).

9.6 Future perspective

Conventional dewatering methods possess several drawbacks, which are being addressed via the introduction of novel hybrid methods such as forward osmotic dewatering (Kim et al., 2018), electro-osmotic dewatering (Li et al., 2018), electrocoagulation flocculants (Elazzouzi et al., 2017), and ozone flotation (Bravo et al., 2019). It is noteworthy that the addition of nanoparticles enhances algal dewatering processes and efficiency. However, the

Table 9.1: Examples of nanoparticles used in algal dewatering processes.

Dewatering method	Nanoparticles	References
Centrifugation	Surface functionalized iron oxide nanoparticles	Toh et al. (2014)
Flocculation	Iron oxide nanoparticles	Matsuda et al. (2016)
	Magnetic nanoparticles grafted with amino-rich dendrimer	Wang et al. (2016)
	Cationic surfactant-decorated iron oxide nanoparticles	Seo et al. (2016)
	Chitosan/magnetic nanoparticles composite	Lee et al. (2013)
	Highly charged cellulose-based nanocrystals	Vandamme et al. (2015b)
Nano-filtration	Crosslinked polyvinylidene fluoride (PVDF)	Van Goethem et al. (2019)
	Nano titanium dioxide-modified hollow fiber membrane	Hu et al. (2015)
	Coal-based carbon membrane	Tao et al. (2017)
	Nylon 6, 6 nanofiber	Azizo et al. (2017)
Sedimentation	Silica nanoparticles	Tan et al. (2018)
	Organo-building blocks of nanoclays	Farooq et al. (2013)
	Needle-shaped hydroxyapatite nanoparticles	MubarakAli (2019)
	Magnetic nanoparticles modified microalgal cells	Safarik et al. (2016)
Flotation	BS-12 assisted silica nanoparticles	Hu et al. (2018)
	Iron oxide nanoparticle incorporated PVC nanocomposites	Liu et al. (2019)
	Surface layered polymeric microspheres for buoy-bead flotation	Xu et al. (2018)
Electrophoresis	Nano-orifice	Zhao and Li (2017)
	Silver nanoparticles	Mozhayeva and Engelhard (2017)
	Titanium dioxide nanoparticles	Morelli et al. (2018)
	Nanoscale thermoplastic columns	Weerakoon-Ratnayake et al. (2016)

toxicity of nanoparticles can become a threat to applications for high-value products development (Suman et al., 2015). Thus, it is necessary to evaluate the toxicity of nanoparticles that are employed as dewatering agents. Green or biosynthesized nanoparticles are highly recommended for these applications as they are comparatively less toxic than chemically synthesized nanoparticles (Roy et al., 2018). Magnetic nanoparticles, synthesized via micro and macroalgal extracts, will be highly beneficial as less toxic agents for algal dewatering (El-Kassas et al., 2016; Siddiqi and Husen, 2016). Bio-flocculation using microbes, enzymes, and extracts as flocculants is also gaining much interest among researchers in the process of dewatering algal biomass (Ummalyma et al., 2017). Hybrid methods such as two-stage dewatering have also been investigated (Soomro et al., 2016). Nanoformulation of microalgal cells with nanoparticles that possess extreme thermal resistance will be beneficial in future to protect the algal cells and high-value products from excess heat during drying. This will facilitate high temperature drying to generate dry biomass powders for high-value products development.

9.7 Conclusion

This chapter gave an overview of the most common and the emerging dewatering and drying processes used to process algal biomass to yield high-value products. Improvements in conventional algal dewatering and drying processes can help in meeting the commercial demand of algal products. The latest reports of the advancements in algal dewatering and drying technologies forecast a better future to offer more effective algal dewatering processes under optimum economy. The introduction of nanotechnology has also enhanced the efficacy of algal dewatering processes.

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CHAPTER 10

Microalgae harvesting techniques

Ana Filipa Esteves, Cíntia Jesus Almeida, Ana Luísa Gonçalves, José Carlos Pires

LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

Chapter outline

10.1 Introduction 225
10.2 Challenges in microalgal biomass harvesting 228
10.3 Effective harvesting techniques for microalgal biomass recovery 229
10.4 Harvesting methods 231
10.4.1 Gravity sedimentation 232
10.4.2 Flocculation 235
10.4.3 Flotation 253
10.4.4 Centrifugation 259
10.4.5 Filtration methods 261
10.5 Selection of the most appropriate harvesting technique 268
10.6 Research needs 270
10.7 Conclusions 271
Acknowledgments 271
References 272

10.1 Introduction

Microalgae are a broad category of photosynthetic microorganisms that comprise the eukaryotic microalgae and the prokaryotic cyanobacteria, also known as blue-green algae. The number of microalgal species is not completely known, but some studies have referred that it may be comprised between 200,000 and some millions of microalgal species (Norton et al., 1996). Microalgae present a unicellular or simple multicellular structure, which determines their ability to grow in both aquatic and terrestrial ecosystems (Gouveia, 2011; John et al., 2011). In addition, microalgae can grow in diverse environmental conditions, even in the most extreme ones (Cellamare et al., 2010; De Morais and Costa, 2007; Mutanda et al., 2011; Pereira et al., 2011; Richardson et al., 2000; Valladares, 2004): (i) they can grow in both dimly lit sites

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and salt-marsh algal mats, exposed to full sunlight; (ii) although most microalgae are mesophilic, some species can grow in extreme temperature conditions, like those found in mountain snowfields, hot springs and desert soil; (iii) microalgae can be found in hypersaline environments; and (iv) some microalgal species are able to grow in extreme acidic and extreme alkaline media.

The huge taxonomic diversity and wide environmental distribution of microalgae make them singular microorganisms, with huge potential in several applications from diversified areas, such as environment, energy, pharmaceutical and cosmetic industries, aquaculture, human food, and animal feed (Allen et al., 2009; Brennan and Owende, 2010; Odjadjare et al., 2017; Spolaore et al., 2006). As photosynthetic microorganisms, microalgae can use carbon dioxide (CO_2) as a carbon source, assimilating it from the atmosphere or flue gas emissions. Accordingly, microalgae can be used as a carbon capture and storage technology, reducing the CO_2 concentration in the atmosphere (which has increased by up to 50% since the Industrial Revolution) (Allen et al., 2009; Costa et al., 2019; Ho et al., 2011; Sepulveda et al., 2019; Singh and Dhar, 2019; Tang et al., 2011). Besides CO_2 uptake, microalgae are also able to uptake nutrients, such as nitrogen and phosphorus, commonly found in wastewaters from different sources (Abdel-Raouf et al., 2012; Cai et al., 2013; Rawat et al., 2011; Silva-Benavides and Torzillo, 2012). Some studies have also referred to the removal of heavy metals and other organic and inorganic pollutants (Abdel-Raouf et al., 2012; Gentili and Fick, 2017; Mehta and Gaur, 2005; Rawat et al., 2011; Silva-Benavides and Torzillo, 2012). Thus, these microorganisms can play an important role in wastewaters' remediation. Finally, the produced biomass can be used as a raw material for different applications (Brennan and Owende, 2010; Hu, 2004; John et al., 2011; Odjadjare et al., 2017; Parmar et al., 2011; Singh et al., 2005; Spolaore et al., 2006): (i) it is suitable for human food and animal feed, as it is an important source of natural vitamins, proteins, minerals, and fatty acids; (ii) it can be used to extract several compounds, such as pigments, antioxidants, β-carotenes, proteins, polysaccharides, triglycerides, fatty acids, and vitamins, with high potential for the production of cosmetics, drugs, and functional food; and (iii) it can be used to answer the current demands for renewable energy resources, as the fatty acids present in microalgal biomass can be used for biodiesel production and the residual biomass can be fermented to produce bioethanol or anaerobically digested to produce methane and electricity.

Besides the biotechnological potential of microalgal biomass, production of these photosynthetic microorganisms presents several advantages over other photosynthetic organisms, such as terrestrial crops (Chisti, 2007; Hoyer et al., 2018; Pulz and Gross, 2004): (i) higher growth rates and biomass productivities can be achieved by microalgae; (ii) microalgal lipid contents and nutritional value are higher than those from terrestrial crops; (iii) microalgae can be produced in non-arable land, thus not competing with that intended for food production; (iv) low-quality waters can be used for microalgal biomass production, thus reducing biomass production costs (associated to nutrients supply) and environmental

impact (associated to freshwater use); and (v) microalgae can tolerate several environmental conditions, being able to grow in such diverse environments. However, microalgal biomass production and processing still present some challenges, especially in terms of process economics. The key steps typically include cultivation, biomass recovery/harvesting, extraction of the compounds with potential interest, and production of the desired products. Taking into account that microalgae grow in very dilute cultures (less than $0.5\,g\,L^{-1}$), the small size of microalgal cells (between 2 and 20 μm), and the negative charge present at their surface (which results in stable dispersed suspensions), microalgal separation from the culture medium represents a major fraction of the production costs (Milledge and Heaven, 2013; Muylaert et al., 2017; Singh and Patidar, 2018; Wang et al., 2015). Typically, microalgal harvesting accounts for 20%–30% of microalgal biomass costs (Barros et al., 2015; Grima et al., 2003), but fractions of up to 50% of microalgal biomass costs were also mentioned in the literature (Greenwell et al., 2009). Additionally, according to Amer et al. (2011), harvesting and dewatering equipment costs may represent 90% of the total equipment costs for microalgal biomass production in open systems.

Taking into account the high process costs associated with microalgal harvesting, researchers worldwide have focused their studies on the optimization of microalgal harvesting processes. Techniques currently applied include gravity sedimentation, flocculation, flotation, centrifugation, filtration, or a combination of these (Barros et al., 2015; Milledge and Heaven, 2013; Muylaert et al., 2017; Pahl et al., 2013; Singh and Patidar, 2018; Wan et al., 2015). Gravity sedimentation is the simplest and cheapest harvesting method, but the time required for microalgal sedimentation may result in biomass degradation, limiting its further applications. Flocculation may be an interesting method to improve harvesting kinetics, but the use of chemical and biological flocculants may contaminate microalgal biomass, also limiting its final application (Barros et al., 2015). Flotation techniques are characterized for high instability, which results in low biomass harvesting efficiencies (Uduman et al., 2010a; Wan et al., 2015). Centrifugation is one of the most commonly used methods for microalgal harvesting, especially when the final application of microalgal biomass is the production of high-added value products. It allows high harvesting efficiencies, but it is an expensive and energy-demanding method (Barros et al., 2015; Grima et al., 2003; Uduman et al., 2010a; Wan et al., 2015). Finally, filtration techniques are far less expensive than centrifugation. However, they are time-consuming and membrane fouling is very common (Chen et al., 2011; Wan et al., 2015). These insights suggest that it is not possible to establish a single method that can be used for all microalgal biomass applications. In addition to process costs, optimization of microalgal harvesting techniques should take into account the harvesting time and final application of microalgal biomass.

This study presents the main challenges faced in microalgal biomass recovery from the culture medium, with an updated overview of the currently applied methods and main modifications that have been performed to improve harvesting efficiencies and reduce

process costs. Since no universal method can be used for all microalgal species and applications, special attention is given to the main advantages and disadvantages of each method and their suitability for different target products.

10.2 Challenges in microalgal biomass harvesting

Microalgal growth in the form of stable dispersed suspensions constitutes one of the main challenges in the separation of microalgal biomass from the culture medium. This dispersed state is mainly related with the presence of functional groups (hydroxyl, —OH, carboxyl, —COOH, and amine, $—NH_2$) on cells' surface, which typically confer a slightly negative surface charge (Gonçalves et al., 2015; Ozkan and Berberoglu, 2013; Pahl et al., 2013). Functional groups present on cells' surface and surface charge are mainly influenced by the pH and ionic strength of the culture medium because these groups are ionizable, being protonated when exposed to low pH values, and deprotonated for high pH values. At the point of zero charge (PZC), which has been reported to be 3 for microalgae, an equilibrium between protonated and deprotonated species is observed, contributing to a neutralized surface charge (Hadjoudja et al., 2010; Stumm and Morgan, 2012). For the range of pH values commonly found in microalgal cultures, functional groups tend to be deprotonated, resulting in a negative surface charge (Grima et al., 2003; Hadjoudja et al., 2010). Evaluation of surface charge can be performed through zeta potential (ZP) measurements. These measurements provide information about the dominant charge present at cells' surface and also about colloids' stability. For high absolute ZP values, repulsive forces between microalgal cells tend to occur, prevailing over van der Waals forces (usually attractive) and resulting in a highly stable dispersed suspension. On the other hand, when the absolute ZP tends to zero, the attractive forces (van der Waals) overlap repulsive ones, negatively influencing the suspensions' stability and promoting aggregates formation (De Schryver et al., 2008; Gonçalves et al., 2015; Zita and Hermansson, 1994). Common ZP values determined for microalgal cells range between −10 and −48 mV (Gonçalves et al., 2015; Henderson et al., 2008), indicating that microalgal species tend to grow in such stable suspensions. However, ZP is highly dependent on the pH and ionic strength of the culture medium (Pahl et al., 2013), meaning that microalgal harvesting can be improved by altering suspensions' stability through changes in the pH and ionic strength of the culture medium. For example, when studying the influence of pH on ZP and flocculation efficiency of the microalga *Desmodesmus* sp. F51, Ndikubwimana et al. (2015) concluded that: (i) at pH 3, surface charge was only slightly protonated (ZP of +7.12 mV), resulting in a flocculation efficiency of 79%; and (ii) at pH 10, absolute ZP values increased (ZP of −18.95 mV), contributing to suspensions' stability and reducing flocculation efficiency to 25%.

Microalgal cells' shape, size, and density also influence the separation from the culture medium, as these properties determine settling rates (Pahl et al., 2013; Show et al., 2013; Uduman et al., 2010a). Microalgal cells are known for their different shapes (spherical,

ring-shaped, rods, or chains) and different mobility abilities (Pahl et al., 2013). In addition, microalgal cells can be organized in different forms: unicellular, colonial, and filamentous (Tomaseli, 2004). Such diversity results in different interactions with the culture medium and settling abilities, thus influencing biomass recovery and the development of a single method that can be universally used. For example, when trying to separate the unicellular microalga *Chlorella ellipsoidea* and the colonial microalga *Botryococcus braunii* through magnetic separation, Xu et al. (2011) concluded that harvesting efficiencies obtained for the unicellular microalga were higher than those obtained for the colonial one, which may be due to the higher specific surface area observed for the unicellular microalga. To achieve similar efficiencies, an increased concentration of magnetic particles was necessary to recover *B. braunii*. Regarding microalgal cells' size, usually these values are small, ranging between 2 and 20 μm, according to microalgal species and growth stage (Barros et al., 2015; Pahl et al., 2013; Show et al., 2013). The small size of microalgal cells results in lower settling velocities and makes the separation of microalgal biomass through some methods (e.g., filtration) more difficult (Gultom and Hu, 2013). Finally, microalgae grow as very dilute cultures, hardly exceeding biomass concentrations of $0.5\,g\,L^{-1}$ in large-scale cultivation systems, and differences between cell density and culture medium density are almost negligible (Das et al., 2016; Laamanen et al., 2016; Ummalyma et al., 2017). The influence of both cell size and density on settling velocity can be explained by Stokes' Law (Eq. 10.1), which states that the settling velocity (υ, in $m\,s^{-1}$) is directly proportional to the square of the particles' radius and to the difference between the densities of microalgal cells and culture medium (Gultom and Hu, 2013; Milledge and Heaven, 2013):

$$\upsilon = \frac{2}{9} \cdot g \cdot \frac{r^2}{\eta} \cdot (\rho_s - \rho_l) \tag{10.1}$$

where g is the gravitational acceleration ($9.81\,m\,s^{-2}$), r is the cell radius (in m), η is the dynamic fluid viscosity (in $N\,s\,m^{-2}$), and ρ_s and ρ_l are the solid (microalgal cells) and liquid densities (in $kg\,m^{-3}$), respectively. Besides the referred physiological parameters, microalgal growth stage also influences the settling ability, as pH, cells' size, and zeta potential vary within the cultivation time (Barros et al., 2015). With the increase of photosynthetic activity and consequent consumption of inorganic carbon by microalgal cells, the pH of the culture medium tends to be high, altering the ionic strength of the culture medium. Furthermore, as microalgal growth tends to reach the stationary growth phase, a decrease in the metabolic activity and in cells' mobility is observed. Finally, an increase in cells' size can be observed in this growth phase, especially due to cell agglomeration (Danquah et al., 2009).

10.3 Effective harvesting techniques for microalgal biomass recovery

An effective harvesting procedure is characterized by high values of recovery efficiency (RE) and concentration factor (CF). These parameters are used to estimate the success of the

harvesting step, and together represent the effectiveness of the separation in terms of mass and volume of recovered biomass (Singh and Patidar, 2018).

RE (Eq. 10.2) can be defined as the mass of cells in the slurry (m_S) divided by the mass of cells present in the culture (m_C). On the other hand, CF (Eq. 10.3) corresponds to the ratio between microalgal biomass concentration in the slurry (X_S) and microalgal biomass concentration in the culture (X_C) (Pahl et al., 2013; Singh and Patidar, 2018).

$$\mathrm{RE}\,(\%) = \frac{m_S}{m_C} \cdot 100 \tag{10.2}$$

$$\mathrm{CF} = \frac{X_S}{X_C} \tag{10.3}$$

The mass and concentration of microalgal biomass can be estimated through the determination of microalgal cell counts, dry weight (DW), ash free dry weight (AFDW), chlorophyll content, and absorbance (optical density, OD). Total suspended solids (TSS) and turbidity measurements can also be performed to estimate the mass and concentration of microalgal cells, especially when the applied technique is flotation (Pahl et al., 2013; Singh and Patidar, 2018). Although RE and CF are important parameters that should be taken into account to determine the success of the recovery method used, other parameters should also be considered: the ability to process large volumes, process economics, processing time, level of moisture achieved, suitability of harvested biomass for different applications, and suitability for recovering different microalgal species in different growth stages.

Owing to the dilute nature of microalgal cultures, large volumes should be processed to achieve considerable biomass concentrations (Muylaert et al., 2017). Accordingly, an effective harvesting procedure should be able to process large culture volumes at reduced costs and with reduced energy requirements (Danquah et al., 2009; Muylaert et al., 2017). The amount of water present in the final product, also known as moisture level, is another important parameter because it can significantly influence further downstream process and economics, especially when dehydration of microalgal biomass is required. The moisture level in the final product differs according to the harvesting method selected. For example, the moisture level in biomass recovered through sedimentation is significantly higher than the one present in biomass recovered through centrifugation (Grima et al., 2003). Accordingly, the selection of an appropriate method should take into account the maximum moisture level allowed, in order not to compromise further downstream processes. Appropriate harvesting technologies should also be as inert as possible, thus not interfering with final applications of microalgal biomass (Barros et al., 2015; Muylaert et al., 2017; Uduman et al., 2010a). For food and feed applications, for example, it is desirable that the harvesting process does not contaminate microalgal biomass or interfere with cells' integrity. Regarding the extraction of some metabolites with potential interest, it is important that the harvesting procedure avoids the use of chemical additives that

may hinder further extraction steps. Due to the huge diversity of microalgal species (Tomaseli, 2004), selection of the most appropriate harvesting technique should take into account their main characteristics, namely cell size, surface charge, motility ability, and growth stage (Barros et al., 2015; Muylaert et al., 2017). Since the selection of an appropriate method for such a diversity of features may be difficult to achieve, adaptation/adjustments to the harvesting techniques may be required, according to the microalgal species being produced and to the culturing conditions used (Muylaert et al., 2017).

Finally, microalgal culturing deals with high amounts of water (Yang et al., 2011), which should be recycled in the harvesting step to reduce the environmental impact of microalgal biomass production associated with freshwater requirements. Accordingly, the selected harvesting procedure may allow culture medium recycling. In addition, this procedure may not contribute to culture medium contamination, so that it can be recycled in the production unit without a pretreatment step (Barros et al., 2015; Muylaert et al., 2017; Uduman et al., 2010a).

10.4 Harvesting methods

Currently used harvesting methodologies include mechanical, chemical, biological, and electrical methods, as exemplified in Fig. 10.1. These methods differ in the separation principle and also on the REs obtained, volume processed, processing cost, and energy requirements. Some of these methods are considered thickening methods, as they allow the recovery of biomass with approximately 2%–7% TSS (corresponding to a CF of 100–200), and others are

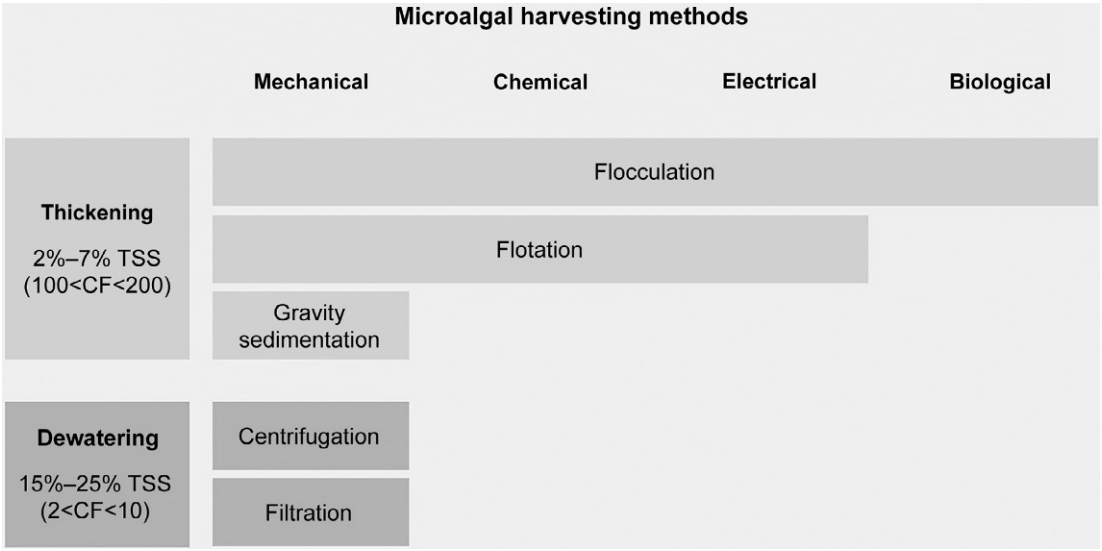

Fig. 10.1
Schematic representation of current harvesting methods used for microalgal biomass recovery.

considered dewatering methods, as biomass recovered presents between 15% and 25% TSS (CF of 2–10). Gravity sedimentation, flocculation, and flotation are examples of thickening methods, whereas centrifugation and filtration are included in dewatering ones (Barros et al., 2015; Brennan and Owende, 2010; Uduman et al., 2010a).

The selection of an appropriate and cost-effective technique for the huge diversity of microalgal species constitutes a major bottleneck in microalgal biomass production. The following sections describe the main microalgal harvesting techniques currently in use, focusing on the most recent developments in this field and on the main advantages and disadvantages of each (which are summarized in Table 10.1).

10.4.1 Gravity sedimentation

Gravity sedimentation is considered one of the simplest methods for microalgal harvesting, and consists of biomass settling by the action of gravitational forces (Pahazri et al., 2016; Salim et al., 2013). This method is very attractive from energetic and economic points of view because it requires very little energy and low equipment and operational costs (Pahl et al., 2013). Regarding equipment requirements, simple settling tanks or inclined settlers, also known as lamella settling tanks, are the most commonly used (Grima et al., 2003; Muylaert et al., 2017). Out of these, lamella settling tanks are usually preferred, as the area available for settling is substantially increased in this configuration (Al Hattab et al., 2015; Muylaert et al., 2017). The main drawback associated with this method relates to the time required for microalgal settling. Taking into account the small size of microalgal cells and the similar densities observed between microalgal cells and the culture medium, sedimentation time is usually high (as demonstrated by Stokes' Law, Eq. 10.1) (Milledge and Heaven, 2013), which can lead to biomass deterioration during the harvesting process and loss of quality in the final product (Singh et al., 2013). Choi et al. (2006) evaluated settling velocities of several microalgal species (*Synedra acus*, *Nitzschia acicularis*, *Nitzschia fruticosa*, *Aulacoseira granulate*, *Asterionella* sp., *Oscillatoria* sp., *Phormidium* sp., *Scenedesmus* sp., and *Ankistrodesmus* sp.), reporting values between 0.1 and 2.6 $cm\,h^{-1}$ for larger microalgal species and values below 1 $cm\,h^{-1}$ for smaller ones. These small values lead to increased harvesting times, which may be unfeasible for food and feed applications, for example. However, a few exceptions can occur, especially for high-size microalgal species, such as *Arthrospira* sp., and for microalgal species that have an innate ability to form aggregates. For example, Wang et al. (2014) concluded that RE obtained in the separation of the unicellular microalga *Chlorella vulgaris* through gravity sedimentation was lower than the one obtained for *Scenedesmus dimorphus*, which occurs naturally in the form of aggregates.

Owing to the high settling times obtained when harvesting microalgae through gravity sedimentation, it is very common to apply a coagulation/flocculation step prior to sedimentation. This preliminary step promotes aggregates/flocs formation, increasing

Table 10.1: Advantages and disadvantages of the most widely used microalgal harvesting methods and most appropriate applications for the recovered biomass.

Harvesting method	Advantages	Disadvantages	Final application of biomass/Target product
Gravity sedimentation	• Simple method • Low equipment and operational costs • Low energy consumption ($<0.1\,kWh\,m^{-3}$) • Chemical-free method	• Low REs (<30%, with final concentrations of 2% TSS) • Time-consuming • Deterioration of microalgal biomass can occur	• Low-valued products - Biofuels
Flocculation	• High REs (60%–99%, with final concentration of 3%–15% TSS) • Simple and cost-effective method (especially in auto- and bioflocculation) • Low energy consumption ($0.35\,kWh\,m^{-3}$) • Able to process large culture volumes • Suitable for almost all microalgal species (in the case of chemical and electroflocculation) • Bioflocculants are biodegradable and non-toxic	• Chemicals used in chemical flocculation may be expensive • Process strongly depends on the pH and ionic strength of the culture medium • Autoflocculation and interaction with flocculant microorganisms is species-specific • Chemical and microbial contamination of microalgal biomass can occur • Water recycling is limited	• Low-valued products - Biofuels
Flotation	• Simple and fast method • Suitable for almost all microalgal species (particle sizes <500 μm)	• Moderate REs (50%–90%, with final concentrations of 7% TSS) • High energy consumption (up to $7.6\,kWh\,m^{-3}$) • High equipment and operational costs • Unable to process large culture volumes	• Low-valued products - Biofuels

Continued

Table 10.1: Advantages and disadvantages of the most widely used microalgal harvesting methods and most appropriate applications for the recovered biomass—cont'd

Harvesting method	Advantages	Disadvantages	Final application of biomass/Target product
		• Requires the use of chemicals (surfactants)	
Centrifugation	• High REs (>90%, with final concentrations of 2%–25% TSS) • Fast method • Able to process large culture volumes • Suitable for almost all microalgal species • Chemical-free method	• High energy consumption (between 0.53 and 20 $kWh\,m^{-3}$) • High operational and maintenance costs • Microalgal cell damage can occur	• High-valued products - Nutraceuticals - Cosmetics - Pharmaceuticals
Filtration	• High REs (70%–90%, with final concentrations of 5%–18% TSS) • Low equipment costs (pumping and membrane replacement, exclusively) • Low energy consumption (gravitational and pressure filtration, between 0.1 and 0.9 $kWh\,m^{-3}$) • Preservation of microalgal biomass • Chemical-free method • Allows water recycling	• Time-consuming (requires pressure or vacuum) • Membrane fouling • Frequent membrane replacement and/or periodic cleaning • High energy consumption (vacuum filtration, between 0.1 and 5.9 $kWh\,m^{-3}$) • Unable to process large culture volumes • Unsuitable for small-sized microalgae	• Moderate-valued products - Human food - Animal feed

RE, recovery efficiency; *TSS*, total suspended solids.

Adapted from Al Hattab, M., Ghaly, A., Hammoud, A., 2015. Microalgae harvesting methods for industrial production of biodiesel: critical review and comparative analysis. J. Fundam. Renew. Energy Appl. 5, 154–179; Collotta, M., Champagne, P., Mabee, W., Tomasoni, G., Leite, G.B., Busi, L., Alberti, M., 2017. Comparative LCA of flocculation for the harvesting of microalgae for biofuels production. Procedia CIRP, 61, 756–760; Laamanen, C.A., Ross, G.M., Scott, J.A., 2016. Flotation harvesting of microalgae. Renew. Sustain. Energy Rev. 58, 75–86; Singh, G., Patidar, S.K., 2018. Microalgae harvesting techniques: a review. J. Environ. Manag. 217, 499–508.

particles' size and hence the settling rate. Japar et al. (2017) studied the effect of a flocculation step prior to sedimentation on the REs of the microalgae *Chlorella* sp. UKM2, *Coelastrella* sp. UKM4, and *Chlamydomonas* sp. UKM6. When applying gravity sedimentation exclusively, REs reported were 27%, 25%, and 21% for *Chlorella* sp. UKM2, *Coelastrella* sp. UKM4, and *Chlamydomonas* sp. UKM6, respectively. These values increased significantly in the experiments where flocculation using aluminum salts, $Al_2(SO_4)_3$, was applied prior to sedimentation (88%, 81%, and 76%, respectively).

10.4.2 Flocculation

Flocculation is a consolidated technique applied in treatment processes to remove colloidal particles present in water and wastewater (Bratby, 2016). As it is a low-cost process, flocculation has been proposed as a technique for harvesting different microalgal species, at both the laboratory and industrial scales (Chen et al., 2011, 2013; Grima et al., 2003; Uduman et al., 2010a). Microalgae harvesting by flocculation consists in the formation of aggregates or flakes from cells and suspended solute particles present in the culture broth by adding different types of flocculants or coagulants (Pragya et al., 2013). The formation of flakes (larger than microalgal cells) facilitates the sedimentation process. However, an additional harvesting step is generally required because the collected biomass contains a significant amount of water. Accordingly, it is common to use a filtration or centrifugation step for biomass dehydration. As mentioned in Section 10.2, in general, microalgal cells' surface is negatively charged, due to the ionizable groups present (Chen et al., 2011; Harun et al., 2010; Uduman et al., 2010b). In addition, microalgal suspensions are typically stable in the dispersed form. Therefore, to allow aggregates formation and promote flocculation, suspensions' stability must be interrupted by the neutralization of the charge load on cells' surface (Chen et al., 2011; Salim et al., 2011). The different flocculation techniques involve (Taher et al., 2011): (i) innovative concepts based on microalgal biology, such as autoflocculation; (ii) traditional flocculation methods commonly applied in other industries, such as chemical flocculation; (iii) ecological methods using bioflocculants; (iv) the interaction between microalgae and other microorganisms; and (v) the use of emerging technologies with physical flocculation methods. These techniques are detailed in the following sections, and Table 10.2 presents microalgal REs already obtained by different researchers.

10.4.2.1 Autoflocculation

The term "autoflocculation" was introduced by Golueke and Oswald (1970). It refers to the mechanism in which, at a certain growth stage, microalgae produce compounds with flocculant activity (González-Fernández and Ballesteros, 2013). This flocculation process may occur due to (Sing et al., 2013): (i) the coprecipitation of ions induced by high pH; (ii) the release of extracellular polymeric substances (EPS); or (iii) the interaction between the cells themselves. In addition to the effect of high pH (due to CO_2 consumption by photosynthetic activity of

Table 10.2: Recovery efficiencies reported for microalgal harvesting through flocculation.

Flocculation type	Microalgae	Method	Volume processed/Flow rate	Operational conditions	RE (%)/CF	Reference
Autoflocculation	*Chlorella vulgaris*	pH induction	V: 100 mL	X_C: 0.5 g DW L^{-1}; pH: 9.7–10.8; CC: 5.75 mM of NaOH/4.00 mM of $Ca(OH)_2$/ 8.00 mM of KOH	RE: 95%	Vandamme et al. (2012)
	Dunaliella tertiolecta	pH induction	V: 50 mL	OM: batch; X_C: 0.25 g DW L^{-1}; pH: 8.6–10.5; CC: 1.0 M of NaOH	RE: 90%	Horiuchi et al. (2003)
Chemical flocculation	*Chlorella minutissima*	Coagulation by addition of metallic salts	V: 20 mL	X_C: 2.20×10^8 cells·mL^{-1}; CC: 0.75 g L^{-1} of $Al_2(SO_4)_3$; RT: 2 h	RE: 80%	Papazi et al. (2010)
	Chlorella minutissima	Coagulation by addition of metallic salts	V: 20 mL	X_C: 2.20×10^8 cells·mL^{-1}; CC: 0.75 g L^{-1} of $Fe_2(SO_4)_3$; RT: 4 h	RE: 80%	Papazi et al. (2010)
	Chlorella minutissima	Coagulation by addition of metallic salts	V: 20 mL	X_C: 2.20×10^8 cells·mL^{-1}; CC: 0.5 g L^{-1} of $AlCl_3$; RT: 1 h	RE: 80%	Papazi et al. (2010)
	Chlorella minutissima	Coagulation by addition of metallic salts	V: 20 mL	X_C: 2.20×10^8 cells·mL^{-1}; CC: 0.75 g L^{-1} of $FeCl_3$; RT: 3 h	RE: 80%	Papazi et al. (2010)
	Nannochloropsis oculata	Coagulation by addition of metallic salts	V: 100 mL	X_C: 1.7 g L^{-1}; pH: 8.3; CC: 438.1 µM of $Al_2(SO_4)_3$	RE: 94%	Shen et al. (2013)
			V: 100 mL	X_C: 2.2 g L^{-1}; pH: 7.9 CC: 383.5 µM of $FeCl_3$	RE: 88%	Shen et al. (2013)

	Dunaliella salina	Coagulation by addition of metallic salts	V: 100 mL	X_C: 0.3 g DW L^{-1}; pH of 7.5; CC: addition of 1 mM $FeCl_3$; RT: 30 min	RE: 90%–97%	Pirwitz et al. (2015)
	Scenedesmus obliquus	Flocculation with polymers	V: 50 mL	X_C: 6.35 g DW L^{-1}; CC: 2 mg L^{-1} of high molecular weight polyacrylamide; RT: 5 min	RE: 90%	Mikulec et al. (2015)
	Tetraselmis chuii	Flocculation with chitosan	V: 1 L	CC: 40 mg L^{-1} of chitosan; pH: 5.03	RE: 80%	Heasman et al. (2000)
	Nannochloropsis sp.	Flocculation with nanochitosan	–	X_C: 1.33×10^8 cells mL^{-1}; CC: 60 mg L^{-1} of nanochitosan; pH: 9	RE: 98%	Farid et al. (2013)
	Parachlorella kessleri	Flocculation with cationic starch	–	X_C: 0.30 g L^{-1}; CC: 20 mg L^{-1} of cationic starch (Greenfloc 120); pH: 10	RE: 90%	Vandamme et al. (2010)
	Scenedesmus obliquus	Flocculation with cationic starch	–	X_C: 0.15 g L^{-1}; CC: 10 mg L^{-1} of cationic starch (Greenfloc 120)	RE: 80%	Vandamme et al. (2010)
	Chlorella vulgaris	Flocculation with *Moringa oleifera* derivatives	–	FAC: 1 g L^{-1} of seed flour; pH: 9.2; RT: 120 min	RE: 89%	Teixeira et al. (2012)
	Chlorella vulgaris	Flocculation with *Moringa oleifera* derivatives	V: 600 mL	OM: batch; FAC: 30 mg L^{-1} of seed flour; pH: 6.9–7.5; RT: 20 min	RE: 95%	Abdul Hamid et al. (2014)

Continued

Table 10.2: Recovery efficiencies reported for microalgal harvesting through flocculation—cont'd

Flocculation type	Microalgae	Method	Volume processed/Flow rate	Operational conditions	RE (%)/CF	Reference
Bioflocculation	*Botryococcus* sp.	Flocculation with cationic inulin (derived from chicory roots)	V: 200 mL	FAC: 60 mg L^{-1}; pH: 7.4; RT: 15 min	RE: 88.6%	Rahul et al. (2015)
	Chlamydomonas sp.	Flocculation with natural bioflocculant (cationic guar gum)	V: 200 mL	X_C: 0.89 mg L^{-1}; FAC: 100 ppm; pH: 7.3; RT: 15 min	RE: 92.1%	Banerjee et al. (2013)
	Chlorella vulgaris	Flocculation with seeds of *Strychnos potatorum*	V: 50 mL	FAC: 100 mg L^{-1}; pH: 7.0; RT: 30 min	RE: 99.7%	Abdul Razack et al. (2015)
	Chlorella vulgaris	Flocculation with poly γ-glutamic acids from *Bacillus subtilis*	V: 150 mL	X_C: 0.57 g L^{-1}; FAC: 22.03 mg L^{-1} of γ-PGA; pH: 8.4; RT: 2 h	RE: 91% CF: 20.5	Zheng et al. (2012)
	Chlorella protothecoides	Flocculation with poly γ–glutamic acids from *Bacillus subtilis*	V: 150 mL	X_C: 0.60 g L^{-1}; FAC: 19.82 mg L^{-1} of γ-PGA; pH: 7.8; RT: 2 h	RE: 98% CF: 29.8	Zheng et al. (2012)
	Botryococcus braunii	Flocculation with bioflocculants from *Pestalotiopsis* sp. KCTC 8637P	V: 50 mL	FAC: 100 mg L^{-1} of Pestan; pH: 11	RE: 90%	Lee et al. (1998)
	Chlorella vulgaris	Flocculation by cocultivation with *Aspergillus niger*	–	X_C: 2.55×10^9 cells·L^{-1}; FIC: 8.50×10^6 spores L^{-1}; pH: 7.1	RE: 90%	Gultom et al. (2014)
	Tetraselmis suecica	Flocculation by cocultivation with *Aspergillus fumigatus*	V: 250 mL	X_C: $7–12 \times 10^8$ cells·mL^{-1}; FIC: $1.5–2.0 \times 10^7$ spores L^{-1}; RT: 24 h	RE: 90%	Muradov et al. (2015)

	Chlorella protothecoides	Flocculation by cocultivation with *Aspergillus fumigatus*	V: 250 mL	X_C: 1–3 × 10^9 cells mL^{-1}; FIC: 1.5–2.0 × 10^7 spores L^{-1}; RT: 24 h	RE: 90%	Muradov et al. (2015)
	Chlorella vulgaris	Flocculation by cocultivation with *Aspergillus nomius*	V: 1 L	X_C: 0.4 g L^{-1}; F/M: 4:1 (*w*/w); pH: 7.0; RT: 4 h	RE: 97%	Talukder et al. (2014)
	Nannochloropsis sp.	Flocculation by cocultivation with *Aspergillus nomius*	V: 1 L	X_C: 0.4 g L^{-1}; F/M: 4:1 (w/w); pH: 6.0; RT: 3 h	RE: 94%	Talukder et al. (2014)
	Chlorella vulgaris CNW11	Flocculation by cocultivation with *Chlorella vulgaris* JSC-7	–	FAC: 0.5 mg L^{-1} of cell wall polysaccharides (crude extract); pH: 7; RT: 1 h	RE: 80%	Alam et al. (2014)
	Scenedesmus obliquus	Flocculation by cocultivation with *Scenedesmus obliquus* AS-6-1	–	X_C: 6 × 10^6 cells mL^{-1}; FAC: of 0.6 mg L^{-1} (purified flocculating agent); pH: 7; RT: 30 min	RE: 88%	Guo et al. (2013)
Physical flocculation	*Monodus subterraneus*	Ultrasound-assisted	FR: 4–6 L·d^{-1}	X_C: 3.3 × 10^8 cells·mL^{-1}; PI: 4 W	RE: 90% CF: 11	Bosma et al. (2003)
	Tetraselmis sp.	Electro-flocculation	V: 150 mL	X_C: 1.36 × 10^6 cells·mL^{-1}; RT: 60 s; El: aluminum (two vertical flat); Vo: 5.2 V; pH: 8.4	RE: 95%	Lee et al. (2013)
	Nannochloropsis maritima	Magnetic separation	V: 100 mL	X_C: 0.1–2 g L^{-1}; CC: 120 mg L^{-1} of Fe_3O_4 nanoparticles; pH: 8	RE: 97.5%	Hu et al. (2013)

RE, recovery efficiency; *CF*, concentration factor; *V*, volume processed; *FR*, flow rate; X_C, biomass concentration in the culture; *CC*, chemicals concentration; *OM*, operation mode; *RT*, residence time; *FAC*, flocculant agent concentration; *FIC*, fungi inoculum concentration; *F/M*, ratio between fungal mycelium and microalgal biomass; *PI*, power input; *El*, electrode; *Vo*, voltage.

microalgae), autoflocculation also occurs by coprecipitation with calcium and magnesium salts, ions naturally available in several microalgal growth media. Therefore, this technique can be described as a chemical flocculation process without the addition of chemical compounds.

Spontaneous alkaline flocculation can be observed in microalgal cultures where the pH increases to values above 9 (Spilling et al., 2011). The pH increase can influence the load of microalgal cells (Harun et al., 2010) and alter the existing forms of metallic cations in suspension, due to their hydrolysis (Gregory and Duan, 2001). Depending on the cultivation conditions, some pH changes can induce the formation of inorganic precipitates (e.g., calcium or magnesium precipitates), leading to alkaline flocculation through load neutralization (Salim et al., 2014; Vandamme et al., 2013, 2014). Autoflocculation at high pH can occur spontaneously in microalgal crops, as a result of photosynthetic CO_2 depletion (and consequent increase in the pH), or through alkalinization of the culture medium. In these conditions, the precipitation of carbonate salts and coprecipitation of magnesium and calcium ions naturally available in the culture broth take place, facilitating the sedimentation process (Knuckey et al., 2006; Sukenik and Shelef, 1984).

Alkaline compounds, such as sodium hydroxide (NaOH) potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), or magnesium hydroxide ($Mg(OH)_2$), can be added to the culture medium as a strategy to force flocculation by increasing the pH (González-Fernández and Ballesteros, 2013). Vandamme et al. (2012) evaluated autoflocculation of *C. vulgaris* by increasing the pH of the medium with the use of different salts. High harvesting efficiencies (greater than 95% at pH 11) were achieved with NaOH ($9\,mg\,g^{-1}$ biomass), followed by $Ca(OH)_2$ ($12\,mg\,g^{-1}$ biomass) and KOH ($18\,mg\,g^{-1}$ biomass). However, in the same study, the authors concluded that sodium carbonate (Na_2CO_3) was not able to flocculate *C. vulgaris* cells at the same pH (Vandamme et al., 2012). Similarly, *Dunaliella tertiolecta* was harvested with an efficiency greater than 90% when the pH increased from 8.6 to 10.5 by the addition of NaOH, obtaining a cell concentration of $15\,g\,L^{-1}$ through gravity sedimentation (Horiuchi et al., 2003).

The flocculation process that involves increasing the pH is fast, inexpensive, and simple, with no or little impact on microalgal cells' integrity (cellular damage can be considered negligible). Additionally, this method allows the reuse of the culture medium after biomass recovery. However, the absence of a fundamental understanding of the alkaline flocculation method may have resulted in low REs and unreliable flocculation for some microalgal species. This method was tested for a limited number of species and only a few data are recorded (Harith et al., 2009; Lee et al., 1998). More specifically, little research has investigated crucial parameters, such as pH, microalgal biomass concentration, and concentration of polysaccharides released in flocculation induced by high pH. Additionally, pH extremes can cause cell damage or death, being limiting for some applications at industrial scale (Milledge and Heaven, 2013).

Regarding microalgal autoflocculation through EPS production, several studies identified the microalgae *C. vulgaris* JSC-7, *Scenedesmus obliquus* AS-6-1, *Ankistrodesmus falcatus*

SAG202-9, and *Ettlia texensis* SAG79.80 as self-flocculating microorganisms (Alam et al., 2014; Guo et al., 2013; Salim et al., 2011, 2012). Alam et al. (2014) and Guo et al. (2013) demonstrated that autoflocculating properties described for the microalgae *C. vulgaris* JSC-7 and *S. obliquus* AS-6-1 are a result of the interaction with polysaccharides produced by these strains. Similarly, Salim et al. (2014) concluded that EPS produced by *E. texensis* SAG 79.80, which are mainly composed of glycoproteins, are responsible for the autoflocculation ability described for this microalga. In autoflocculation mediated by EPS release, microalgal harvesting is favored in conditions where EPS production is increased—that is, under stress conditions or as a result of cell lysis. EPS are macromolecules that act (Sigee, 2004): (i) in the prevention against desiccation and pathogens; (ii) as receptor molecules for communication between microalgae; and (iii) as flocculant agents that promote microalgal cells aggregation. EPS secreted by microalgae can lead to the neutralization of cells' surface charges and thus to flocculation. In addition, it was suggested that oxygen produced in photosynthesis dominates attractive and repulsive forces between EPS (Barsky et al., 1984), meaning that bulky flakes can be obtained under high dissolved oxygen concentrations (Wilen and Balmer, 1999). As published by Andreadakis (1993), cells' aggregation is governed by the composition and properties of EPS, rather than the amount released. Carbohydrates and proteins are the main constituents of EPS (Dugdale et al., 2006; Sigee, 2004). The concentration ratio between these macromolecules are the main factor for microalgal cells' surface properties. For example, the surface charge tends to become less negative as the carbohydrate:protein ratio increases (Shin et al., 2000), since proteins are the main source of negative charges (Jorand et al., 1998). Similarly, proteins play an important role in the hydrophobicity of microalgal cells' surface. The most important stress conditions that induce EPS production include high temperature, high light intensity, and deficient nutrients concentration (Sing et al., 2013). Moreno et al. (1998) observed a 4-fold increase in EPS production by *Anabaena* sp. when increasing the irradiance from 345 to 460 $\mu mol\, m^{-2}\, s^{-1}$. This is in agreement with Wilen and Balmer (1999), who reported maximum release of EPS by *Porphyridium cruentum* during periods of high irradiance (between 3000 and 4000 $\mu E\, m^{-2}\, s^{-1}$). Temperature is another factor that leads to the production of EPS. Temperatures above 35°C are considered stress conditions, while low temperatures result in the interruption of EPS production, due to decreased metabolic activities. The ideal temperature for EPS excretion is species-specific. The release of EPS by *B. braunii* was insignificant below 23°C, while the opposite was observed at an optimal temperature range of about 30–33°C (Lupi et al., 1991). In the case of *Anabaena* sp., lower amounts of EPS were produced at a temperature of 30–35°C; maximum excretion of these compounds occurred for temperatures above 40°C (Moreno et al., 1998). Nutritional deficiency is also an important stress factor that influences the production of EPS (Lee et al., 2009). With CO_2 concentration being the basis of autotrophic microalgal growth (carbon is consumed 6.5–100 times more than other nutrients), concentration of this nutrient may significantly affect EPS production by microalgae (Shelef and Sukenik, 1984). Córdoba-Castro et al. (2012) observed that EPS production increased concomitantly with the CO_2 supplied to a *S. obliquus* culture. In this

study, EPS production was related to microalgal growth, since when high CO_2 concentration (4% v/v) was supplied, both microalgal growth and EPS production were maximal. On the other hand, low CO_2 concentrations decreased EPS release by half (González-Fernández and Ballesteros, 2013).

Taking into account that this process can naturally occur with certain microalgal species, it can be considered as low cost, ecofriendly, and energy efficient, with the additional advantage of allowing the reuse of the culture medium (Horiuchi et al., 2003). However, autoflocculation mediated by EPS becomes limiting as it may take time for EPS secretion to occur and it is not applicable for all microalgal species.

10.4.2.2 Chemical flocculation

Chemical flocculation is a technique commonly used in some industrial processes. In this process, chemical compounds with flocculant activity are added to the culture medium to promote aggregates formation. Depending on their chemical nature, these flocculants can be distinguished between inorganic and organic flocculants, cationic and anionic (Taher et al., 2011). Cationic flocculants are the most commonly applied, as they promote the neutralization of cells' surface charge, enabling the spontaneous formation of cellular aggregates or flakes. Multivalent or polyvalent salts of aluminum and iron, such as aluminum sulfate ($Al_2(SO_4)_3$), iron sulfate ($Fe_2(SO_4)_3$), and iron chloride ($FeCl_3$), are widely used as coagulants/flocculants to separate microalgal cells from the culture medium (Barros et al., 2015; De La Noüe et al., 1992). The presence of these salts in the culture medium results in their dissociation and consequent release of the respective cations, which interact with hydroxyl and carboxyl groups present on cells' surface and contribute to its neutralization, thus reducing electrostatic repulsive forces between cells and promoting aggregates formation (Chalmers et al., 1998; Chen et al., 2011; Papazi et al., 2010; Vandamme et al., 2013). Taking into account that the efficiency of these flocculants is strongly dependent on the ionic strength of the culture medium, the use of trivalent salts instead of divalent ones results in higher flocculant activity (Christenson and Sims, 2011). Trivalent salts allow a wider molecular conformation and cell bonding, leading to the rapid neutralization of cells' surface charge (Mathimani and Mallick, 2018). Cationic polymers are other widely used and effective flocculants, but usually they are much more expensive than metallic salts (Vandamme et al., 2010; Granados et al., 2012; Roselet et al., 2015; Wu et al., 2015). Flocculation can also be obtained using acids (Pezzolesi et al., 2015), but generally a large amount is required.

The efficiency of 12 inorganic salts in *Chlorella minutissima* harvesting was evaluated by Papazi et al. (2010). To obtain a coagulation efficiency of 80%, the authors reported optimal concentrations of $0.75\,g\,L^{-1}$ for sulfate salts and $0.5\,g\,L^{-1}$ for chloride salts. Considering the process kinetics, for the same coagulation efficiency (80%), a residence time of 4 h was required when using $Fe_2(SO_4)_3$, whereas for $Al_2(SO_4)_3$, a residence time of 2 h was sufficient.

A reduction in these residence times by 50% was further obtained, when using chloride salts instead of sulfate ones. Based on these results, it was demonstrated that chloride salts were more efficient than sulfate salts, requiring lower concentrations than the latter for *C. minutissima* coagulation. Sánchez et al. (2013) observed the efficiency of using $AlCl_3$ as a flocculant for the harvesting of the marine microalga *Isochrysis galbana*, emphasizing the importance of mixing time in the process of flakes formation. With this study, the authors reported optimal mixing times between 2 and 3 min (at a rotation speed of 100–200 rotations per minute, rpm). Shen et al. (2013) developed polynomial models to optimize the flocculation of the marine microalga *Nannochloropsis oculata* by adding the cationic salts $Al_2(SO_4)_3$ and $FeCl_3$. The effects of initial biomass concentration, pH, and flocculant dose were evaluated. Average REs predicted in this study were 94.4% and 87.9% for $Al_2(SO_4)_3$ and $FeCl_3$, respectively, using the following optimal conditions: (i) initial biomass concentration of 1.7 g L^{-1}, pH 8.3 and $Al_2(SO_4)_3$ concentration of 383.5 μM; and (ii) initial biomass concentration of 2.2 g L^{-1}, pH 7.9 and $FeCl_3$ concentration of 438.1 μM. Vergini et al. (2016) evaluated the potential of $Al_2(SO_4)_3$, $AlCl_3$, and $FeCl_3.6H_2O$ in the flocculation of *Scenedesmus rubescens* and *D. tertiolecta*. From the tested chemical salts, $Al_2(SO_4)_3$ resulted in 99% turbidity removal in both cultures. On the other hand, REs of 20% and 93% were determined using $FeCl_3{\cdot}6H_2O$ for *S. rubescens* and *D. tertiolecta*, respectively. To flocculate *Dunaliella salina* effectively, Pirwitz et al. (2015) tested different types of inorganic salts, and concluded that $FeCl_3$ was the most promising for *D. salina* harvesting, in terms of REs obtained (around 90%–97%), concentration factor, and the possibility for culture medium reuse. In addition to metallic flocculants, polymeric metal salts also function as flocculants, because they cover an extensive range of pH. Polyferric sulfate is a type of prepolymerized salt that has been identified to recover effectively (95%) the microalgae *Anabaena flosaquae* and *Asterionella formosa* (Grima et al., 2003; Jiang et al., 1993). The efficiency of chemical flocculation is also species-dependent. In a study performed by Jiang et al. (1993), RE obtained when harvesting *Chlorella stigmatophora* using 25 mg L^{-1} of $FeCl_3$ was 90%. The authors reported that harvesting of *A. flosaquae* and *A. formosa* required 58 mg L^{-1} of $FeCl_3$, and REs obtained ranged between 63% and 74%. Among aluminum, iron, and zinc salts, aluminum stands out as the most efficient because it is a trivalent cation with low molecular weight, presenting higher solubility in water. According to Grima et al. (2003) and Papazi et al. (2010), aluminum salts have shown better results in the harvesting of microalgal species from the genera *Chlorella* and *Scenedesmus*. Regarding iron and zinc cations, iron cations are more effective than zinc because the latter has a lower load (+2) and higher molecular weight (Papazi et al., 2010). Although aluminum salts are considered good flocculants, they can cause cell damage because the cell viability of the obtained flakes is low. Moreover, the use of iron salts at concentrations greater than 1 g L^{-1} can change microalgal cells' color, and the use of zinc salts may result in flakes adhesion to the container walls (Papazi et al., 2010). Among the salts, chlorides are considered more effective than sulfate ones, due to their higher solubility in aqueous media. Besides the chemical nature of the chemical flocculants used, other parameters, such as electronegativity and ionic strength of the culture

medium, also influence the flocculation efficiency. High electronegativity leads to faster flocculation with higher efficiency. On the other hand, the ionic strength of the culture medium also determines the flocculation efficiency and the flocculant dose required. Due to differences in conductivity between freshwater and seawater, the flocculation of saline species requires flocculant concentrations 5–10 times higher than the flocculation of freshwater microalgae (Milledge and Heaven, 2013; Uduman et al., 2010b). Thus, the efficiency of chemical flocculation techniques decreases considerably with marine microalgae, due to the high ionic strength of seawater (Teixeira et al., 2012). Taking into account these considerations, a good chemical or inorganic flocculant must address the following criteria (Grima et al., 2003): (i) avoid biomass contamination; (ii) allow high REs; (iii) enable the rapid sedimentation of microalgal biomass; (iv) allow the reuse of the culture medium; and (v) enable the use of low-cost, low-concentration, and sustainable flocculants (Grima et al., 2003). Although metallic salts are being applied in microalgal biomass harvesting at a relatively improved efficiency, high doses of chemical flocculants make the process expensive (per unit of microalgal biomass) and result in high concentrations of metals in the harvested biomass (Vandamme et al., 2013). These metals remain in the biomass residue and may hinder the extraction of lipids or carotenoids in certain microalgal species (Rwehumbiza et al., 2012). In addition, metal residues in biomass alter biochemical composition and proteins profile of microalgal biomass, limiting its application for animal feed and other food applications. Finally, chemical flocculation may not be favorable for the sustainable harvesting of large amounts of microalgae because high amounts of metal residues must be discarded for further processing of microalgal biomass (Schenk et al., 2008).

Although metallic salts and polymeric metal salts are the most studied chemical flocculants, other commonly used chemical compounds include synthetic polymers. Being another significant group of flocculants, charged polymers can bind to the surface of different particles and enable flocculation. This mechanism can occur through the formation of bridges or the neutralization of charges and even reversal of the surface load (Vandamme et al., 2013). Chitosan, cellulose, surfactants, polyacrylamides, and certain synthetic fibers are examples of organic cationic polymers usually applied (Grima et al., 2003). Factors such as the polymer molecular weight, molecular loading density, polymer dosage, microalgal biomass concentration, ionic strength, and pH of the culture medium and residence time are identified as the major contributors to the success of the flocculation process using these polymers (Grima et al., 2003). Generally, high molecular weight and high loading density polyelectrolytes are better bonding agents, with better ability to neutralize cells' surface charge. By the addition of a high molecular weight polymer, bridges formation can occur, and it is possible to increase flakes' size to values as high as 100 μm, thus promoting the rapid sedimentation of microalgal cells (Edzwald, 1993). High density cultures also enable higher flocculation efficiencies. Increased mixing level and time also play an important role, as these factors promote the cells

aggregation. However, excessive shear forces caused by high mixing intensities may induce cells'/flakes' damage (Chisti, 2009).

Using Praestol (a cationic polyacrylamide) at a dose of 1 mg L^{-1}, Pushparaj et al. (1993) were able to harvest the microalgae *Tetraselmis suecica* and *Spirulina platensis*, reporting REs of 70%. At the same time, the authors referred the possibility of culture medium reuse. Similarly, the polyacrylamide polymers Flopam and Zetag were effectively applied in the recovery of the marine microalga *N. oculata*, achieving an RE of 75% (Van Haver and Nayar, 2017). Following the line of polyacrylamide flocculants, the high molecular weight and high load density (2–4 meq g^{-1}) of polyacrylamide at low doses (2–5 mg L^{-1}) was very effective in the recovery of *S. obliquus*, *Scenedesmus subspicatus*, *Synechococcus nidulans*, and *Chlorella sorokiniana* (REs of 95%) (Mikulec et al., 2015). Tilton et al. (1972) used anionic polystyrene, cationic polyethyleneimine, and non-ionic and anionic polyacrylamides (at concentrations from 0.01 to 1000 mg L^{-1} and at a pH range between 4 and 7) for the flocculation of *C. ellipsoidea* (biomass concentrations of 50–3000 mg L^{-1}). In this study, the authors reported that the use of cationic polyethyleneimine resulted in the highest REs, especially in the case of high molecular weight polyethyleneimine flocculants. When testing the polyelectrolyte crystalline nanocellulose (produced from the acid hydrolysis of cellulose fibers) in a 200 mg L^{-1} *C. vulgaris* culture, Van Haver and Nayar (2017) reported an RE of 90%. Synthetic polyacrylamide polymers are flocculants commonly used in different industries, but the toxic acrylamide residues can contaminate microalgal biomass (Bratby, 2016). Polymeric flocculants are unsuitable for flocculation of marine microalgae or brackish water, because they do not work on a large conductivity range and when the ionic strength of the culture medium is high (Grima et al., 2003; Schlesinger et al., 2012; Uduman et al., 2010b).

The utilization of biopolymers as flocculants can be a natural and safe alternative to synthetic polymers. These biopolymers must be positively charged, which is rare in nature, so that they can interact with the surface charge of microalgal cells (Vandamme et al., 2013). A well-known positively charged biopolymer is chitosan, an acetyl glucosamine polymer obtained by the deacetylation of chitin, present in crustacean shells and in fungi. Biodegradability is the main positive characteristic of chitosan (Brennan and Owende, 2010; Harith et al., 2009; Schlesinger et al., 2012; Vandamme et al., 2013). After cellulose, chitosan is an abundant natural polymer that can be used in the flocculation process (Honarkar and Barikani, 2008), being a good solution to reduce the environmental impact and operational costs. Compared to inorganic flocculants, chitosan does not contaminate microalgal biomass, enabling its use in food and feed applications (Ahmad et al., 2011). Being a non-toxic flocculant, chitosan has proven to be effective when tested with several microalgal species, but its flocculant activity may be significantly reduced in salt water (Farid et al., 2013). The ideal dose of chitosan varies greatly with microalgal species. Optimal flocculation of *Tetraselmis chuii*, *Thalassiosira pseudonana*, and *Isochrysis* sp. (REs between 80% and 90%) was observed for a chitosan dosage of 40 mg L^{-1} (Heasman et al., 2000). In contrast, 150 mg L^{-1} of chitosan was necessary for

optimal flocculation (RE of 95%) of *Chaetoceros muelleri* (Heasman et al., 2000). In a study carried out with the microalga *Nannochloropsis* sp., higher REs (around 97%) were obtained when using 60 mg L^{-1} of nanoquitosan, instead of 60 mg L^{-1} of chitosan. Ahmad et al. (2011) also used chitosan as a flocculant agent for the recovery of *Chlorella* sp. In this study, different parameters were evaluated: (i) optimal flocculant dosage; (ii) optimal mixing speed and time; and (iii) sedimentation time. REs of 99% were obtained for an ideal dose of chitosan of 100 ppm, a mixing time of 20 min at 150 rpm and a sedimentation time of 20 min. Despite being a very efficient flocculant, chitosan only works at low pH, which is the opposite condition from those typically observed in microalgal cultures (Chang and Lee, 2012). Additionally, the use of chitosan requires high dosage (between 20 and 150 mg L^{-1}), when compared to other synthetic organic flocculants (Grima et al., 2003; Harith et al., 2009), which limits its application at industrial scale due to the high costs of this natural polymer (Chen et al., 2011).

Another important member of natural polymers is starch, the most abundant storage polysaccharide, commonly used in various branches of industry. Natural starch is a mixture of two natural polymers: amylose, which is present at levels of up to 25%, depending on the source, and amylopectin, which corresponds to the main fraction (levels of up to 95%) (Pal et al., 2005). However, the use of natural starch for flocculation is inadequate, due to its insolubility in water and its tendency to form gels (Pi-Xin et al., 2009). Consequently, cornstarch is chemically modified to achieve specific properties and to suit the requirements of an effective coagulant/flocculant. Of these, cationic starch, which is prepared from the addition of quaternary ammonium groups to the glucose hydroxyl groups (Pal et al., 2005), constitutes an alternative to chitosan. The charge of these quaternary ammonium groups is independent of the pH. Thus, cationic starch works in a wider pH range than chitosan, but requires greater concentration (Vandamme et al., 2010). The process of flocculation based on cationic starch is gradually attracting the attention of researchers because it is non-toxic, biodegradable, and has a low production cost (about $1–3 per kg) (El-Naggar et al., 2018). Chemically modified starches have very different properties from the original starch and can be used in different industrial applications (Prakash et al., 2007). Vandamme et al. (2010) used the commercial starch Greenfloc 120 to evaluate its flocculation potential in the recovery of the microalgae *Parachlorella kessleri*, *S. obliquus*, *Phaeodactylum tricornutum*, and *Nannochloropsis salina*. In this study, REs obtained for *S. obliquus* and *P. kessleri* using Greenfloc 120 at optimal dose (around 10–20 mg·L^{-1}) were 90% and 80%, respectively. For the marine microalgae, *N. salina* and *P. tricornutum*, cationic starch was inefficient, due to the high concentration of NaCl in the culture medium. Additionally, this study demonstrated that harvesting of *S. obliquus* required a lower dose of Greenfloc 120, which may be related to the larger size of *S. obliquus* cells (Bratby, 2016). The efficiency of flocculation with the cationic starch Greenfloc 120 was also tested with the microalga *Chlorella protothecoides*, under different pH conditions and different flocculant concentrations. Satisfactory results (REs of 96%) were obtained for a flocculant concentration of 40 mg L^{-1} and pH values of 7.7 and 10 (Letelier-Gordo et al., 2014).

The use of plant-based biopolymers has also been highlighted as an inexpensive and viable alternative for flocculation of microalgal biomass. For example, *Moringa oleifera* derivatives are seen as an example of natural coagulants, as they present high molecular weight cationic proteins that can promote the destabilization of microalgal suspension and hence aggregates formation (Teixeira et al., 2012). The use of *M. oleifera* derivatives as bioflocculants offers several benefits, such as (Abdul Hamid et al., 2014): (i) low environmental impact; (ii) reduced harvesting costs, as costs associated to chemical flocculants can be avoided; and (iii) almost negligible toxicity for microalgal cells. Teixeira et al. (2012) used *M. oleifera* seed flour ($1\,g\,L^{-1}$) to recover *C. vulgaris*, obtaining an RE of 89% after a residence time of 120 min at pH 9.2. Similarly, Abdul Hamid et al. (2014) proved the efficiency of *M. oleifera* derivatives in microalgal harvesting, reporting an RE of 95% for a residence time of 20 min. Inulin (a plant reserve polysaccharide) is a renewable and biodegradable compound that, alongside its derivatives, has several applications. Cationic inulin is used as a natural flocculant, whose flocculation mechanism is based on electrostatic interactions between the opposite charges of cationic inulin and microalgal cells, which enable microalgal cells' neutralization and hence their sedimentation. The positively charged polysaccharide binds to several microalgal cells at the same time and this interaction results in a structural complex in the form of bulky flakes (Ummalyma et al., 2017). Cationic inulin ($60\,mg\,L^{-1}$ at pH 7.4) was tested in the harvesting of *Botryococcus* sp., reaching an RE of 88.6% in 15 min (Rahul et al., 2015). In another report on microalgal flocculation with plant derivatives, the cationic guar gum was applied to cultures of *Chlorella* sp. and *Chlamydomonas* sp., exhibiting flocculation efficiencies of 92% and 94%, respectively (Banerjee et al., 2013). *C. vulgaris* harvesting using *Strychnos potatorum* powder seeds revealed an RE of 99.68% for a flocculant concentration of $100\,mg\,L^{-1}$ and a settling time of 30 min (Abdul Razack et al., 2015). Polymer- and biopolymer-mediated flocculation is a less toxic, fast, and economical method for microalgal biomass harvesting. Even though there is still minimal concern associated with the addition of the quaternary cationic amine group to some polymers, they are a feasible option in comparison with expensive and unsafe chemical flocculants.

10.4.2.3 Bioflocculation

As mentioned in previous sections, autoflocculation and chemical flocculation techniques have some limitations, mainly regarding the cost and dosage of flocculants used and the contamination of microalgal biomass, which may limit further biotechnological applications. Taking into account these limitations, bioflocculation methods emerged as a viable alternative because they involve the presence of flocculating microorganisms or natural flocculants, such as polysaccharides and proteins, produced by them (Ndikubwimana et al., 2015; Salehizadeh et al., 2000; Wan et al., 2015). Bioflocculation promoted by co-cultivation of microalgae with other microorganisms, such as other microalgae or cyanobacteria, fungi, bacteria, and yeasts, as well as by the action of substances excreted by them, has been explored as a possible alternative to improve microalgal harvesting. The symbiotic association between

microalgae, cyanobacteria, and fungi can naturally occur in the environment or be artificially engineered to take advantage of these positive interactions.

In the case of microalgal interactions, several studies have reported flocculant activities of EPS produced by other microalgae, either in co-culture or not. For example, soluble extracts present in *Skeletonema marinoi* cultures acted as flocculants of the microalga *N. oculata*, resulting in a flocculation efficiency of 95%, achieved after 6 h of settling time (Taylor et al., 2012). Alam et al. (2014) reported that polysaccharides present in the cell wall of self-flocculating microalga *C. vulgaris* JSC-7 may act as flocculating agents for the microalga *S. obliquus* FSP, since this process resulted in an RE of 80%. Salim et al. (2014) demonstrated that *E. texensis* EPS were responsible for a flocculation efficiency of 55% when applied to the microalga *C. vulgaris*.

In the symbiotic relation between microalgae and fungi, fungi use the sugars and other organic compounds produced by microalgae in the photosynthetic process, providing, in turn, microalgal protection, maintenance in water, and an amplified area for nutrients uptake (Zhou et al., 2012). The process of entrapment of microalgal cells within filamentous fungi is called co-pelletization and constitutes a potential technique for microalgal harvesting. In this process, fungal hyphae and mycelium containing polysaccharides with active sites may attract the negatively charged microalgal cells, promoting the interaction between fungi and microalgae and surface cells charge neutralization, and hence co-pelletization (Zhang and Hu, 2012; Zhou et al., 2012). Fungi-mediated harvesting technology does not require the addition of toxic compounds or energy, and several studies have shown that many microalgal cells were effectively recovered by interactions with fungi (Xie et al., 2013; Zhang and Hu, 2012; Zhou et al., 2012). In a study carried out with the microalga *C. vulgaris* UMN235 cultivated with *Aspergillus* sp. spores ($10^8\,L^{-1}$), it was possible to obtain the complete pelletization of microalgal cells (Zhou et al., 2012). Similar results were obtained when the microalga *C. vulgaris* (at an initial concentration of 6.9×10^9 cells L^{-1}) was cultivated with *Aspergillus niger* spores (7.6×10^9 spores L^{-1}) (Zhang and Hu, 2012). When *Cunninghamella echinulata* was co-cultivated with the microalga *C. vulgaris* for 2 days, 99% of microalgal biomass was recovered (Xie et al., 2013). Muradov et al. (2015) demonstrated that the co-cultivation of *Aspergillus fumigatus* pellets with high cell density cultures of *C. protothecoides* and *T. suecica* resulted in a biomass RE of up to 90% after 24 h of co-cultivation. Another study showed that co-cultivation of *A. niger* with *C. vulgaris* (at initial concentrations of 8.50×10^6 spores L^{-1} and 2.55×10^9 cells L^{-1}, respectively) resulted in a microalgal biomass RE of 90% (Gultom et al., 2014). Inoculation of *C. sorokiniana* with spores produced from *Isaria fumosorosea* resulted in the formation of large pellets (with diameters of 1–2 mm) and improved biomass recovery through filtration (REs obtained ranged between 94% and 97%) (Mackay et al., 2015). The co-cultivation of *C. vulgaris* and *Nannochloropsis* sp. in the fungal mycelium of *Aspergillus nomius* resulted in biomass recoveries of 97% and 94%, respectively (Talukder et al., 2014). EPS excreted by yeasts can also be used as flocculant agents for

microalgal harvesting. Prochazkova et al. (2015) used the brewing industry yeast *Saccharomyces pastorianus* to harvest the microalga *C. vulgaris*. In this study, the authors showed that the modification of the yeast surface with a positively charged functional group resulted in an RE of 90%.

Interactions between microalgae and bacteria can also favor microalgal flocculation, by promoting aggregates formation. This improvement in the speed of flakes formation can be achieved in different ways: (i) through flocculant agents extracted from bacteria; and (ii) through flocculant agents excreted by bacteria when in co-cultivation with microalgae, which may promote microalgal-bacterial flocs formation (Gutzeit et al., 2005; Lee et al., 2009; Sing et al., 2013). Regarding bioflocculation in the presence of bacterial-based EPS, several studies demonstrated high biomass REs, especially when poly γ-glutamic acid (γ-PGA) is used as a flocculant agent (Zheng et al., 2012). In the work performed by Zheng et al. (2012), γ-PGA from *Bacillus subtilis* proved to be an effective microbial flocculant, being able to harvest the microalgae *C. vulgaris* (RE = 91%; CF = 20.5) and *C. protothecoides* (RE = 98%; CF = 29.8) after 2 h. These results were obtained in the following conditions: (i) γ-PGA dosage of 22.03 mg L^{-1} and initial microalgal biomass concentration of 0.57 g L^{-1} for *C. vulgaris*; and (ii) γ-PGA dosage of 19.82 mg L^{-1} and initial microalgal biomass concentration of 0.60 g L^{-1} for *C. protothecoides*. To verify the suitability of this method for different microalgal species, the authors also applied the previously mentioned optimized conditions to the microalgae *N. oculata*, *P. tricornutum*, *C. vulgaris*, and *B. braunii*, achieving REs of 96%, 97%, 90%, and 92%, respectively (with CFs ranging between 20.1 and 28.2). Ndikubwimana et al. (2014) obtained an RE of 92% for the microalga *Desmodesmus* sp. F51 when using γ-PGA from *Bacillus licheniformis* culture broth. Bioflocculants extracted from *Pestalotiopsis* sp. KCTC 8637P (Pestan) (at a concentration of 100 mg L^{-1}) demonstrated a satisfactory efficiency (90%) in the recovery of *B. braunii* biomass with initial cell density of 0.3 g L^{-1} (Lee et al., 1998). The presence of ions Ca^{2+} facilitates the bioflocculation process, as they contribute to cells' surface charge neutralization. When bioflocculants from *Paenibacillus* sp. were used at a concentration of 7–8 g L^{-1} for the recovery of a low-density *C. vulgaris* culture, maximum RE of 83% was obtained in the presence of 6.8 mM of $CaCl_2$ (Oh et al., 2001). Nie et al. (2011) also observed that the efficiency of bioflocculants produced by *Klebsiella pneumoniae* in the recovery of cyanobacteria increased in the presence of Ca^{2+} ions.

Co-cultivation of microalgae and bacteria can also be very advantageous in microalgal flocculation because in these conditions, large microalgal-bacterial flocs (50–800 μm) can be formed (Gutzeit et al., 2005). For this reason, several research studies have focused on the study of microalgal-bacterial co-cultures to improve biomass recovery. A high aggregation potential was obtained in the co-culture of a new isolated bacteria belonging to the order *Pseudomonadales* with *Nannochloropsis oceanica* in the proportion of 30:1 (microalgae: bacteria) during 3 d (Wang et al., 2012). Although the production of bacterial bioflocculants has proven to be beneficial by improving the formation of large microalgal-bacterial flakes, the

challenge of harvesting still remains, due to the additional cost involved in requiring a carbon source for bacterial growth (González-Fernández and Ballesteros, 2013). The large volume of bacterial inoculation and the prolonged co-culture time seem to be the main concerns of the harvesting process, which need to be addressed further.

Bioflocculation techniques are well accepted, especially because these methods do not require the addition of chemical flocculants. In addition, compared to other flocculation methods, bioflocculation is easy, economically viable, and allows the reuse of the culture medium (Ummalyma et al., 2017). However, these methods require a deep understanding about the conditions in which the co-cultivated microorganisms present flocculant activity or excrete EPS with flocculant activity, which may limit their application at an industrial scale. Furthermore, bioflocculation may require the purification of bioflocculants, as well as the presence of organic substrates for the co-cultivation of bacteria or fungi with microalgae, which could restrict their further application (Powell and Hill, 2014; Ummalyma et al., 2017; Zhou et al., 2012). Another factor that must be considered is the presence of contaminating microorganisms in the collected biomass, which is somewhat limiting when the final application of microalgal biomass is the production of human food and animal feed, pharmaceuticals, or cosmetics (Wan et al., 2015). If the application of biomass is directed to biofuels production, the presence of microorganisms may even help to increase lipid yields and fatty acids contents.

10.4.2.4 Physical flocculation

The possible contamination of microalgal biomass and the costs associated with some flocculants can be avoided by applying flocculation methods mediated by physical forces. In this field, methods based on electrophoresis, ultrasound flocculation, and magnetic separation can be used to form clusters of microalgal cells (Milledge and Heaven, 2013).

Ultrasound-assisted flocculation is characterized by the high frequency (order of MHz) and low amplitude of ultrasound pressure, which results in almost negligible cell damage. In this method, maximum potential energy fields and minimum potential energy fields (nodes) are created by the application of an acoustic field precisely defined to form a stationary wave. In this field, mean temporal forces (primary radiation force) act on the cells by instantly directing them toward the planes of the nodes, an energy region with lower acoustic potential (Coakley et al., 2000; Hawkes et al., 1997). The effect of the interaction between particles results in an attractive force between these cells that slowly unites them in the nodes of the ultrasonic wave. Then, the concentration of cells is driven by the forces of acoustic interaction and the forces of particle-particle interaction. Once the ultrasonic field is annulled, these large microalgal clusters tend to sediment due to gravitational forces (Bosma et al., 2003). Ultrasound with high frequency (in the order of MHz) and low amplitude allows the cells to aggregate, while ultrasound with low frequency (in the order of kHz) and high amplitude induce cell lysis. Bosma et al. (2003) evaluated the performance of the acoustic technique (resonance frequency of

2.1 MHz) in the harvesting of *Monodus subterraneus*. The experiments were conducted in continuous mode at a flow rate of 4–6 $L\,d^{-1}$ and REs higher than 90% were obtained. With this study, the authors demonstrated the potential of this technique in microalgal harvesting, also concluding that for high biomass concentration, higher REs can be obtained by increasing the ultrasonic field frequency. Zhang et al. (2009) combined ultrasonic-assisted flocculation with coagulation using polyaluminum chloride (PAC) to harvest *Microcystis aeruginosa*. In this study, the authors concluded that REs increased from 35%, when PAC coagulation was applied, to 67%, when ultrasonic radiation was applied for 5 s in combination with PAC coagulation. In addition, the authors concluded that sonication promoted the rupture of gaseous vacuoles present in *M. aeruginosa* cells, leading to the loss of buoyancy and greater sedimentation ability. The advantages associated to the use of ultrasound-assisted techniques for microalgal cells separation include (Benes et al., 2001; Bierau et al., 1998): (i) the maintenance of microalgal cells' integrity; (ii) high mechanical robustness, as this device has no moving parts; and (iii) the suitability for continuous operation.

Electrical methods follow the principle of electrophoresis (Vandamme et al., 2011), where negatively charged microalgal cells can be concentrated by moving from an electric field (Zhang et al., 2012). This technique promotes the destabilization of microalgal suspensions by the use of electrodes to form an electric field, which is responsible for microalgal cells separation (Uduman et al., 2010a). When the electric field is applied, microalgal surface charge tends to be less positive, promoting the movement of microalgal cells toward the anode. At this stage, flakes formation may occur both on the electrodes (electrophoresis) and at the bottom (electroflocculation) (Barros et al., 2015). Microalgal REs of 80%–95% have already been reported using this method (Chen et al., 2011). Electroflocculation methods differ according to the electrode type used: sacrificial or non-sacrificial. When sacrificial electrodes are used, metal ions that can act as coagulants are released into the culture medium, promoting the destabilization of microalgal suspension and aggregates formation. Accordingly, this method is governed by physical and chemical stimuli (Uduman et al., 2010b): the positively charged metals bind to microalgal cells and promote their movement toward the anode, in an electrophoretic motion (Kim et al., 2012). In electroflocculation based on non-sacrificial electrodes, microalgal cells movement toward the anode is exclusively mediated by the negative charges present on microalgal cells' surface. Once in the anode, microalgal cells lose their charge, resulting in flakes formation (Wan et al., 2015). These methods are strongly influenced by the type of electrode material, with aluminum electrodes being considered the most effective in microalgal harvesting through electroflocculation (Dassey and Theegala, 2013; Lee et al., 2013). Additionally, this method depends heavily on the pH and ionic strength of the culture medium. Electrolytic recovery of marine microalgae requires less energy than freshwater species because seawater presents higher conductivity and ionic strength, considerably reducing the energy consumption required to release the ions from the electrodes. However, electrolyte recovery from marine species may be difficult, due to the high chloride concentration present in seawater (approximately 19 $g\,L^{-1}$) and to the similar redox potential of

chlorine, chlorine dioxide, and oxygen (1.36, 1.57, and 1.23 V, respectively), which may result in the formation of reactive chlorine species (Ghernaout et al., 2011). Kim et al. (2012) observed a reduction in *N. oculata* cells' viability when the residence time of microalgal harvesting through electroflocculation was increased, and concluded that cells' viability loss was promoted by the accumulation of reactive chlorine species, which resulted in biomass bleaching. In addition to microalgal biomass degradation, the accumulation of reactive chlorine species hinders the reuse of the culture medium after the harvesting procedure and promotes fouling in the electrodes (Kim et al., 2013; Milledge and Heaven, 2013). When applying electroflocculation for 600 s using an aluminum electrode adjusted to 5 V, Uduman et al. (2013) obtained REs of 93.3% and 87.3% for the microalgae *Tetraselmis* sp. and *Chloroccum* sp., respectively. Poelman et al. (1997) tested the efficacy of electrolytic flocculation in the harvesting of 100-L microalgal cultures from different taxonomic groups and obtained REs between 80% and 95% in 35 min. With this study, the authors also demonstrated that a decrease in operating voltage results in decreased REs and that energy requirements could be reduced through the reduction of electrodes' surface area or the reduction of the distance between electrodes. Xu et al. (2010) reported an RE of 93.6% when harvesting the microalga *B. braunii* through electroflocculation (6 W source) for 30 min. When applying electroflocculation (using two vertical flat aluminum electrodes with a voltage of 5.2 V) to harvest the marine species *Tetraselmis* sp., Lee et al. (2010) reported an RE of 95% after a residence time of 60 s. Zenouzi et al. (2013) concluded that electroflocculation is an efficient and cost-effective method for *D. salina* harvesting. The authors reported an RE of 97.4% after 3 min of operation at a current density of 90 $A\,m^{-2}$, with a maximum energy consumption of 0.621 $kW\,h\,m^{-3}$. Vandamme et al. (2011) demonstrated that the release and consumption of aluminum is lower than those reported for chemical flocculation. In addition, energy consumption associated to these electrolytic methods is significantly lower than the one from centrifugation methods, especially when electroflocculation is applied to marine species (Vandamme et al., 2011). The advantages of electroflocculation and electrophoresis methods include flexibility, energy efficiency, security, environmental friendliness, and cost-effectiveness (Poelman et al., 1997). Even if electrical energy is required in these methods, harvesting costs of electrolytic methods are much lower when compared to other techniques. Like other processes, electrolytic methods also have some disadvantages (Vandamme et al., 2011; Wan et al., 2015): (i) cathode fouling, requiring electrode replacement and maintenance; (ii) changes in cell composition, which can occur at high current densities (Wan et al., 2015); (iii) increase in the temperature of microalgal suspension; (iv) changes in pH; and (v) possible contamination of microalgal biomass with metals from the electrodes.

The use of magnetic separation for microalgal harvesting constitutes a promising alternative, as this method lies in the non-destructive nature of the magnetic field, particles' biocompatibility, and the easy manipulation and regeneration of magnetic particles (Cerff et al., 2012; Prochazkova et al., 2013). In magnetic separation, flocculation and separation occur

simultaneously (Vandamme et al., 2013). This method involves the use of both magnetic particle functionality (a magnetic agent that adsorbs to microalgal cells) and an external magnetic field. Because both microalgal cells and magnetic particles have negative surface charges in aqueous media, cationic polyelectrolytes (also known as cationic binders) are required as bridges between magnetic particles and microalgal cells (Lim et al., 2012). Once bonded to magnetic particles, microalgal cells can be recovered through an external magnetic field. Magnetic particles for harvesting microalgae may be in the form of uncoated iron oxide (Fe_3O_4) magnetic particles or as compounds consisting of a magnetic core coated with silica (Hu et al., 2013; Prochazkova et al., 2013). When using Fe_3O_4 magnetic nanoparticles to separate the microalgae *B. braunii* and *C. ellipsoidea*, Xu et al. (2011) reported REs higher than 98% after 1 min. Cerff et al. (2012) proposed that magnetic separation of microalgae depends on the pH and composition of the culture medium. The presence of di- and trivalent ions (e.g., Ca^{2+}, PO_4^{3-} and Mg^{2+}) results in an increased interaction between microalgae and magnetic particles, thus enabling higher REs. Hu et al. (2013) used magnetic separation with Fe_3O_4 nanoparticles to harvest the microalga *Nannochloropsis maritima*, obtaining a maximum RE of 95% after 4 min at pH 8. However, the maximum recovery capacity using these magnetic nanoparticles was only achieved after 18 d. The main advantages of magnetic separation include: (i) effective separation of microalgal cells in a short period of time; (ii) the biocompatibility and non-toxic character of magnetic particles; (iii) the possibility of particles' regeneration; (iv) the possibility of culture medium recycling; and (v) low energetic requirements. However, biomass harvesting using magnetic separation technology on a large scale remains a challenge, due to the high costs of magnetic particles (Branyikova et al., 2018).

10.4.3 Flotation

Flotation is a method that uses air bubbles to attach small particles (<500 μm). The size of suspended particles and the tendency of collision and adhesion are factors that affect the process of attachment between bubbles and particles. The hydrophilic and hydrophobic particles are separated, since the hydrophobic particles are captured by the air bubbles that float to the surface. Once floating in the surface, the particles can be collected by the skimming process. Flotation is a method that: (i) needs little space; (ii) has a short operation time; (iii) is suitable for processing large culture volumes; (iv) involves low associated costs; and (v) does not require high amounts of energy (approximately 0.8 $kW\,h\,m^{-3}$ of processed culture). One way to enhance the efficiency of this method is to use collectors—reagents that can make the particles more hydrophobic—thus facilitating the union between particles and air bubbles. These reagents can be either surfactants or flocculants and are classified into cationic, nonionic, thio-compounds, and anionic (Naghdi and Schenk, 2016; Singh and Patidar, 2018). Since this method is ruled by the particles' hydrophobicity and their interaction/collision with air bubbles, its efficiency is influenced by many factors, such as the type of collector, pH, ionic strength of the culture medium, size of the produced bubbles, and hydraulic retention time (Singh and

Patidar, 2018). The type of collector influences the efficiency of the process because different collectors have several abilities for floc formation and different charge densities, resulting in the formation of larger or smaller flocs (Zhang et al., 2014). pH affects the collector adsorption to the particles and microalgal surface charge, thus playing an important role in the flotation efficiency (Garg et al., 2015). As mentioned above, microalgal surface has a negative charge when pH is alkaline, and at acidic pH this surface becomes neutral or positively charged. Due to the electrostatic interactions between the collector and microalgal cells, the collectors have an optimum pH range. For instance, if a cationic collector is used, flotation efficiencies tend to be higher in alkaline conditions. On the other hand, anionic collectors have better performances for low pH values (acidic conditions) (Chen et al., 1998; Garg et al., 2015; Phoochinda and White, 2003). The ionic strength of the culture medium is related with the presence of inert salt in the solution. When the ionic strength increases, ZP decreases and the electrostatic interactions between microalgal cells and the collector become weaker, leading to lower flotation efficiencies. Furthermore, for high concentrations of inert salt, the bubbles tend to be larger and rupture can occur with greater ease (Chen et al., 1998; Liu et al., 1999). Finally, the efficiency of this process is inversely connected to bubble size. To achieve high efficiency values, the bubble size has to be small, due to the fact that bubbles with small dimensions have a higher surface-area-to-volume ratio and their rise velocity is lower, which allows them to have a high residence time in the liquid (Garg et al., 2015). Depending on the methods used for bubbles formation, flotation can be divided into different types, such as dissolved air flotation, dispersed air flotation, electrolytic flotation, and dispersed ozone flotation. Table 10.3 presents REs obtained in microalgal harvesting through these techniques.

10.4.3.1 Dissolved air flotation

In dissolved air flotation (DAF), bubbles are produced when the dissolution of air in water occurs under very high pressure. In this method, bubbles' diameter typically ranges between 10 and 100 μm (Chen et al., 2011; Naghdi and Schenk, 2016). Some of the factors that influence the efficiency of this technique include bubbles' size, saturator pressure, pH, hydraulic retention time, and recycle flow (Fuad et al., 2018). To promote aggregates formation and an increase in microalgal particles' size (and thus improve the efficiency of the process), it is possible to use collectors (Pragya et al., 2013). This method is more effective than dispersed air flotation because the bubbles produced are smaller than those produced in dispersed air flotation. However, this method is more expensive, mainly because it requires pressurized air (Laamanen et al., 2016). Besson and Guiraud (2013) reported an efficiency of 90% when harvesting *D. salina* using DAF with sodium hydroxide (0.1–1 M) as a surfactant. Zhang et al. (2014) harvested *Chlorella zofingiensis* using DAF and tested several surfactants at different concentrations. In this study, REs of 81%, 86%, 91%, and 87% were obtained when using as collectors chitosan (70 mg g^{-1}), Al^{3+} (180 mg g^{-1}), Fe^{3+} (250 mg g^{-1}), and cetyltrimethylammonium bromide (CTAB, 500 mg g^{-1}), respectively. Zhang et al. (2016) used DAF for 10 min to harvest *Nannochloropsis* sp. and tested different concentrations of the

Table 10.3: Recovery efficiencies reported for microalgal harvesting through flotation.

Flotation type	Microalgae	Volume processed (mL)	Operational conditions	RE (%)	Reference
Dissolved air flotation	Mixed culture (*Chlorella* and *Scenedesmus*)	V: 1.2 L	OM: batch; EC: 0.76 Wh L^{-1}; CC: C-floc 60	84.9%	Wiley et al. (2009)
	Scenedesmus dimorphus	–	RT: 10 min; CC: 45.6 mg L^{-1} of Mg^{2+}	85%	Zhang et al. (2016)
	Nannochloropsis sp.	–	RT: 10 min; CC: 1330 mg L^{-1} Mg^{2+}	92%	Zhang et al. (2016)
	Chlorella sp. XJ-445	–	OM: batch; RT: 15 min; CC: 40 mg g^{-1} of Al^{3+} + 60 mg g^{-1} of CTAB; GFR: 50 mL min^{-1}	98.7%	Xia et al. (2017)
Dispersed air flotation	*Scenedesmus quadricauda*	–	RT: 20 min; X_C: 7.4×10^4 cells mg^{-1}; CC: 10 mg L^{-1} of Triton X-100; GFR: 114 mL min^{-1}	<10%	Chen et al. (1998)
	Scenedesmus quadricauda	–	RT: 20 min; X_C: 7.4×10^4 cells mg^{-1}; CC: 10 mg L^{-1} of CTAB; GFR: 114 mL min^{-1}	50%	Chen et al. (1998)
	Scenedesmus quadricauda	–	RT: 20 min; X_C: 7.4×10^4 cells mg^{-1}; CC: 40 mg L^{-1} of CTAB; GFR: 114 mL min^{-1}	90%	Chen et al. (1998)
	Tetraselmis sp. M8	–	RT: 15 min; CC: 25 ppm of DAH; GFR: 10 L min^{-1}	97%	Garg et al. (2015)
	Tetraselmis sp. M8	–	RT: 15 min; CC: 15 ppm of DPC; GFR: 10 L min^{-1}	99%	Garg et al. (2015)
	Chlorella vulgaris; *Scenedesmus obliquus*	–	RT: 20 min; CC: 20 mg L^{-1} of saponin +5 mg L^{-1} of chitosan	>93%	Kurniawati et al. (2014)

Continued

Table 10.3: Recovery efficiencies reported for microalgal harvesting through flotation—cont'd

Flotation type	Microalgae	Volume processed (mL)	Operational conditions	RE (%)	Reference
Electrolytic flotation	Mixed culture	–	OM: batch; RT: 140 min	95%–99%	Neto et al. (2014)
	Mixed culture	–	OM: batch; RT: 40 min	75%–88%	Neto et al. (2014)
	Chlorella sorokiniana	–	OM: batch; EC: 4 kW h kg^{-1}; CC: 0 mg L^{-1} of NaCl; C: 1 A; Vo: 6.6 V; El: carbon	66%	Misra et al. (2014)
	Chlorella sorokiniana	–	OM: batch; EC: 1.6 kW h kg^{-1}; CC: 6 mg L^{-1} of NaCl; C: 1 A; Vo: 3.7 V; El: carbon	95%	Misra et al. (2014)
	Aurantiochytrium sp. KRS101	–	OM: batch; RT: 15 min; EC: 0.125 kW h kg^{-1}; C: 17.2 mA cm^{-2}; El: aluminum	55.6%	Kim et al. (2015)
	Chlorella sp.	–	EC: 0.43 kWh kg^{-1}; CC: 15 mg L^{-1} chitosan; GFR: 1000 L h^{-1}; C: 5.9 A; Vo: 4 V; El: carbon	90%	Zhou et al. (2016)
Dispersed ozone flotation	*Chlorella vulgaris*	–	OM: batch; GC: 0.024–0.05 mg O_3 mg^{-1}; GFR: 0.6 L min^{-1}	98%	Cheng et al. (2010)
	Scenedesmus obliquus FSP-3	–	OM: batch; RT: 4 min; GC: 0.2–0.52 mg O_3 mg^{-1}; GFR: 0.6 L min^{-1}	95%	Cheng et al. (2011)
	Mixed culture	–	OM: batch; RT: 5 min; GC: 45 mg O_3 L^{-1}; GFR: 0.4 L min^{-1}	79.6%	Velasquez-Orta et al. (2014)

RE, recovery efficiency; *OM*, operation mode; *RT*, residence time; *EC*, energy consumption; X_C, biomass concentration in the culture; *CC*, chemicals concentration; *GC*, gas concentration/dose; *GFR*, gas flow rate; *C*, electric current density; *Vo*, voltage; *El*, electrode.

surfactant magnesium, obtaining a flotation efficiency of 92% without extra addition of the surfactant, since this microalga was from marine water and presented a high concentration of this cation (1330 mg L^{-1}) in the beginning of the experiment. The authors also harvested *S. dimorphus*, a freshwater microalga, that grows in a culture medium with low magnesium concentration (45.6 mg L^{-1}), and obtained a flotation efficiency of 85%. Wiley et al. (2009), with the goal of comparing DAF and suspended air flotation, harvested a mixed culture (mainly composed by *Chlorella* and *Scenedesmus*) using DAF on batch mode, and reported an RE of 84.9% and an energy consumption of 0.76 W h L^{-1}. Xia et al. (2017) used a combination of 40 mg g^{-1} of Al^{3+} as coagulant and 60 mg g^{-1} of CTAB as a collector to harvest *Chlorella* sp. XJ-445 through DAF. The experiment was carried out in batch mode for 15 min with a gas flow rate of 50 mL min^{-1}, achieving an RE of 98.7%.

In addition to the conventional DAF process, there is a modified version of this process, called PosiDAF. In this process, bubbles produced are positively charged, due to the addition of chemicals in the saturator. The chemicals used in the saturator can be surfactants, coagulants, or polymers that have a hydrophobic and hydrophilic part, to promote the bonding between cells and bubbles (Fuad et al., 2018).

10.4.3.2 Dispersed air flotation

In dispersed air flotation (DiAF), bubbles are formed in two possible ways: (i) when air passes continuously and at high speed through a porous material or a mechanical agitator; and (ii) through the production of foam using a surfactant. Usually, the diameter of the formed bubbles is between 700 and 1500 μm. This technique can be used to process large culture volumes (Naghdi and Schenk, 2016) and is characterized by low energy consumption. However, the equipment required is expensive (Singh and Patidar, 2018). DiAF differs from DAF because in this case the air injected in the flotation tank is non-pressurized (Fuad et al., 2018). Chen et al. (1998) used DiAF to harvest *Scenedesmus quadricauda* with different collectors (CTAB and Triton X-100) to evaluate which one results in higher efficiencies. When Triton X-100 (10 mg L^{-1}) was used, the efficiency was only 10%. However, when CTAB was applied in the same concentration, the RE increased to 50%. The authors then used a higher concentration of CTAB (40 mg L^{-1}) and obtained an RE of 90%. Kurniawati et al. (2014) harvested *C. vulgaris* and *S. obliquus* using a mixture of 20 mg L^{-1} of a natural biosurfactant (saponin) and 5 mg L^{-1} of a collector (chitosan) through DiAF for 20 min, obtaining an RE of 93%. Garg et al. (2015) tested different concentrations and several types of surfactants to harvest *Tetraselmis* sp. M8 through DiAF with a residence time of 15 min. The surfactants used in this study were dodecyl amine hydrochloride (DAH) and dodecyl pyridinium chloride (DPC). The authors reported that when DAH was used at a concentration of 25 ppm, the efficiency of the process was 97%, but for lower concentrations of DPD (15 ppm), a higher RE was obtained (99%).

10.4.3.3 Electrolytic flotation

In electrolytic flotation, bubbles are composed of hydrogen and produced through electrolysis. As collector/flocculating agents, it is possible to use active metal anodes. Cost-effectiveness and suitability for all types of microalgae are the main advantages of this method. However, cathode fouling can occur and high power input is required (Fuad et al., 2018; Pragya et al., 2013; Singh and Patidar, 2018). Neto et al. (2014) used electrolytic flotation to harvest a mixed microalgal culture and tested different residence times (40, 70, and 140 min). For the residence time of 40 min, the REs obstained were 75% and 88% (in terms of chlorophyll-a and turbidity, respectively). For a residence time of 70 min, the chlorophyll-a removal was 93% and the turbidity removal was 91%. At 140 min, REs reported were 99% and 95%, respectively. Zhou et al. (2016) harvested *Chlorella* sp. using electrolytic flotation with chitosan (15 mg L^{-1}) and reported an efficiency of 90% and an energy consumption of 0.43 kW h kg^{-1}. Kim et al. (2015) tested different methods to harvest *Aurantiochytrium* sp. KRS101. One of the methods chosen was electrolytic flotation. The retention time was 15 min and the authors obtained an efficiency of 55.6% and an energy consumption of 0.125 kW h kg^{-1}. Although this technique is suitable for harvesting all microalgal types, it is more efficient when applied to separate marine microalgae (instead of freshwater microalgae), due to the high electrical conductivity of seawater (Muylaert et al., 2017). Misra et al. (2014) investigated the use of NaCl to enhance the electrochemical reaction rate. The authors harvested *C. sorokiniana* using different concentrations of NaCl and concluded that the harvesting efficiency was higher (95%) and the power consumption was lower (1.6 kW h kg^{-1}) in the experiments conducted with a NaCl concentration of 6 g L^{-1}, when compared to the results obtained with no NaCl addition (66% and 4 kW h kg^{-1}, respectively).

10.4.3.4 Dispersed ozone flotation

Dispersed ozone flotation uses ozone to generate charged bubbles, as an alternative to atmospheric air. Ozone is used because it is an oxidizing agent that oxidizes soluble organic compounds, enhancing the connection between bubbles and microalgae. Furthermore, ozone can promote cell lysis, leading to the release of biopolymers that can act as coagulants/flocculants, thus improving the separation efficiency (Cheng et al., 2010; Singh and Patidar, 2018). Despite these advantages, this process is expensive, due to the use of ozone instead of atmospheric air (Laamanen et al., 2016; Pragya et al., 2013). Some parameters that are important to take into account in this process are the ozone flotation time, ozone gas concentration, and flow rate (Velasquez-Orta et al., 2014). Velasquez-Orta et al. (2014) tested the effects of ozone flow rate (0.2, 0.4, and 0.6 L min^{-1}), ozone gas concentration (25, 35, and 45 mg O_3 L^{-1}), and ozone flotation time (5, 10, and 15 min) on TSS and turbidity removal of a mixed microalgal culture (composed of *Oscilatoria* sp., *Scenedesmus* sp., *Diatomea* sp., *Eugiena* sp., *Merismopedia* sp., *Chlorella* sp., *Pediastrum* sp., *Cyclotella* sp., *Anabaena* sp., and *Arthospira* sp.). With this study, the authors concluded that when the gas concentration was

45 mg O_3 L^{-1}, TSS removal percentage was independent from the ozone flotation time and flow rate: TSS removal efficiencies were the same for ozone flotation times of 5 and 15 min, and no differences were observed when using gas flow rates of 0.4 and 0.6 mL min^{-1}. Accordingly, the authors determined that for an ozone concentration of 45 mg O_3 L^{-1}, it is only necessary to use a gas flow rate of 0.4 mL min^{-1} and an ozone flotation time of 5 min to obtain high TSS and turbidity removal efficiencies (79.6% and 97.8%, respectively). Cheng et al. (2010) used dispersed ozone flotation to harvest *C. vulgaris* with an ozone dose between 0.024 and 0.050 mg O_3 mg^{-1}, and obtained a reduction in turbidity of 98%. Similarly, Cheng et al. (2011) harvested *S. obliquus* FSP-3 using an ozone dose ranging between 0.2 and 0.52 mg O_3 mg^{-1}, and achieved turbidity removal efficiencies of 95%.

10.4.4 Centrifugation

Through centrifugation, microalgae are separated from the culture medium by the action of a centrifugal force. This is the most applied and one of the fastest harvesting methods; it does not require the use of chemicals, it is appropriate for almost all types of microalgae, and it provides high biomass recoveries (Mantzorou and Ververidis, 2018; Suali and Sarbatly, 2012; Alam et al., 2017). Despite the high biomass REs reported, this method requires high energy input and expensive equipment, being used mainly to recover biomass intended for extraction of high-valued products (Rawat et al., 2011; Zhou et al., 2013; Mantzorou and Ververidis, 2018). In addition, the centrifugation process is ruled by high gravitational forces and shear stress, which can cause damage in microalgal cells' structure, and this process is not suitable for processing large culture volumes because the volume and flow rate processed in the centrifuge can significantly influence the capture efficiency (Barros et al., 2015). For high flow rates, the retention time required to obtain high capture efficiencies needs to be increased, thus increasing the energy requirements. On the other hand, when processing lower flow rates, attention should be paid to the retention time and energy input, to reduce the amount of energy supplied per unit of microalgal biomass. In addition to the influence of the volume to be processed, other parameters that are important to take into account in the centrifugation process are the particle size and density, since particle diameter and the difference between microalgal cell density and culture medium density are inversely proportional to the time required to settle a microalgal cell (Eq. 10.4):

$$t = \frac{18\,\eta\,s}{\omega^2\,R\,\Delta\rho\,D_p^2} \tag{10.4}$$

where t is the total time needed to settle a cell (in s), η is the medium viscosity (in N s m^{-2}), s is the settling path (in m), ω is the rotational velocity (in s^{-1}), R is the radial distance from centrifuge axis (in m), $\Delta\rho$ is the difference between microalgal cells' density and culture medium density (in kg m^{-3}), and D_p is the microalgal cells' diameter (in m) (Gerardo et al., 2015b).

Several studies have referred the use of centrifugation for the recovery of microalgal biomass produced at laboratory scale (Table 10.4). For example, Eppink et al. (2017) reported biomass CFs of up to 25% DW using this technique and Ahmad et al. (2014) tested centrifugation (4000 rpm, 10 min) to harvest *Chlorella* sp. and the RE obtained was 99%. Anthony et al. (2013)

Table 10.4: Recovery efficiencies reported for microalgal harvesting through centrifugation (laboratory-scale examples).

Microalgae	Volume processed (mL)	Operational conditions	RE (%)	Reference
Chaetoceros calcitrans	–	OM: batch; v: 1300 × *g*	48%	Heasman et al. (2000)
Chaetoceros muelleri	–	OM: batch; v: 1300 × *g*	15%	Heasman et al. (2000)
Chlorella sp.	600 mL	OM: batch; RT: 30 min; v: 4000 rpm; X_C: 4.86×10^9 cells mL^{-1}	99%	Ahmad et al. (2014)
Isochrysis sp.	–	OM: batch; v: 1300 × *g*	54%	Heasman et al. (2000)
Nannochloropsis oculata	–	OM: batch; v: 1300 × *g*	67%	Heasman et al. (2000)
Pavlova lutheri	–	OM: batch; v: 1300 × *g*	66%	Heasman et al. (2000)
Phaeodactylum tricornutum	–	OM: batch; v: 1300 × *g*	56%	Heasman et al. (2000)
Scenedesmus obliquus	–	OM: batch; RT: 10 min; v: 8000 rpm	≈100%	Anthony et al. (2013)
Scenedesmus spinosus	300 mL	OM: batch; RT: 15 min; v: 1500 rpm; X_C: 0.4 gL^{-1}	95.2%	Reyes and Labra (2016)
Scenedesmus spinosus	300 mL	OM: batch; RT: 15 min; v: 1800 rpm; X_C: 0.4 gL^{-1}	95.2%	Reyes and Labra (2016)
Scenedesmus spinosus	300 mL	OM: batch; RT: 15 min; v: 2200 rpm; X_C: 0.4 gL^{-1}	96.0%	Reyes and Labra (2016)
Skeletonema costatum	–	OM: batch; v: 1300 × *g*	39%	Heasman et al. (2000)
Tetraselmis chuii	–	OM: batch; v: 1300 × *g*	5%	Heasman et al. (2000)
Thalassiosira pseudonana	–	OM: batch; v: 1300 × *g*	57%	Heasman et al. (2000)

RE, recovery efficiency; *OM*, operation mode; *RT*, residence time; *v*, velocity; X_C, biomass concentration in the culture.

harvested *S. obliquus* through centrifugation at 8000 rpm for 10 min and obtained an RE of 100%. Chen et al. (2011) concluded that biomass REs can reach values between 80% and 90% within 2–5 min, at velocities between 500 and 1000 $\times g$. Similarly, Heasman et al. (2000) applied centrifugation for harvesting cultures of eight microalgal species: *Pavlova lutheri*, I. galbana, C. *muelleri*, *Chaetoceros calcitrans*, *Skeletonema costatum*, *T. pseudonana*, *P. tricornutum*, *T. chuii*, and *N. oculata*. Using a velocity of 1300 $\times g$, the authors reported REs of 5%–66% and an apparent cell viability between 95% and 100%.

Regarding microalgal harvesting at an industrial scale, there are many types of industrial centrifuges: disc stack, tubular and multi-chamber centrifuges, and also decanters and hydrocyclones (Pahl et al., 2013). From these, the most commonly used in large-scale applications is disc stack centrifuge, because it does not require much manual intervention, the separation time is short and, compared with other types of centrifuges, it is considered the most suitable for microalgal harvesting. However, this technology is expensive due to the high equipment costs (Singh and Patidar, 2018; Alam et al., 2017). Tubular and multi-chamber centrifuges allow the achievement of high REs, but they are not able to work continuously because periodic cleaning is required (Mathimani and Mallick, 2018). On the other hand, a decanter can work continuously and is mainly used when the suspension has high solids' content (10%–50% DW) (Muylaert et al., 2017). Finally, hydrocyclones can be used to harvest large volumes of microalgal cultures continuously, but the capital costs and energy demand are high (Singh and Patidar, 2018). Table 10.5 presents some examples of successful application of centrifugation to harvest microalgae at industrial scale. Acién et al. (2012) harvested *Scenedesmus almeriensis* with a decanter centrifuge, operating at a flow rate of 2 m^3 h^{-1} and 9500 rpm for 6 h, and obtained a sludge concentration between 15% and 20% DW. Mohn (1980) harvested two microalgae (*Scenedesmus* sp. *and Coelastrum proboscideum*) using a disc stack centrifuge and obtained a final concentration of 12% TSS and a CF of 120. With the same microalgal species, but using a nozzle discharge centrifuge, sludge concentration obtained was between 2% and 15% TSS and the CF between 20 and 150. When using a decanter bowl, the results obtained were 22% TSS and a CF of 11. Finally, the authors harvested *C. proboscideum* using hydrocyclones and concluded that it is possible to obtain a suspension with 0.4% TSS and a CF of 4.

To reduce the operational costs associated with the centrifugation process, several authors have reported the use of a pre-concentration step (through coagulation/flocculation, for example). This pre-concentration step results in a reduction of the volume to be processed in the centrifugation step (up to 65%) and thus a reduction in the speed and time of the process (Barros et al., 2015; Schlesinger et al., 2012).

10.4.5 Filtration methods

Filtration is a method that requires the maintenance of a pressure drop across the system, so that the fluid can flow through specific filters, such as membranes, screens, or microstrainers, where microalgal biomass is retained. The required pressure drop can be achieved by the use of

Table 10.5: Recovery efficiencies reported for microalgal harvesting through centrifugation (industrial-scale examples).

Microalgae	Equipment	Volume processed/Flow rate	Operational conditions	RE (%)/CF	Reference
Coelastrum proboscideum	Hydrocyclone	–	OM: continuous; EC: 0.3 kWh m^{-3}	CF: 4	Mohn (1980)
Coelastrum proboscideum, *Scenedesmus* sp.	Disc stack centrifuge	–	OM: continuous; EC: 1 kWh m^{-3}	CF: 120	Mohn (1980)
Coelastrum proboscideum, *Scenedesmus* sp.	Nozzle Disc Centrifuge	–	OM: continuous; EC: 0.9 kWh m^{-3}	CF: 20–150	Mohn (1980)
Coelastrum proboscideum, *Scenedesmus* sp.	Decanter centrifuge	–	OM: continuous; EC: 8 kWh m^{-3}	CF: 11	Mohn (1980)
Nannochloris sp.	Disc stack centrifuge	FR: 0.94 L min^{-1}	OM: continuous; RT: 10 min; v: 3000 × *g*; EC: 20 kWh m^{-3}; X_C: 100 mg L^{-1}	RE: 94%	Dassey and Theegala (2013)
Nannochloris sp.	Disc stack centrifuge	FR: 23.2 L min^{-1}	OM: continuous; RT: 10 min; v: 3000 × *g*; EC: 0.80 kWh m^{-3}; X_C: 100 mg L^{-1}	RE: 17%	Dassey and Theegala (2013)
Scenedesmus almeriensis	Decanter centrifuge	FR: 2 m^3 h^{-1}	OM: continuous; RT: 10 min; v: 9500 rpm; EC: 2.75 kWh m^{-3}; X_C: 0.7–2.0 g L^{-1}	CF: ≈135	Acién et al. (2012)
Scenedesmus spinosus	Nozzle disc centrifuge	FR: 14 L min^{-1}	OM: continuous; RT: 10 min; v: 5500 rpm; X_C: 0.4 g L^{-1}	RE: 82.1%	Reyes and Labra (2016)

RE, recovery efficiency; *CF*, concentration factor; *FR*, flow rate; *OM*, operation mode; *RT*, residence time; *v*, velocity; *EC*, energy consumption; X_C, biomass concentration in the culture.

different types of forces like gravity, pressure, and vacuum (Barros et al., 2015). Table 10.6 presents the main improvements achieved in microalgal biomass harvesting through filtration techniques.

In membrane filtration, a membrane is used as a selective barrier (Cheryan, 1998; Singh et al., 2011), and it allows the concentration of biomass to values up to 5% DW (Fasaei et al., 2018).

Table 10.6: Recovery efficiencies reported for microalgal harvesting through filtration methods.

Filtration type	Microalgae	Process	Volume processed/Flow rate	Operational conditions	RE (%)/CF	Reference
Filtration	*Coelastrum proboscideum*	Pressure filtration; Netzsch chamber filter	–	OM: batch, one step; EC: 0.88 kWh m^{-3}	CF: 245	Mohn (1980)
	Coelastrum proboscideum	Pressure filtration; suction filter	–	OM: batch	CF: 160	Mohn (1980)
	Coelastrum proboscideum	Vacuum filtration; suction filter	–	OM: batch; one step; EC: 0.1 kWh m^{-3}	CF: 80	Mohn (1980)
	Coelastrum proboscideum	Vacuum filtration; belt filter	–	OM: continuous; EC: 0.45 kWh m^{-3}	CF: 95	Mohn (1980)
	Chlorella sp.	Microfiltration; tangential flow	–	OM: continuous; RT: 6 h	RE: 98%	Ahmad et al. (2014)
	Nannochloropsis oculata	Ultrafiltration; tangential flow	–	RT: 30 min; X_C: 3.38×10^6 cell mL^{-1}	RE: 79.5%	Devianto et al. (2018)
	Aurantiochytrium sp. KRS101	Microfiltration; membrane PVDF 0.2 µm	FR: 8 L min^{-1}	RT: 240 min	RE: 97.3%	Kim et al. (2015)
	Aurantiochytrium sp. KRS101	Ultrafiltration; membrane PVDF 150 kDa	FR: 8 L min^{-1}	RT: 180 min	RE: 99.8%	Kim et al. (2015)
	Aurantiochytrium sp. KRS101	Ultrafiltration; membrane PES 150 kDa	FR: 8 L min^{-1}	RT: 240 min	RE: 99.9%	Kim et al. (2015)
	Chlorella vulgaris	Submerged microfiltration; membrane PVDF-9	FR: 38.3 L m^{-2} h^{-1}	EC: 0.27 kWh m^{-3}; X_C: 0.41 g L^{-1}	RE: 98%	Bilad et al. (2012)
	Phaeodactylum tricornutum	Submerged microfiltration; membrane PVDF-9	FR: 42.5 L m^{-2} h^{-1}	EC: 0.25 kWh m^{-3}; X_C: 0.23 g L^{-1}	RE: 70%	Bilad et al. (2012)
	Phaeodactylum tricornutum	Submerged microfiltration; membrane PVDF-12	–	X_C: 0.23 g L^{-1}	RE: 77%	Bilad et al. (2012)

Continued

Table 10.6: Recovery efficiencies reported for microalgal harvesting through filtration methods—cont'd

Filtration type	Microalgae	Process	Volume processed/Flow rate	Operational conditions	RE (%)/CF	Reference
	Phaeodactylum tricornutum	Submerged microfiltration; membrane PVDF-15	–	X_C: 0.23 g L^{-1}	RE: 90%	Bilad et al. (2012)
	Scenedesmus quadricauda	Ultrafiltration; membrane PVC 50 kDa	–	OM: batch; v: 0.17 m s^{-1}; X_C: 1.0 g L^{-1}	CF: 150	Zhang et al. (2010)
Vibrating screen	*Coelastrum proboscideum*	Vibrating screen	–	OM: continuous	CF: ≈110	Mohn (1980)
	Coelastrum proboscideum	Vibrating screen	–	OM: batch	CF: ≈150	Mohn (1980)
Microstraining	*Coelastrum proboscideum*	Microstraining	–	EC: 0.2 kWh m^{-3}	CF: ≈30	Mohn (1980)

RE, recovery efficiency (%); *CF*, concentration factor; *V*, volume processed; *FR*, flow rate; *OM*, operation mode; *RT*, residence time; *v*, velocity; *EC*, energy consumption; X_C, biomass concentration in the culture.

Taking into account the membrane pore size, the direction of the feed flow, and the forces used in the filtration process, different membrane techniques can be used (see Fig. 10.2). Regarding pore size, macrofiltration, microfiltration, and ultrafiltration can be applied. Macrofiltration (membrane pore size greater than 10 μm) is used for separation of large microalgal cells, such as filamentous microalgae. In microfiltration (membrane pore size between 0.1 and 10 μm), it is possible to separate microalgal cells from the culture medium with an operational pressure of 1–2 bar. On the other hand, in ultrafiltration (pore size range of 0.02–0.2 μm), the membrane is able to retain organic molecules, such as proteins and carbohydrates, at an operational pressure of 5 bar (Cheryan, 1998; Fasaei et al., 2018). However, this technique has high operational and maintenance costs, so it is not commonly used in microalgal harvesting (Singh and Patidar, 2018). In terms of the flow of feed water, the filtration can be

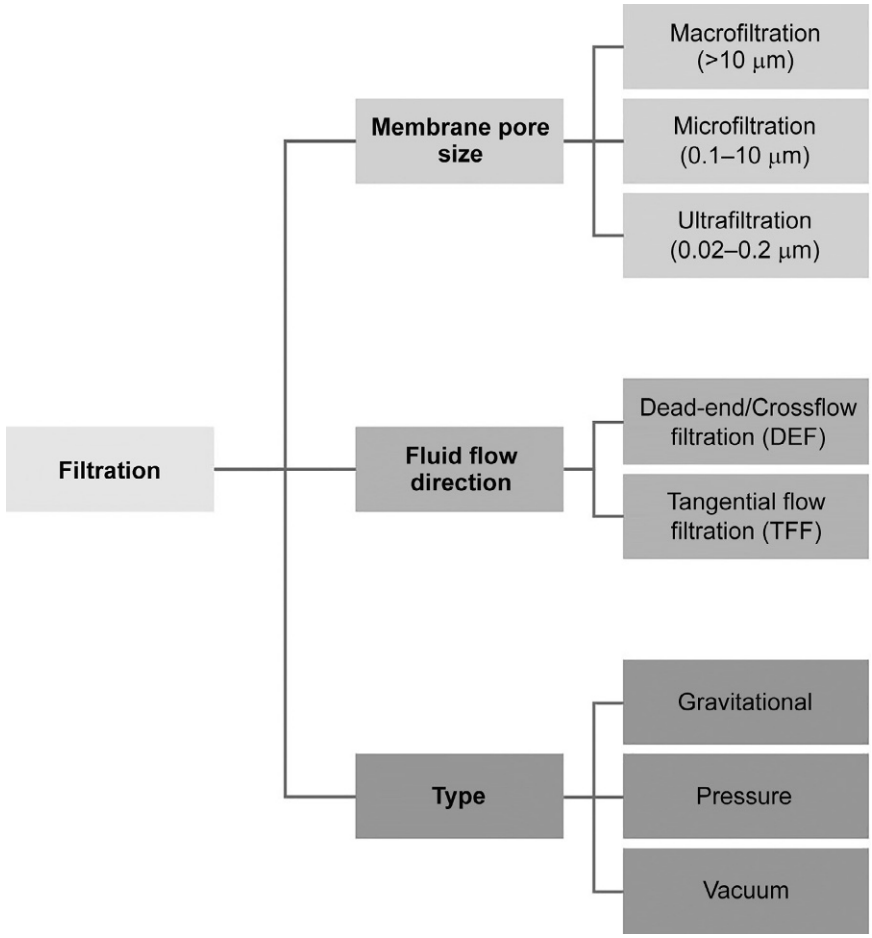

Fig. 10.2
Classification of the different types of filtration that can be applied according to the membrane pore size, fluid flow direction, and type of pressure applied.

divided into dead end filtration (DEF) or tangential flow filtration (TFF), also known as crossflow filtration (Gerardo et al., 2015a). In DEF, microalgal culture passes perpendicularly to the membrane, causing fouling and the rapid clogging of the membrane. Therefore, this flow type is not used very often in microalgal harvesting. On the other hand, in TFF, microalgal culture passes in parallel with the membrane and the filtrate passes perpendicularly (Muylaert et al., 2017). Accordingly, the filter cake is washed away and the rapid fouling and clogging of the membrane can be avoided (Fuad et al., 2018). TFF is more appropriate than DEF, especially for small microalgal cells. With this technique, it is possible to maintain microalgal cells' viability and obtain high REs (between 70% and 89%) (Christenson and Sims, 2011; Fuad et al., 2018; Pragya et al., 2013). Vacuum filtration and pressure filtration are used to harvest microalgae that have large cell dimensions (such as the filamentous *S. platensis*) (Harun et al., 2010). To harvest microalgae by pressure filtration, it is common to use plate-and-frame filter presses or pressure vessels with filters. The plate-and-frame filter presses are a succession of rectangular plates positioned face to face vertically on a frame. Each plate is covered by a filter cloth and microalgal culture is pumped into the space between the plates, where pressure is applied, allowing the liquid to pass through the filter cloth. Regarding pressure vessels, these systems have different configurations, with the chamber filter press, cylindrical sieve, and filter basket being most commonly used for microalgal filtration, due to their concentration capability and reliability (Show et al., 2013). In vacuum filtration, a suction force is applied on the filtrate side. The recommended configurations used to harvest microalgae are vacuum drum filter, suction filter, and belt filter (Show and Lee, 2013). Several studies have reported the use of these membrane filtration techniques. Ahmad et al. (2014) used microfiltration with tangential flow to harvest the microalga *Chlorella* sp., and obtained an RE of 98%, working in continuous mode for 6 h. Bilad et al. (2012) harvested *C. vulgaris* and *P. tricornutum* using submerged microfiltration. For *C. vulgaris*, the processed flow rate was 38.3 $L\,m^{-2}\,h^{-1}$ and the efficiency obtained was 98%, with an energy consumption of 0.27 $kWh\,m^{-3}$; for *P. tricornutum*, the applied flow rate was 42.5 $L\,m^{-2}\,h^{-1}$ and the RE obtained was 70%, with an energy consumption of 0.25 $kWh\,m^{-3}$. Devianto et al. (2018) harvested *N. oculata* through ultrafiltration in tangential flow mode. The higher efficiency (79.5%) was obtained with a filtration time of 30 min with a backwashed treatment at 2 bar. Zhang et al. (2010) used ultrafiltration to harvest *S. quadricauda* in batch mode and obtained a CF of 150. Das et al. (2019) were able to harvest *Tetraselmis* sp. using TFF with a permeate flux ranging from 40 to 120 $L\,m^{-2}\,h^{-1}$, obtaining an RE of 100%. Mohn (1980) used different types of pressure and vacuum filters to harvest *C. proboscideum*. The author reported a final concentration of 22%–27% TSS (CF of 245) when using pressure filtration with a Netzsch chamber filter in batch mode and a final concentration of 16% TSS (CF of 160) when using a suction filter, also in batch mode. The author also evaluated vacuum filtration with a suction filter in batch mode and the results obtained were 8% TSS, corresponding to a CF of 80. Finally, when using a belt filter operating in continuous mode, the author reported a final concentration of 9.5% TSS and a CF of 95. In addition to the different membrane techniques identified, different

membrane materials can also be used. These materials include polyethersulfone polyvinyl-pyrollidone (PES-PVP), polyvinylidene fluoride (PVDF), polyvinyl chloride (PVC), polyacrylonitrile (PAN), polyether sulfone (PES), polytetrafluoroethylene (PTFE), and polyethylene terephthalate (PET). Additionally, there are different membrane configurations available, such as spiral wound, tubular membranes, flat sheet, and fiber, which have different costs and different resistances to fouling (Fasaei et al., 2018; Gerardo et al., 2015a). The efficiency of the membranes can be negatively affected by the increase of dry matter content, because this can cause fouling and concentration polarization. To decrease and/or avoid membranes' fouling, it is possible to cover the membrane with a thin layer of hydrophilic polyvinyl alcohol (PVA) polymer, which makes the membrane surface more hydrophilic, thus reducing the contact between microalgal cells and the membrane (Singh and Patidar, 2018). Hwang et al. (2013) harvested *Chlorella* sp. KR-1 using TFF with a PET membrane coated with PVA and obtained an increase in maximum flux of about 36% when compared to the original PET membrane. Kim et al. (2015) tested three types of membranes to harvest *Aurantiochytrium* sp. KRS101: a microfiltration membrane made of PVDF 0.2 μm and two ultrafiltration membranes, one made of PVDF 150 kDa and the other made of PES 150 kDa. In this configuration, the filtration system was equipped with a rotational disk to avoid fouling problems. With this study, the authors obtained the following REs: (i) 97.3% when a PVDF membrane was used for 240 min; (ii) 99.8% when a PES membrane was used for 180 min and the velocity of the disk was 1600 rpm; and (iii) 99.9% when a PVDF 150 kDa membrane was used for 240 min.

Filtration can also be performed using screens with a specific pore size, where the flux passes through and particles with higher diameter than the pore size are retained. To harvest microalgae, it is common to use two types of screens: microstrainers and vibrating screen filters (Show and Lee, 2013). Microstrainers consist of rotating filters with a continuous backwash and are made of fine mesh screens that retain large particles. This process is highly dependent on microalgal cells' size. For small microalgal cells, the opening of the screen will be smaller, leading to lower flow rates and hence higher costs (as the volume of culture processed per microstrainer is higher) (Uduman et al., 2010a). This process was used to harvest *C. proboscideum*, resulting in a final concentration of 1.5% TSS with an energy consumption of 0.2 $kWh\,m^{-3}$ (Mohn, 1980). In the case of vibrating screens, these systems are used as separating device in many industries, such as food and paper. This technology can also be used to harvest microalgae. When harvesting *C. proboscideum* using this method in both continuous and batch mode, Mohn (1980) reported final concentrations of 5%–6% TSS and 7%–8% TSS, respectively. Vibrating screens were also used by the Food and Agriculture Organization of the United Nations to harvest *Spirulina* for food production (Habib et al., 2008). The main disadvantage of using microstrainers and vibrating screens is that they are not suitable for all microalgal cells, being more appropriate for larger cells. In addition, due to the build-up of microalgal biofilm on the mesh, periodic cleaning is required (Show et al., 2013).

10.5 Selection of the most appropriate harvesting technique

As reported in Section 10.3, selection of the most appropriate harvesting technique should be based on several factors, such as RE/CF, quality of the produced biomass, cost, processing time, toxicity, suitability for large-scale application, and level of moisture achieved. Furthermore, the final application of the recovered biomass is an important factor to consider, as the referred parameters should be prioritized in different orders, according to the final use of microalgal biomass. Taking into account the wide range of parameters to consider, as well as the huge diversity of microalgal species, selection of a single method to be widely applied is almost impossible. One possible approach that can be followed to determine the most suitable method for each application is a ponderation between: (i) the most important criteria that should be taken into account for each application; and (ii) the most satisfactory harvesting methods regarding each criterion. A similar analysis was performed by Al Hattab et al. (2015) and Singh and Patidar (2018). In these studies, the analysis was performed assuming as important criteria biomass quantity and quality, cost, processing time, toxicity, and suitability for large-scale application. In addition, the main applications of biomass considered were biofuels production, human food and animal feed, and production of high-valued compounds. Accordingly, the ponderation was performed by: (i) defining the prioritization list of the selected criteria for each application; (ii) attributing the level of satisfaction provided by each harvesting method for each criterion; and (iii) selection of the most suitable technique for each application (based on the results from steps (i) and (ii)). Regarding the prioritization of the selected criteria, taking into account the current demand for low-cost biofuels, biomass quantity, cost, and processing time were considered as the most important criteria for biofuels production. In terms of food and feed production, biomass quality, toxicity, and suitability for large-scale application were considered key factors. Finally, toxicity, and biomass quality and quantity were selected as the most important criteria for the production of high-valued compounds. The complete order of prioritization defined for each application of microalgal biomass is shown in Table 10.7. Based on the main advantages and disadvantages of each harvesting method described throughout this chapter, Fig. 10.3

Table 10.7: Prioritization order of the most important criteria to take into account for different applications of microalgal biomass.

Biofuels production	Human food/Animal feed	Production of high-valued compounds
1. Biomass quantity	1. Biomass quality	1. Toxicity
2. Cost	2. Toxicity	2. Biomass quality
3. Processing time	3. Large-scale application	3. Biomass quantity
4. Large-scale application	4. Biomass quantity	4. Large-scale application
5. Biomass quality	5. Processing time	5. Cost
6. Toxicity	6. Cost	6. Processing time

	Biomass quantity	Biomass quality	Cost	Processing time	Toxicity	Large-scale application
Unsatisfactory	Sedimentation	Sedimentation	Centrifugation	Sedimentation	Flocculation Flotation	Sedimentation Flocculation Flotation
Fair	Flocculation Flotation Filtration	Flocculation Flotation Centrifugation	Flocculation Flotation Filtration	Flocculation Flotation Filtration		Filtration
Satisfactory	Centrifugation	Filtration	Sedimentation	Centrifugation	Sedimentation Centrifugation Filtration	Centrifugation

Fig. 10.3

Level of suitability of each harvesting method according to some important factors, such as biomass quantity and quality, cost, processing time toxicity, and possibility for large-scale application.

presents the evaluation of these methods taking into account each criterion. In summary, centrifugation is considered the most satisfactory method in terms of biomass quantity, processing time, and suitability for large-scale application. For biomass quality, filtration is considered the most appropriate method. Taking into account the almost negligible costs associated with gravity sedimentation, this is considered the most satisfactory method for the cost criterion. Finally, in terms of toxicity to final biomass, the most suitable methods include sedimentation, centrifugation, and filtration (when applied as single-step procedures). This analysis has shown that: (i) gravity sedimentation, flocculation, and flotation methods are more suitable for biofuels production; (ii) filtration-based techniques are more appropriate for food and feed applications; and (iii) centrifugation is the most suitable choice for high-valued compounds production (as also shown in Table 10.1).

Even though this analysis can give important tools for the selection of the most appropriate harvesting technique for each application of microalgal biomass, further improvements in REs, process economics, and processing time can be achieved by using a combination of two or more methods. Typical combinations of harvesting techniques include a pre-concentration/thickening step followed by dewatering. In the first step, microalgal suspension with approximately 0.05% TSS is concentrated by a factor of 100–200, achieving a concentration of about 2%–7% TSS. In the dewatering step, biomass is further concentrated (CF $\approx$ 2–10), achieving a final concentration of 15%–25% TSS. Several studies have reported the advantages of combining different harvesting methods. Grima et al. (2003) reported that for extremely low-value products (biofuels production, for example), flocculation followed by sedimentation would be a feasible option. Batch experiments performed by Chatsungnoen and Chisti (2016) for the microalgae *C. vulgaris*, *Choricystis minor*, *Cylindrotheca fusiformis*, *Neochloris* sp., and *N. salina* (microalgae presenting different cell sizes, morphologies, and ionic strength

requirements) showed that these microalgae could be effectively harvested from the culture medium using chemical flocculation (with both aluminum sulfate and ferric chloride) followed by sedimentation. To reduce microalgal harvesting costs, Hapońska et al. (2018) evaluated the performance of pH-induced flocculation in combination with dynamic filtration in the pilot-scale harvesting of *D. tertiolecta* and *C. sorokiniana*. With these experiments, the authors reported CFs as high as 207 and 245 for *D. tertiolecta* and *C. sorokiniana*, respectively. In addition, the authors concluded that this combined process was successfully applied in pilot-scale experiments, thus avoiding the use of an energy-demanding centrifugation step. These results evidence the potential benefits of using a combination of methods for microalgal harvesting, from the recovery efficiency point of view to processing time and process economics.

10.6 Research needs

According to the advantages of using a combination of procedures for the development of a cost-effective harvesting method, research studies should focus on the optimization and integration of processes, such as the application of a pre-concentration or thickening process, followed by a dewatering one. Researchers should also take into account process sustainability. Accordingly, methods that allow culture medium/water recycling and avoid biomass contamination or cell damage should be in the priority list.

Regarding specific issues of the most commonly used methods, application of gravity sedimentation, centrifugation, and filtration in a two-step concentration procedure should be studied. Prior concentration of microalgal slurry will allow: (i) the increase of settling rates and consequent decrease of harvesting time (in the case of gravity sedimentation); (ii) the possibility of working at lower shear forces, thus not damaging microalgal cells (in the centrifugation process); and (iii) the improvement of harvesting efficiencies to values close to those obtained with centrifugation, but with lower energetic requirements (in the case of filtration). Alternative filtration procedures should also be a research focus, as membrane fouling and clogging is still a challenge in this process, limiting its application for large culture volumes. In the case of bioflocculation procedures, little is known about the ability of microorganisms to autoflocculate and the interactions between microalgae and the microorganisms used as flocculating agents or as flocculating agents' providers. Therefore, attention should be directed to the complete understanding of: (i) which mechanisms are involved in microalgal autoflocculation and in which conditions this phenomenon occurs; (ii) which microorganisms have flocculating ability, which interactions are established between these microorganisms and microalgae, and in which conditions they exhibit this property; and (iii) which biomolecules have flocculating activity, which microorganisms produce them, and in which conditions they are produced. In addition, although bioflocculation seems to be an important alternative to the use of chemical flocculants, large-scale

cultivation of microalgae with flocculating microorganisms or with microorganisms excreting flocculant substances has not been studied yet. Accordingly, it is of extreme importance to understand their behavior in these conditions for an effective scale-up. Regarding flotation processes, research should be directed to the understanding of how different microalgal species and culture medium composition influence the flotation procedure and harvesting efficiencies. Process scale-up is also an important issue to address in future research studies. Finally, electrical-based methods can be a valuable alternative for microalgal harvesting, but their usage in large-scale applications is also a challenge.

10.7 Conclusions

Microalgal harvesting constitutes one of the most challenging steps of biomass production, due to the associated costs and difficulties in obtaining an effective and reliable method suitable for large-scale harvesting of different microalgal species. Due to these difficulties, several researchers focused their studies on the optimization and improvement of the recovery process. As a result, several technologies and appropriate adaptations have emerged, and nowadays different technologies can be employed in microalgal harvesting. However, selection of the most appropriate technique for microalgal harvesting remains a difficult task, as these technologies differ in several parameters, such as REs achieved, processing time, energy consumption, equipment and operational costs, species-specificity, and suitability for processing large volumes. Additionally, selection of the most appropriate technique should consider final application of microalgal biomass. This chapter provided an up-to-date overview of current harvesting technologies applied to microalgae, emphasizing the main advantages and disadvantages of each one. To help in the selection of the most suitable harvesting techniques for different microalgal species and final applications, an analysis of the most suitable method for the three main applications of microalgal biomass (biofuels production, human food and animal feed, and production of high-valued compounds) was also provided. Weighting the key factors that should be considered for each biomass application with the most effective technologies for each factor, this study identified the most suitable techniques as follows: (i) gravity sedimentation, flocculation, and flotation for biofuels production; (ii) filtration for food and feed applications; and (iii) centrifugation for the production of high-valued compounds. As a way to improve REs further and reduce harvesting costs and processing time, this study also concluded that a combination of thickening and dewatering processes could be a good option.

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CHAPTER 11

Extraction of biomolecules from microalgae

Yulin Hu, Amarjeet Bassi

Department of Chemical and Biochemical Engineering, University of Western Ontario, London, ON, Canada

Chapter outline

11.1 Introduction 283
11.2 Extraction approaches for carotenoids 285
11.2.1 Cell disruption 285
11.2.2 Extraction methods 290
11.2.3 Purification 292
11.3 Biofuels production 293
11.3.1 Biodiesel 293
11.3.2 Bioethanol 298
11.3.3 Bio-crude oil 299
11.4 Future perspectives and conclusions 302
Acknowledgment 303
References 303

11.1 Introduction

In general, both freshwater and marine eukaryotic microalgae and the prokaryotic cyanobacteria have been investigated for the recovery of several biomolecules. In addition, microalgae found on soil or land surfaces and macroalgae (red or brown) have also been utilized. However, these latter types are not the focus of this review. As biological systems, microalgae can provide many types of byproducts, e.g., carbohydrates, proteins, and amino acids, lipids, poly-unsaturated fatty acids (omega-3 or -6), carotenoids (carotene or xanthophyll based), and vitamins. There is also recent interest in the production of bioplastics from microalgae.

It is important to understand the biochemical composition of the microalgae before strategies can be developed for biomolecule extraction. While the internal structure of all photosynthetic eukaryotic microalgae is largely similar, key differences exist in the cell wall and also in the photosynthetic machinery. The chlorophytes (green microalgae) contain chlorophylls a and b and both xanthophylls and carotene pigments. Starch is used as an energy storage molecule in these microalgae and the cell wall contains polysaccharides and/or

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cellulose. The Bacillariophyta (diatoms) have chlorophylls a and c and the pigments carotenes and fucoxanthins. The diatoms utilize carbohydrates such as chrysolaminarin as energy storage materials. They have unique cell walls containing silica and cellulose. The group of microalgae known as Dinoflagellates also contain chlorophyll *a* and c and carotene pigments. Starch is additionally used as an energy storage molecule in these microalgae while cell walls contain cellulose. Finally, another group of the eukaryotic microalgae, the euglenophytes, have chloroplasts containing chlorophyll *a* and c in and carotene pigments. A type of starch (paramylon) is utilized for energy storage in this group. These microalgae lack cell walls; however, they have protein-rich pellicles instead.

Currently, there is significant commercial interest in the nutraceutical market for developing economical manufacturing approaches for one group of molecules from microalgae, namely carotenoids. These molecules are known for their antioxidant properties to protect against oxidative damage. To date, more than 600 carotenoids pigments have been extracted and β-carotene, lutein, and astaxanthin are identified among the most important carotenoids (Ambati et al., 2019). In recent decades, due to their adaptability to cultivation in various conditions, microalgae have been considered as alternative sources to produce carotenoids with antioxidant and antiinflammatory properties (Herrero et al., 2019). In this regard, extensive studies have been carried out toward the extraction of carotenoids from microalgae using different extraction approaches, such as organic solvent extraction and supercritical fluids (Guedes et al., 2013; Millao and Uquiche, 2016a; Tirado and Calvo, 2019).

In another important application, microalgae have been widely investigated as promising feedstocks for biofuel production. This is because of some inherent advantages: (i) faster and continuous production compared to seasonal oil-crops; (ii) high lipid content per unit mass; (iii) carbon neutral processing by sequestering of atmospheric CO_2; (iv) cultivation in wastewater and saline waters; and (v) the ability to accumulate industrially valuable bioactive compounds (Sudhakar et al., 2019). As a result, numerous studies have been carried out to convert microalgae into different types of biofuels, such as biodiesel, bioethanol, biochar, bio-crude oil, bio-hydrogen, and biogas via biochemical, chemical, or thermochemical conversion techniques (Caputo et al., 2016; Cheng et al., 2018; Choi et al., 2014; Sivaramakrishnan and Incharoensakdi, 2018).

Taking these advantages into consideration, a variety of potential applications have been developed for microalgae, including bioactive molecule extraction, biofuel production, and wastewater treatment. An overall possible schematic for microalgal applications is depicted in Fig. 11.1. In this chapter, the extraction of industrially important bioactive molecules (i.e., carotenoids) from microalgae is provided. Following this, the utilization of microalgae as the feedstock for producing biodiesel, bioethanol, and bio-crude oil via different biochemical, chemical, and thermochemical conversions is presented. Finally, the main conclusions and possible future directions are provided.

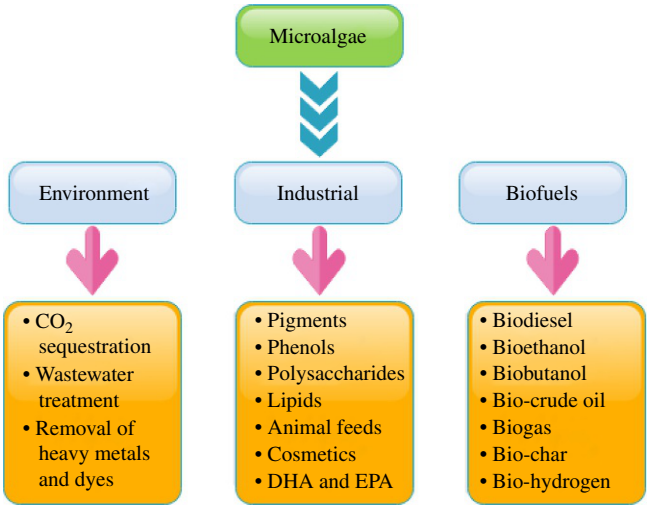

Fig. 11.1
The numerous applications for microalgae (Sudhakar et al., 2019).

11.2 Extraction approaches for carotenoids

At the present time, carotenoids are the focus for the development of various extraction approaches due to their economic potential. These compounds are lipophilic and are composed of a C40 backbone structure of isoprene units, which can be categorized into carotenes and xanthophylls (Gong and Bassi, 2016). The chemical structure of the most commonly studied carotenoids (i.e., β-carotene, lutein, and astaxanthin) in microalgae is shown in Fig. 11.2. The current cell disruption and extraction techniques used for carotenoid recovery from microalgae are discussed in the following sections. It should be noted that these approaches are general and can be adapted for the recovery of a myriad of other products as well from the microalgae.

11.2.1 Cell disruption

Microalgae are covered by rigid cell walls and thus can be highly resistant to either physical or chemical stress, thereby affecting the release of intracellular components. To solve this problem, a number of cell disruption methods have been developed, which can be broadly divided into mechanical and nonmechanical approaches. Table 11.1 summarizes the main advantages and limitations of each cell disruption technique. In general, the mechanical-based approaches are more energy-intensive, and therefore more expensive. Chemical-based approaches may result in environmental waste stream generation. In most cases, the economics of the process and environmental considerations will drive the process selection.

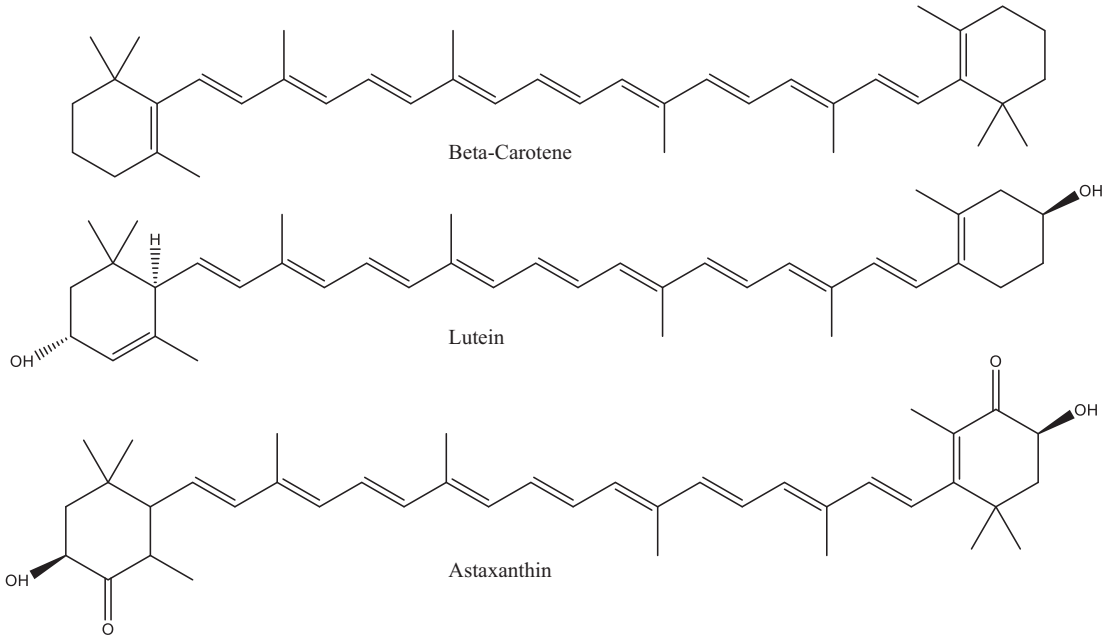

Fig. 11.2
The chemical structure of the most commonly identified carotenoids in microalgae.

Table 11.1: The main advantages and disadvantages for each cell disruption method (Gong and Bassi, 2016; Günerken et al., 2015; Lee et al., 2017).

Approaches	Advantages	Disadvantages
	Mechanical	
Bead milling	High disruption efficiency in single-pass operating; high biomass concentration; easy to control reaction temperature; commercially available bead miller; easy to scale up; less labor-intensive	High energy input; heat can be generated and thus damage bioactive molecules; the requirement for downstream processing
High-pressure homogenization	High disruption efficiency; easy to scale up	Low biomass loading; the requirement for downstream processing; nonselective release of intra-molecules
Ultrasonication	Superior disruption efficiency; short processing time; low toxicity; simple operation	Temperature control is needed; high energy demand
Pulsed electric field	Can be combined with other pretreatment methods; easy to scale up	Additional prewashing and deionization steps are required; higher energy input
Microwave	High disruption efficiency; easy to scale up; less solvent is required	The design for large-scale reactor is needed; temperature control

Table 11.1: The main advantages and disadvantages for each cell disruption method—cont'd

Approaches	Advantages	Disadvantages
Chemical		
Acid and alkaline	Easy to scale-up; low energy consumption; mild capital cost	Possibility to destroy carotenoids
Osmotic shock	Easy to scale-up; low energy consumption; mild capital cost	Cannot extract pigment from microalgae
Ionic liquid	High chemical and thermal stability; low volatility; tunability; can dissolve lignin	High cost of ionic liquid; high energy demand; toxicity; cause damage to carotenoid
Enzyme based approaches	High selectivity; moderate processing conditions; no corrosion problems	High cost of enzyme; the requirement to maintain stable processing conditions; time-consuming

11.2.1.1 Mechanical methods

11.2.1.1.1 Bead milling

The cell disruption by bead milling is achieved by applying compressive and shear stresses resulting from the high-speed movements of solid beads inside the grinding chamber. The major advantages of bead milling are high disruption efficiency, low operational costs, and ease of scale-up, leading to potential for industrialization (Lee et al., 2017). In general, the disruption rate obtained from bead milling is dependent on the filling ratio of the grinding media, bead density and diameter, the velocity of the impeller tip, and the flow rate of the feedstock (Zinkoné et al., 2018). Recently, Kulkarni and Nikolov (2018) compared three different cell disruption methods (high-pressure homogenization, ultrasonication, and bead milling) in terms of cell disintegration rate and protein extraction for the microalgae *Chlorella vulgaris*. It was observed by the authors that the most effective protein recovery (>76%) was obtained from either the pretreatment by bead milling at 15 min or high-pressure homogenization at 15,000 psi. Nevertheless, the extraction by bead milling has disadvantages of high energy consumption and heat generation. In consideration of the heat-sensitive properties of the carotenoids, cell disruption by bead milling might not be a suitable pretreatment approach.

11.2.1.1.2 High-pressure homogenization

The principle behind high-pressure homogenization is the sudden formation of turbulence, shear stress, and cavitation, caused by the mechanically induced movements of the algal cell suspension being forced to pass through a small-sized orifice (Fig. 11.3). As suggested by the

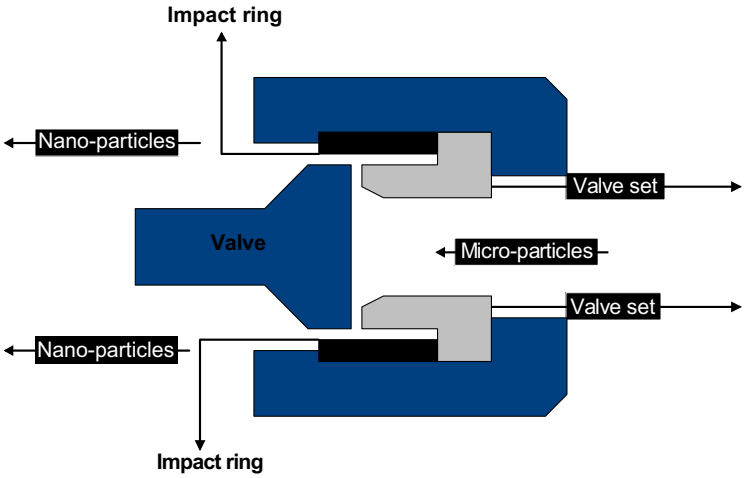

Fig. 11.3
A schematic diagram of a high-pressure homogenizer (Sutradhar et al., 2013).

reported literature, the combination of high-pressure homogenizer with other pretreatment techniques like acid, alkali, or enzyme resulted in the extraction of the valuable carotenoid, astaxanthin, at yields three times larger than that obtained from chemicals such as HCI, NaOH, or enzyme-assisted techniques (Show et al., 2015). The cell disruption efficiency of high-pressure homogenization is a function of a number of factors including the system design, the range of applied pressure, and the microalgal cell dry weight. In a study by Carullo et al. (2018), the rate of cell disintegration obtained from high-pressure homogenization was compared with another approach: pulsed electric field. It was found that high-pressure homogenization was capable of simultaneously disrupting microalgal cell structure and releasing intra-molecules (e.g., protein) from microalgae. However, the formation of large concentrations of fine cell debris also appeared; therefore, further downstream processing was required for the high-pressure homogenization method. On the contrary, the pulsed electric field was found to be a mild pretreatment technology without the formation of cell debris. Apart from the requirement for downstream processing, the high energy input and expensive facility remain as significant challenges for the industrialization of high-pressure homogenization approach.

11.2.1.1.3 Ultrasonication

The basic mechanism for ultrasonication-assisted cell disruption involves the formation of microbubbles in the liquid medium, and these bubbles then violently collapse to create micro-turbulence and pressure shock (cavitation), during which the microalgal cells can be mechanically disrupted, and consequently, the intra-molecular components are rapidly released. In a previous study, Singh et al. (2013) applied various cell disruption techniques to

improve carotenoid extraction efficiency from *Chlorella saccharophila*. Among all the investigated physical methods (ultrasonication, homogenization, bead milling, and maceration), ultrasonication resulted in the highest yield of carotenoid extracted (16.39 mg/g) consisting of 31% β-carotene and 69% zeaxanthin. Grimi et al. (2014) pretreated *Nannochloropsis* sp. using ultrasonication, high-pressure homogenization, high voltage electrical discharge, and pulsed electric field, and their effects on the cell disruption rate and intra-molecules recovery efficiency were studied. The authors reported that pretreatment by either ultrasonication or high-pressure homogenization was effective for pigments recovery from microalgae, whereas the electrically induced disruption methods—high voltage electrical discharge and pulsed electric field—were found to be inefficient to extract pigments, and the use of other cell disruption methods was needed. The main drawbacks of ultrasonication-based disruption include the low disruption efficiency to some microalgal species (Widjaja et al., 2009) and the need for temperature control.

11.2.1.1.4 Microwave

The underlying working principle for microwave-assisted cell disruption is the generation of heat and a sudden increase in the internal pressure. The heat is generated by the movements of inter- and intra-molecules when the liquid medium (e.g., water) is subjected to microwave irradiation. Meanwhile, the internal pressure increases since water can readily reach its boiling point and, therefore, results in the expansion of microalgal cells. Even though microwave-based disruption is easy to operate and a low amount of solvent is required, the reactor design and a good understanding of basic working principles are still needed to address the cell disruption of microalgae.

11.2.1.2 Nonmechanical disruption methods

11.2.1.2.1 Acid, alkali, osmotic shock, and ionic liquids

Acid- or alkali-assisted cell disruption methods are the most commonly used because of their advantages of low energy consumption, moderate capital cost, and ease in scale-up. The underlying mechanism of osmotic shock is related to the differences in the concentration of water across the cell wall/membrane by adding salts, which results in an improvement in the extraction of intra-molecules from microalgae (Hu et al., 2019). However, the extraction by osmotic shock is not able to recover pigments from microalgae owing to the rigid and complex microalgal cell structure. Ionic liquids (ILs) are composed of large asymmetric organic cations and small organic/inorganic anions. ILs exhibit several benefits, including: (i) high chemical and thermal stability; (ii) low volatility; (iii) tunability; and (iv) the ability to dissolve recalcitrant biomass components (e.g., lignin). It should be noted that the extraction treatment by ILs might cause damage to extracted carotenoids as they are not inert

solvents; therefore ILs-assisted extraction is undesirable for carotenoids recovery from microalgae (Gong and Bassi, 2016).

11.2.1.2.2 Enzyme-based approaches

The enzyme-based approach is an effective pretreatment for microalgal cell wall disruption, due to its biological selectivity, moderate reaction conditions, and scale-up potential. Consequently, this pretreatment might be suitable to extract carotenoids from microalgae. However, the high cost of enzyme and the requirement for controlling the operating conditions throughout the pretreatment process are currently limiting its large-scale applications (Lee et al., 2017). Several enzymes such as proteases, lipases, and cellulase can be applied. In a recent study, Coelho et al. (2019) utilized 200 preselected carbohydrate-active enzyme (CAZymes) and sulfatases, individually or together, to pretreat *C. vulgaris*, and the effects of these enzymatic pretreatments on the further recovery of carotenoids were assessed. Coelho et al. observed that the yield of carotenoids extracted was improved when microalgae underwent pretreatment by enzyme mixtures. This could be attributed to the disruption of microalgal cell wall by mixed enzymes, thereby promoting the release of microalgal intramolecular compounds.

11.2.2 Extraction methods

In the following sections, two extraction methods, namely organic solvent and supercritical fluid extraction, are fully discussed, and their relating case studies are summarized in Table 11.2.

Table 11.2: A summary of the most recent studies investigating carotenoid extraction from microalgae.

Extraction approaches	Microalgae strains	References
Organic solvent		
	H. pluvialis *Heterochlorella luteoviridis* *C. sorokiniana* MB-1 *Nannochloropsis* spp.	Molino et al. (2018) Jaeschke et al. (2016) Chen et al. (2016) Parniakov et al. (2015)
Supercritical CO_2		
	T. obliquus *D. salina* *N. gaditana* *D. salina* *Neochloris oleoabundans* *N. gaditana* *U. pinnatifida* *S. obliquus* M2-1	Chronopoulou et al. (2019) Tirado and Calvo (2019) Millao and Uquiche (2016a) Mouahid et al. (2016) Reyes et al. (2016) Millao and Uquiche (2016b) Goto et al. (2015) Guedes et al. (2013)

11.2.2.1 Organic solvent extraction

The extraction of carotenoids from microalgae follows the basic chemistry, "like dissolves like," and the commonly adopted solvents are chloroform/methanol, hexane/isopropanol, and dichloromethane/methanol. For instance, Molino et al. (2018) carried out lutein extraction from *Haematococcus pluvialis* using four different organic solvents (e.g., chloroform/methanol, hexane, acetone, and ethanol) under various reaction conditions (temperature: 50–100°C; pressure: 5–10 MPa; processing time: 20–80 min). At 50°C, the yield of lutein extracted was increasing with increasing pressure, i.e., the use of chloroform/methanol slightly increased the lutein extraction from 2 mg/g at 5 MPa to 2.2 mg/g at 10 MPa. In comparison, the highest yield of lutein was obtained by using acetone as the extraction solvent, i.e., acetone: 3.9–4.3 mg/g; ethanol: 2.3–2.6 mg/g; hexane: 3.5–3.8 mg/g; chloroform/methanol: 2–2.2 mg/g. In addition, the yield of extracted lutein increased with an increase in the temperature; however, further increases in the temperature lowered the lutein recovery. This phenomenon might be due to the thermal instability of carotenoids at higher temperatures. At pressure of 10 MPa, chloroform/methanol resulted in the highest lutein yield of 5.9 mg/g at 67°C for 80 min, whereas the optimal lutein recovery rate of 72% was achieved by using ethanol as the extraction solvent. Moreover, Chen et al. (2016) extracted lutein from *Chlorella sorokiniana* MB-1 using different cell disruption pretreatments (e.g., bead milling, French pressing, and freeze-drying), followed by solvent extraction under varying processing conditions. As expected, the yield of lutein extracted from pretreated microalgae was higher than that obtained from raw feedstock. Specifically, the pretreatment by French pressing coupled with tetrahydrofuran (THF) extraction led to a recovery rate of lutein of 87%, which was much higher compared to that obtained from bead milling pretreated microalgae (58.7%). THF was observed to be the most effective solvent in terms of lutein recovery from *C. sorokiniana* MB-1, when compared with ether, ethanol, acetone, and hexane. It was also observed that the lutein recovery by THF extraction improved from 87% at 20 min to 99.5% at 40 min, suggesting that the processing time of 40 min could completely extract lutein from microalgae.

Although the extraction by organic solvent provides benefits, such as low cost and ease of scale-up, the prolonged processing time, requirement for downstream processing, and the use of large amounts of organic solvents make this extraction technique undesirable for the future biorefinery.

11.2.2.2 Supercritical fluid extraction

Extraction by supercritical fluids is regarded to be a desirable approach for recovery of bioactive compounds from natural sources like microalgae. This technology offers several inherent benefits: (i) easy to scale-up; (ii) can be combined with other extraction methods; and (iii) fulfills the criteria for the concept of Green Chemistry (Herrero et al., 2019). Until now, supercritical CO_2 has been one of the common solvents to extract carotenoids from microalgae (Tirado and Calvo, 2019).

Chronopoulou et al. (2019) explored the influences of temperature, pressure, the addition of a cosolvent on the carotenoids extraction from microalgae *Tetradesmus obliquus* using supercritical CO_2 treatment. The tested reaction conditions included: temperature of 40–60°C; pressure of 10–35 MPa; and methanol and ethanol as cosolvents. It was observed that increasing pressure at a constant temperature led to an increase in the extraction yield of carotenoids, which could be caused by the improvement in the solvent power with increasing pressure. On the contrary, an opposite trend was found between the extraction rate of carotenoids and temperature at a constant pressure. Similarly, Millao and Uquiche (2016a,b) carried out a systematic investigation on the carotenoids recovery by supercritical CO_2 under varying reaction conditions, i.e., temperature: 36–64°C; pressure: 31.7–54.3 MPa; and CO_2 density: 914–56 kg/m^3. The results showed that the highest yield of extracted carotenoids was in the range of 393–773.7 mg/kg dry substrate. The authors also reported that the recovery efficiency of carotenoids was proportional to temperature and CO_2 density. Specifically, the effect of temperature on the carotenoids extraction was found to be stronger than that of CO_2 density. In another study, Mouahid et al. (2016) studied the effects of water containing the microalgae and the drying pretreatment on the extraction rate and kinetics of carotenoids from microalgae, *Dunaliella salina*, via supercritical CO_2. They found that all three tested drying methods, including air flow drying, microwave-assisted air flow drying, and freeze-drying, promoted the recovery of carotenoids from microalgae. Among them the combined pretreatment consisting of air flow drying and microwave was determined as the most desirable method for *D. salina*. Additionally, it was reported that the water content of microalgal biomass did not negatively affect the extraction process, but served as a cosolvent for promoting carotenoids recovery.

11.2.3 Purification

After the completion of extraction, a series of purification stages (Fig. 11.4) are often conducted in order to obtain highly purified carotenoids. Initially, NaOH or KOH, as the saponification agent, is introduced into the microalgae-derived extracts to release carotenoids. Following this, the extraction by an organic solvent (e.g., hexane and ethanol-water-dichloromethane) is carried out to create two phases, during which the target products are partitioned into organic solvent phase. Afterwards, the organic solvent is removed and the obtained crude carotenoids undergo recrystallization to produce highly purified carotenoid.

It is worth mentioning that the conventional purification methods have the following limitations: (i) extensive time required; (ii) labor-intensive; and (iii) the requirement for a large amount of organic solvent. In this sense, several modern purification techniques have been proposed, such as expanded bed coupled column chromatography and reversed phase high performance liquid chromatography (HPLC); however, the high costs of these newly developed approaches impede their large-scale applications.

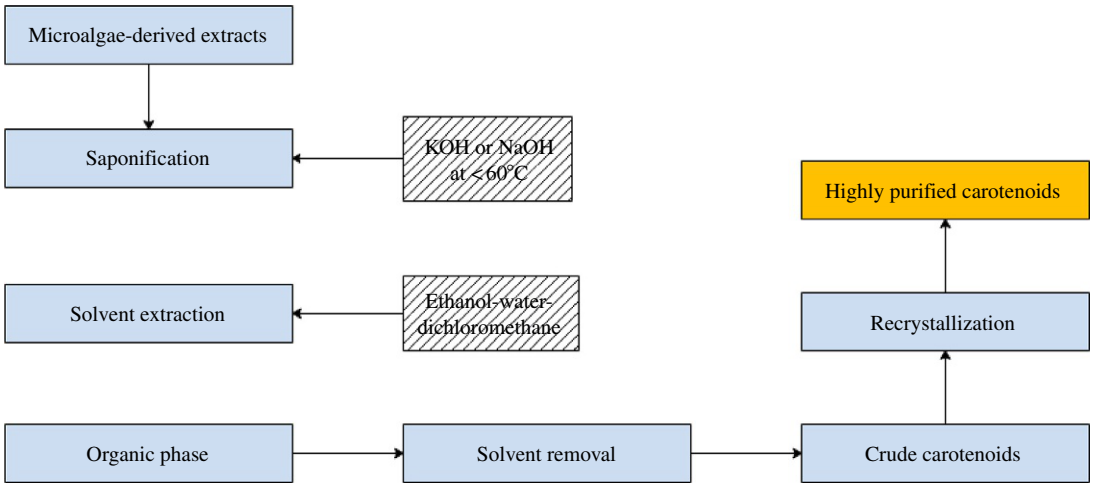

Fig. 11.4
A schematic diagram of conventional purification technology used for carotenoids from microalgae (Gong and Bassi, 2016).

11.3 Biofuels production

General speaking, microalgae-derived bioenergy can be presented in the form of liquid, solid, or gas, like biodiesel, biochar, and syngas. In this section, biodiesel, bioethanol, and bio-crude oil from microalgae by biochemical, chemical, or thermochemical conversion routes are discussed, and the most recent studies are summarized in Table 11.3.

11.3.1 Biodiesel

As indicated by Deshmukh et al. (2019), around 19,000–57,000 oil/acre can be accumulated by microalgae on a yearly basis, which is higher than other biodiesel sources. In common, there are several stages required for producing biodiesel from microalgae, among which lipid extraction is considered as the most critical step. The lipid extraction approaches used for microalgae are shown in Fig. 11.5.

11.3.1.1 Lipid extraction

11.3.1.1.1 Organic solvent

Conventionally, organic solvent extraction has been extensively used for extracting lipids from microalgae, i.e., polar solvent for dissolving cell structure-related lipids like phospholipids and nonpolar solvent for dissolving neutral lipids. To improve lipid extraction efficiency, the cell disruption by a mechanical method is usually conducted prior to organic solvent extraction. Consequently, the selection of the proper cell disintegration technique and organic solvent are the key elements for microalgal biodiesel production. Broadly, a cosolvent

Table 11.3: A summary of the most recent studies on the production of biodiesel, bioethanol, and bio-crude oil from microalgae.

Biofuels	Microalgae strains	Approach	References
Biodiesel			
	Schizochytrium sp.	Hydrolysis—ethanolysis	Kuan et al. (2019)
	Scenedesmus sp.	Supercritical CO_2 with methanol	Shomal et al. (2019)
	C. pyrenoidosa	In situ transesterification	Bindra and Kulshrestha (2019)
	Botryococcus sp.	Ultrasonication-assisted transesterification	Ashokkumar et al. (2019)
	Anabaena sp. OCC7120	Organic solvent extraction—transesterification	Singh et al. (2019)
	Shizochitrium limacinum	Transesterification by supercritical fluids	Mani Rathnam and Madras (2019)
	N. oleoabundans	Transesterification by Fe_2O_3 nanocatalyst	Banerjee et al. (2019)
	C. vulgaris	In situ transesterification	Felix et al. (2019)
	C. saccharophila	Supercritical CO_2—transesterification	Alhattab et al. (2019)
	N. oculate	Ultrasonication assisted-organic solvent extraction—transesterification	Vinoth Arul Raj et al. (2019)
	C. vulgaris	Transesterification with CaO catalyst	Pandit and Fulekar (2019)
Bioethanol			
	Chlorella sp.	Acid hydrothermal treatment/enzymatic hydrolysis—fermentation	Ngamsirisomsakul et al. (2019)
	S. platensis	Freeze drying—enzymatic hydrolysis—fermentation	Rempel et al. (2019)
	H. tetrachotoma ME03	Chemical/enzymatic hydrolysis—fermentation	Onay (2019)
	Monoraphidium sp.	Acid-base transesterification	Mishra and Mohanty (2019)
Bio-crude oil			
	C. vulgaris; *Arthrospira platensis*	HTL	Palomino et al. (2020)
	Chlorella	Co-liquefaction with sewage sludge	Xu et al. (2019b)
	C. vulgaris; *N. gaditana*	HTL	Guo et al. (2019)
	C. vulgaris	HTL	Rathsack et al. (2019)
	Tetraselmis sp.	HTL	Das et al. (2019)
	Chlorella	HTL	Xu et al. (2019a)

Table 11.3: A summary of the most recent studies on the production of biodiesel, bioethanol, and bio-crude oil from microalgae—cont'd

Biofuels	Microalgae strains	Approach	References
	Tetraselmis sp.	HTL	Eboibi (2019)
	C. pyrenoidosa	Co-liquefaction with sweet potato residue	Wang et al. (2019)
	S. platensis	Co-liquefaction with α-cellulose	Feng et al. (2019)
	Tetraselmis sp.	Liquefaction in isopropyl alcohol-ethylene glycol cosolvents	Han et al. (2019)
	S. obliquus	HTL	Arun et al. (2019)
	C. vulgaris	Co-pyrolysis with wood and scrap tire	Azizi et al. (2019)
	Chlorella sp.; *Bracteacoccus* sp.	Pyrolysis	Shahid et al. (2019)
	S. platensis	Co-pyrolysis with swine manure digestate	Vuppaladadiyam et al. (2019)
	Defatted *Euglena gracilis*	Pyrolysis	Jung et al. (2019)
	Chlorella	Pyrolysis	Palumbo et al. (2019)
	Nannochloropsis sp.	*Co*-pyrolysis with low-density polyethylene	Tang et al. (2019)

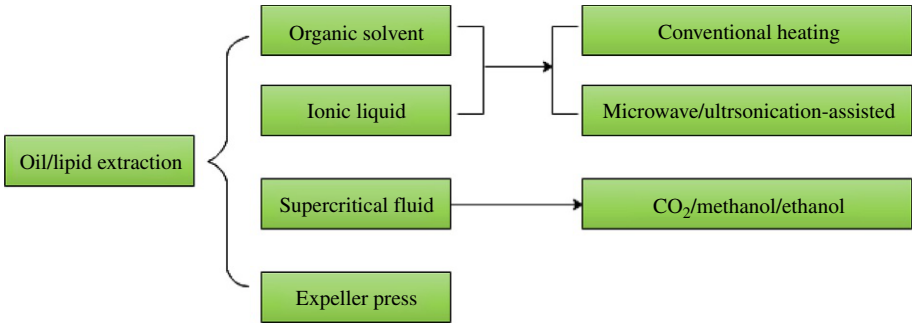

Fig. 11.5
The lipid/oil extraction approaches used for microalgae (Deshmukh et al., 2019).

system consisting of a polar and nonpolar solvent has been used for various types of microalgae. Polar solvents like methanol are capable of breaking down the hydrogen bonds (forming the structural lipids) and this allows nonpolar solvents, such as chloroform, to penetrate the cell cytoplasm, during which neutral lipids present inside the microalgal cell can be extracted. At the end of the extraction process, water is added into the mixture to create two layers; i.e., hydrophobic neutral lipids can be found in the chloroform-containing phase and hydrophilic structural lipids are observed in the methanol-containing phase. Cheng et al. (2011), for instance, used an organic cosolvent mixture of hexane/toluene/ethyl acetate and

method (2/1, v/v) for the extraction of lipid from *Pavlova* sp., which was coupled with an ultrasonication treatment. The results showed that the yield of crude lipid from hexane/methanol, toluene/methanol, and ethyl acetate/methanol was 13.8 wt%, 17.6 wt%, and 44.7 wt%, respectively. This large variation in the crude lipid yield might be due to the polarity of each solvent.

11.3.1.1.2 Ionic liquids

Recently, a number of investigations have been performed on lipid extraction by ILs from microalgae. Lu et al. (2019) utilized [BMIM]Cl for the extraction of lipid from *Chlorella pyrenoidosa*, and approximately 89% (w/w) of lipid was recovered by using the IL extraction approach, whereas around 26% (w/w) of protein and 99% (w/w) of chlorophyll were found to be dissolved in the IL because of the disruption of the microalgal cell wall/membrane. Furthermore, a high stability of [BMIM]Cl solvent was reported during the recycling studies by analyzing its molecular structure. Chua et al. (2019) investigated the lipid extraction efficiency from *Nannochloropsis* sp. using cholinium [Ch] amino acid-based ILs, including cholinium arginate [Ch][Arg], cholinium prolinate [Ch][Pro], cholinium lysinate [Ch][Lys], cholinium tryptophanate [Ch][Trp], cholinium leucinate [Ch][Leu], cholinium glycinate [Ch][Gly], cholinium phenylalaniate [Ch][Phe], cholinium valinate [Ch][Val], cholinium alaninate [Ch][Ala], cholinium histidinate [Ch][His], cholinium serinate [Ch][Ser], cholinium glutaminate [Ch][Gln], cholinium asparaginate [Ch][Asp], cholinium aspartate [Ch][Asp], cholinium glutamate [Ch][Glu], and 1-ethyl-3-methyl limidazolium acetate [emim][Ac]. The results indicated that nearly 100 wt% of lipid were extracted when using [Ch][Arg], during which only 1.4 wt% of lipid remaining in the microalgal residue.

Even though lipid extraction using ILs have been found to be more effective for microalgae when compared with conventional organic solvent extraction (Kim et al., 2013), the high cost of ILs and related environmental concerns remain as technical barriers to its industrial implementation (Hu et al., 2019).

11.3.1.1.3 Supercritical fluid extraction

As indicated by the previous literature, the most commonly used solvents in the extraction by supercritical fluid include CO_2, methanol, and ethanol. The main advantages of using supercritical fluid are the short period of processing time, no addition of catalyst, and recoverability. Crampon et al. (2017) extracted lipid and antioxidants (chlorophylls and β-carotene) from *Spirulina platensis* using supercritical CO_2, and the effects of temperature (45–65°C), pressure (28–46 MPa), and CO_2/microalgae ratio (80–100 (w/w)) on the lipid recovery efficiency and antioxidants concentration were examined. They found that pressure was identified as the most important reaction variable in the lipid-extract yield, whereas temperature appeared to be the most influential parameter on the recovery of antioxidants. Taher et al. (2014) optimized the reaction conditions for extracting lipids from *Scenedesmus* sp.

by supercritical CO_2 extraction, and compared this with conventional organic solvent extraction (hexane/methanol and chloroform/methanol). The authors observed that the extraction by supercritical CO_2 was more efficient than an organic solvent, and the highest lipid yield (7.4 wt%, based on dry weight) was obtained from supercritical CO_2 extraction at a temperature of 53°C and pressure of 50 MPa, and a CO_2 flow rate of 1.9 g/min. Apart from CO_2, methanol and ethanol have also been widely used, particular for directly producing biodiesel from microalgae (Mohamadzadeh Shirazi et al., 2017; Nan et al., 2015; Reddy et al., 2014).

11.3.1.2 Transesterification

At the end of the lipid/oil extraction stage, direct transesterification between triglycerides (TAGs) and methanol with the use of catalyst is carried out to produce fatty acid alkyl ester (or biodiesel), which is accompanied by the formation of glycerol as a by-product. As a contrast, more recent research has focused on the direct conversion of wet microalgae (water content: 60–80 wt%) into biodiesel using in situ transesterification (IST) technology. Fig. 11.6 depicts a schematic diagram of IST treatment of microalgae with an organic solvent and acid catalyst. Nevertheless, future work on performing the continuous economic evaluation of in situ transesterification using wet microalgae is still needed (Kim et al., 2019).

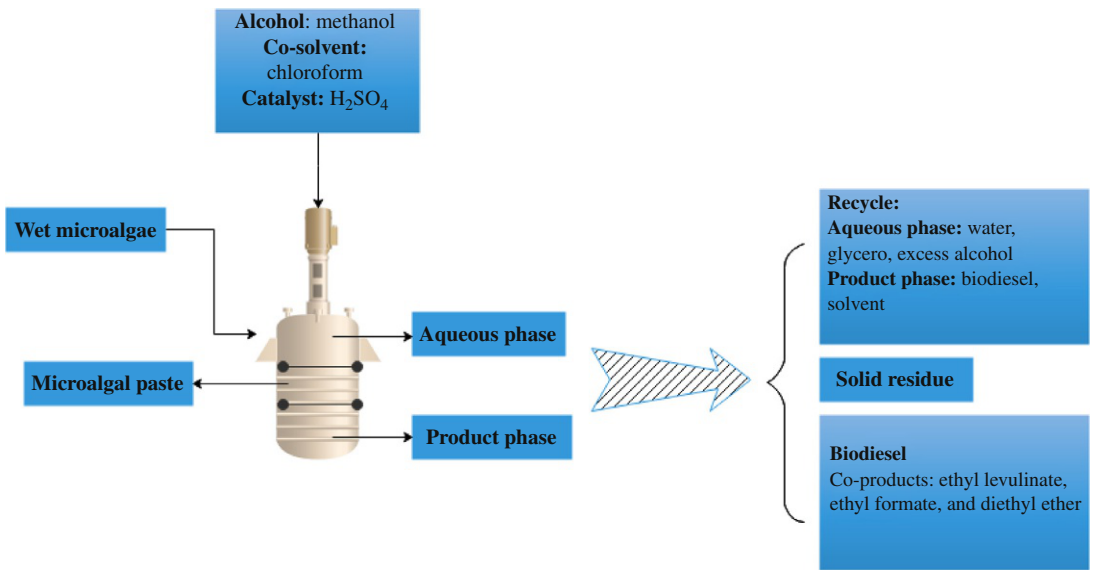

Fig. 11.6
A schematic diagram of in situ transesterification of wet microalgae for biodiesel production (Kim et al., 2019).

11.3.2 Bioethanol

To compare with conventional lignocellulose biomass, microalgae containing no or little lignin are widely considered as the promising sources for bioethanol production. Conventionally, there are three primary stages involved in the production of bioethanol: (i) pretreatment of biomass; (ii) hydrolysis of polysaccharides into simple sugars; and (iii) conversion of these sugars into bioethanol by fermentation (Fig. 11.7).

11.3.2.1 Pretreatment

Until now, a number of pretreatment methods have been developed for microalgae to improve bioethanol production, which can be broadly categorized into chemical (e.g., acid and alkali), physical (e.g., bead milling, ultrasonication, and microwave), enzymatic, and combined approaches. Among them, chemical pretreatment by H_2SO_4, HCl, NaOH, or KOH are among the most accessible approaches for disrupting the cell wall/membrane and releasing simple sugars from microalgae. Cell disruption by acid pretreatment is achieved through the hydrolysis of sugar polymers in the microalgal cell wall, while, in contrast, the saponification of membrane lipid is attributed to the cell disruption during alkaline pretreatment (Hu et al., 2019). Sert et al. (2018) adopted acid and alkaline pretreatment approaches to enhance bioethanol production from *Chlorella minutissima*. The pretreatment by acid was performed at 100–140°C for 15–60 min with 0.5 N, 1 N, 2 N, 3 N, and 5 N H_2SO_4 solution, and alkaline pretreatment was carried out using 0.5%, 0.75%, 1%, 1.5%, or 2% (v/v) KOH solution at 80–120°C for 15–60 min. The results showed that the yield of bioethanol from H_2SO_4 pretreatment (2.92%–18.52%) was higher than that from KOH pretreatment (1.01%–6.11%). Specifically, the bioethanol yield was proportional to acid concentration up to 1 N, while further increases in the acid content lowered the yield of bioethanol. This reduction could be due to the formation of furaldehyde, acetate, and 5-hydroxymethyl-2-furaldehyde, which have been reported to limit fermentation processes.

A similar trend was also observed in the yield of bioethanol from alkaline pretreatment. It should be noted that moderate temperature and low concentration of chemical addition are

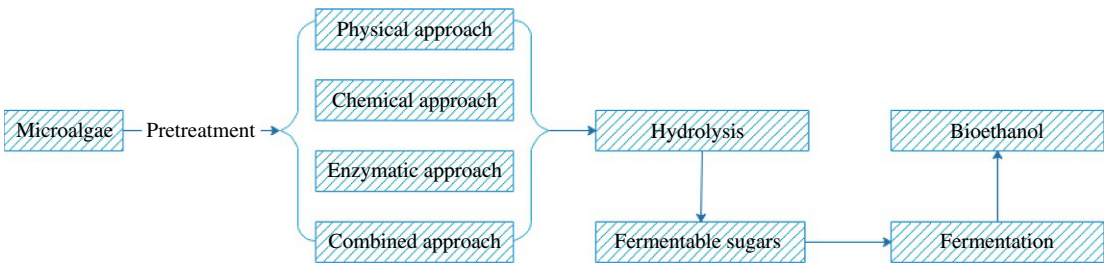

Fig. 11.7
A schematic diagram of bioethanol production from microalgae (Phwan et al., 2018).

needed when recovering bioactive microalgal molecules. Except for chemical pretreatment, physical methods such as bead milling, freeze-drying, microwave, and ultrasonication have been widely employed for various microalgal strains (Gong and Bassi, 2016). Although the physical pretreatment methods have the advantages of easy scale-up and high disruption efficiency, heat can be generated during pretreatment, which might damage fragile bioactive compounds, and the methods' requirement for high energy demand must be addressed.

11.3.2.2 Hydrolysis and fermentation

Rempel et al. (2019) conducted the enzyme-assisted saccharification of *S. platensis* after pretreatment by freeze-drying, and the resulting sugars were used as the feedstock for producing bioethanol via fermentation at 30°C using yeast *Saccharomyces cerevisiae* CAT-1. The results indicated that the carbohydrates conversion efficiency at 120 min was ~ 50% and then remained stable after 10 h of saccharification (82%), leading to an amount of 70.45 g/L of reducing sugars. This high hydrolyzed efficiency caused by the use of combined α-amylase and amyloglucosidase enzymes might be due to their strong affinity to the substrate and thus improves the formation of fermentable sugars. Onay (2019) hydrolyzed microalgae, *Hindakia tetrachotoma* ME03, using enzymes, acids, or alkali, followed by the fermentation of the obtained reducing sugars into bioethanol using the yeast *S. cerevisiae* S288C at 30°C and 150 rpm for 12–48 h anaerobically. It was observed that the pretreatment by the combined enzymes of β-glucosidase/cellulase and α-amylase led to an optimal saccharification rate of 92.3%.

In a similar study, Hernández et al. (2015) compared the extraction efficiency of physical, chemical, and enzymatic hydrolysis for three microalgae strains, *C. sorokiniana*, *Nannochloropsis gaditana*, and *Scenedesmus almeriensis*. Additionally, the effect of the combination of two different pretreatment methods on the amount of simple sugars from microalgae was explored. The authors reported that the most effective hydrolysis approach for both *C. sorokiniana* (128 mg/g) and *N. gaditana* (129 mg/g) was acid hydrolysis at 121°C for 30 min by 7% and 10% H_2SO_4, respectively, coupled with enzymatic hydrolysis by cellulases (Celluclast and Novozyme), whereas, in the case of *S. almeriensis*, the highest yield of monosaccharide (88 mg/g) was achieved from acid hydrolysis by H_2SO_4 at 121°C for 60 min alone. These results might be attributed to the fact that the use of acid before enzymatic hydrolysis could allow enzymes to be accessible to carbohydrate chains, thereby facilitating the disruption of carbohydrates and promoting their conversion into monosaccharides.

11.3.3 Bio-crude oil

Bio-crude oil is a dark brown and viscous liquid containing numerous organic components, such as N- and O-containing compounds, organic acids, alcohols, straight and branched hydrocarbons, and aromatic compounds. Generally, the chemical and physical properties of

Table 11.4: The major chemical compounds (relative peak area > 1%) of bio-crude oil obtained from *S. platensi* at 300°C with the use of KOH as the catalyst, as determined by GC–MS analysis (Zhang et al., 2018).

Compounds	Area (%)
Ketones	
7-Ethyl-4,6-heptadecandione	4.41
6-Hydroxy-3-methyl-9-oxabicyclo[3.3.1]nonan-2-one	16.23
5,8-Heptadecanedione	5.77
Amides	
N-(3-methylbutyl)-myristamide	1.28
Hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-α] pyrazine-1,4-dione	6.32
3,6-bis(2-methylpropyl)-2,5-piperazinedione	7.13
Tetradecanamide	5.83
(3*S*,6*S*)-3-butyl-6-methylpiperazine-2,5-dione	7.11
Hydrocarbons	
Heptadecane	1.78
Organic acids	
n-Hexadecanoic acid	24.84
Palmitoleic acid	1.46
Total (%)	82.16

microalgae-based crude oil are determined by feedstock characteristics, temperature, residence time, and catalyst type/dosage (Hu et al., 2019). The chemical composition of bio-crude oil from *S. platensis* is shown in Table 11.4. So far, the most common conversion technologies adopted for converting microalgae into bio-crude oil include pyrolysis and hydrothermal liquefaction (HTL).

11.3.3.1 Pyrolysis

Pyrolysis is a thermochemical conversion technique conducted at 350–700°C in oxygen-free conditions, during which bio-crude oil, biochar, and gaseous products can be generated (Saber et al., 2016). Owing to the differences in the operation conditions including residence time, heating rate, and reaction temperature, pyrolysis can be divided into slow, flash, or fast pyrolysis. Among these, flash and fast pyrolysis aim for optimizing liquid crude oil production, while in contrast, a high yield of biochar is often produced from slow pyrolysis. Miao et al. (2004) performed fast pyrolysis of two different microalgae species (i.e., *Chlorella protothecoides* and *Microcystis aeruginosa*) in a fluid bed reactor at 500°C for 2–3 s and a heating rate of 600°C/s. The yield of bio-crude oil from *C. protothecoides* and *M. aeruginosa* was 18 wt% and 24 wt%, respectively, which was accompanied by a heating value (HHV) of 30 MJ/kg (*C. protothecoides*) and 29 MJ/kg (*M. aeruginosa*). Although the O-content of bio-crude oil obtained from pyrolysis of microalgae (20.19%) was considerably higher than that

of petroleum crude oil (0.05–1.5%), higher HHV (29–30 MJ/kg) and lower density (1.16 kg/L) were found for microalgae-derived oil product, when compared with crude oil from woody biomass (HHV: 21 MJ/kg; density: 1.2 kg/L). Hence, it can be inferred that microalgae might be a more suitable feedstock for producing high-quality bio-crude oil than lignocelluloses.

In a more recent study, Babich et al. (2011) carried out pyrolysis experiments of *Chlorella* in a fixed-bed reactor at 300–450°C, with or without the addition of Na_2CO_3 as the catalyst, and the resulting bio-crude oil from catalytic pyrolysis was observed to contain higher HHV and lower acidity than those obtained from the noncatalytic run. Most importantly, the authors claimed that both catalytic and noncatalytic pyrolysis resulted in significantly higher energy recovery efficiency (42% and 44%, respectively) than that from biodiesel production (13%), thereby confirming pyrolysis as an efficient conversion method for microalgae, particularly the low lipid-containing species. Recently, some studies have evaluated microwave-assisted pyrolysis (MAP) for converting microalgae into bio-crude oil, which is noted for its advantages of uniform internal heating, instant response, and nonrequirement for agitation/fluidization (Borges et al., 2014; Du et al., 2011; Huang et al., 2017). Du et al. (2011) reported that the highest yield of bio-crude oil obtained from MAP of *Chlorella* sp. was 28.6 wt%, which was accompanied by the density, viscosity, and HHV of 0.98 kg/L, 61.2 cSt, and 30.7 MJ/kg, respectively.

11.3.3.2 HTL

HTL can be defined as the conversion of biomass into four different products (bio-crude oil, solid residue, water-solubles, and gas) at 200–400°C and 5–20 MPa by using water as the reaction medium. When considering the high water content of microalgae, HTL has been broadly employed as the conversion technique for producing biofuel from microalgae due to its elimination for drying the feedstock (Hu et al., 2019). According to a previous study, the authors claimed that around half of the energy is required in the process of distillation and drying (Peterson et al., 2008). In comparison, the bio-crude oil obtained from HTL contained less oxygen than that obtained from pyrolysis, thereby resulting in a higher energy density (Guo et al., 2015). In addition, not only lipid but also protein and carbohydrates present in the microalgae can be liquefied all together into bio-crude oil.

Guo et al. (2019) liquefied *C. vulgaris* and *N. gaditana* in a continuous stirred tank (CSTR) or batch reactor at 350°C and 24 MPa for 15 min. For the CSTR reactor, it was found that the bio-crude oil yield from *C. vulgaris* (36.2 wt%) was higher than that from *N. gaditana* (31.5 wt%), which might be due to the higher lipid content in the *C. vulgaris* (23.6 wt%) than that in the *N. gaditana* (18.0 wt%). On the contrary, a higher yield of bio-crude oil was observed in the HTL of *N. gaditana* (47.9 wt%) compared to that obtained from HTL of *C. vulgaris* (42.5 wt%) in a batch reactor. This difference in the oil yield could be attributed to the variations in the reaction time between the CSTR (15 min) and batch reactor (30 min including heating and cooling period). At a longer residence time, protein can be further decomposed and react

with carbohydrates, thereby promoting bio-crude oil formation. Another reason could be related to the effect of back mixing occurring in the CSTR reactor.

Hietala et al. (2019) investigated the effects of temperature, species identity, feedstock characteristics, residence time, and feedstock loading on HTL of microalgae in terms of bio-crude oil yield and properties. Among all tested reaction variables, feedstock composition and temperature showed the most important impact on the bio-crude oil formation. It was also found that the effect of feedstock concentration and species identity was similar to that of residence time. Additionally, the reaction between amino acids from protein and reducing sugars from carbohydrates via a series of Maillard reactions was reported to be affected by the feedstock loading and the ratio of protein and carbohydrates. Remarkably, a high concentration of saturated, monounsaturated, and polyunsaturated fatty acid in the oil product was observed at a short residence time, which implies that fast HTL could be a promising route for producing value-added unsaturated fatty acids.

However, HTL is still at an early development stage compared to pyrolysis, and a thorough understanding of its reaction chemistry is lacking. Another challenge relating to HTL is the requirement for high-pressure working conditions, which leads to high-cost and serious technical barriers (Saber et al., 2016).

11.4 Future perspectives and conclusions

In this chapter, the cell disruption and extraction techniques adopted specially for carotenoid recovery or for biofuel chemicals from microalgae were discussed. While there is extensive research on extraction approaches, at this time, the commercial viability of these approaches is still lacking until a stable market demand develops for microalgae base byproducts. The current cell disruption methods can be broadly categorized into mechanical (bead milling, high-pressure homogenization, ultrasonication, and microwave) and nonmechanical (acid, alkali, osmotic shock, ionic liquid, and enzyme) methods. In general, mechanical technologies offer high disruption efficiency and are easy to scale-up; however, high energy input and the requirement for heat control need to be addressed. In addition, chemical-based methods might cause damage to carotenoids and a large amount of chemicals is commonly required. Thus, future work should focus on the development of cell disruption techniques for extracting carotenoids from microalgae with improved process control, lower energy input and solvent usage, and no contamination of the disruption agent.

Moreover, the working principles and relating case studies of two commonly used carotenoids extraction approaches, namely organic solvent and supercritical fluid extraction, were provided. It can be concluded that supercritical fluid extraction techniques have potential advantages over conventional organic solvent methods; however, this area needs further research on different aspects: (i) a thorough understanding of the physiochemical properties

of bioactive compounds; (ii) the development of novel green solvents; and (iii) the scale-up of supercritical fluid is another perspective of future work since most current studies are carried out in lab scale (Herrero et al., 2019).

Compared to conventional lignocellulose biomass, microalgae contain no or little lignin. Thus microalgae can also be used an alternative sources for biofuels production, such as biodiesel, bioethanol, and bio-crude oil, via different conversion technologies (e.g., transesterification, in situ transesterification, fermentation, pyrolysis, and HTL). HTL approaches may offer more advantages due to their better fuel quality, as the high cost of current biofuel production methods and low price of crude oil have severely impacted the biofuel industry. Although HTL offers advantages of eliminating the feedstock drying stage, the low quality of microalgae-derived bio-crude oil, particularly the high N-content, remains the most important technical barrier. The batch reactor system is widely employed in the current HTL investigations, and therefore future work should focus on the liquefaction performing in a continuous system.

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PART 3

Microalgae-based products

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CHAPTER 12

Biogas from microalgae

Bárbara Rincón

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain

Chapter outline

12.1 Introduction 311
12.2 Anaerobic digestion of microalgae 312
12.3 Bacteria and archaea in the AD of microalgae 315
12.4 Main drawbacks affecting AD of microalgae 315
12.4.1 Complex cell walls 315
12.4.2 Low C/N ratios 317
12.4.3 Humidity of microalgal biomass 319
12.4.4 Salinity 319
12.5 AD of spent microalgae 320
12.6 Nutrient and CO_2 recycling: Toward a sustainable closed loop 321
12.7 Biorefinery concept 323
12.8 Conclusions 323
References 323

12.1 Introduction

Global warming and the need to reduce the use of fossil fuels have motivated the search of alternative fuels. In this sense, biofuels are promising alternatives. Technologies associated to the first-generation biofuels are very mature but feedstock supply is one of the main drawbacks. The growth of corn or sugarcane for this purpose competes with food producing. In this regard, they cannot act in response to the current energy demand for the substitution of oil. In this sense, second-generation biofuels, obtained from wastes and biomass, together with third-generation biofuels, obtained from microalgae, are seen as one of the renewable energies that will be used in the future (Appels et al., 2011; Borja and Rincón, 2017). Third-generation biofuels appear to be a promising alternative for energy production and to mitigate climate change. There is an increasing number of studies about the life-cycle assessment of biofuel deriving from microalgae (Quinn et al., 2014a; Morales et al., 2019) and the cost of microalgae production (Acién Fernández et al., 2019). Microalgae have revealed their potential for biofuels generation such as biodiesel, biohydrogen, bioethanol,

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and biogas. Among the main advantages associated with the use of microalgae as substrates for AD processes are that they have high growth rates and they do not compete with food crops for arable land (De Schamphelaire and Verstraete, 2009; Stephens et al., 2010). They can be grown in wastewater and in seawater. Furthermore, they have low lignin content and can be easily introduced into the biorefinery approach (Montingelli et al., 2015). Other advantages linked to microalgal AD are that it is possible to exploit the entire organic biomass for energy production, and wet biomass can be used (Milledge and Heaven, 2014). The use of wet biomass is a major advantage when compared with other methods of microalgal utilization for biofuel production like biodiesel production. In addition, the operation of the AD reactors does not require high energy inputs.

12.2 Anaerobic digestion of microalgae

Anaerobic digestion (AD) is a very interesting and complex process which allows for obtaining biogas from organic matter. AD processes have been widely studied and applied to large quantities of substrates such as food wastes, grass, crops, wastes, wastewaters, etc. AD is a biological process which is carried out in the absence of oxygen by a consortium of microorganisms, bacteria, and archaea, which work in a coordinated and interdependent metabolism (Zeikus, 1980). The main steps of AD are hydrolysis-acidogenesis, acetogenesis, and methanogenesis (Gujer and Zehnder, 1983). Microorganisms involved in the AD process have the capacity to transform a substrate that is rich in organic matter into biogas. The first step of AD, or hydrolysis-acidogenesis, is accomplished by bacteria. Hydrolytic bacteria break down high molecular weight compounds, such as polysaccharides, lipids, and proteins, into soluble compounds. During acidogenesis, these soluble compounds are transformed by fermentative bacteria into volatile fatty acids, H_2, CO_2, NH_3, H_2S, and other intermediate compounds. In a second step, acetogens convert the intermediates produced by acidogenesis into acetic acid, CO_2, and hydrogen. Lastly, archaea transform these intermediate products into biogas. Biogas is mainly a mixture of methane (65%–70%) and carbon dioxide (35%–30%), with traces (usually less than 1%) of other constituents (hydrogen sulfide, hydrogen, nitrogen oxides, nitrogen, etc.) (Angelidaki and Sanders, 2004). Biogas has a very high calorific value of 20–25 MJ/m^3, which can be used in diverse ways: as heat and electricity, using cogeneration engines with electrical efficiencies of 33% and thermal efficiencies of 45% (Appels et al., 2011); being injected into the natural gas grid (Angelidaki et al., 2018; Ullah Khan et al., 2017); or being used as fuel for vehicles after an upgrading process into biomethane (Börjesson and Mattiasson, 2008).

The theoretical methane yields of carbohydrates, proteins, and lipids in AD are 0.415 L CH_4/g VS (volatile solids), 0.851 L CH_4/g VS, and 1.014 L CH_4/g VS, respectively (Sialve et al., 2009). The carbohydrate content in microalgae is around 20%, but microalgae can be found that are rich in carbohydrates, i.e., around 64% (dry weight) (Renaud et al., 2002). The composition

of microalgae is highly dependent on growing conditions such as temperature, light (Singh and Singh, 2015), photoperiods, etc. This knowledge is important because by using certain growing conditions, the enrichment and accumulation of compounds of interest can be induced, hence increasing methane yields. Markou et al. (2013) pointed out that the use of carbohydrate-enriched cyanobacterias was a successful way to improve the methane contained in the biogas. They obtained an increase in methane yield from 0.123 to 0.203 L CH_4/g COD (chemical oxygen demand) after inducing an increase in the carbohydrate content from 20% to 60% for the microalga *Arthrospira platensis*, which was grown with phosphorous limitation. Carbohydrates may have a lower methane potential than other compounds like lipids or proteins, but when they are nonstructural they are easily available to bacteria and yield more than other structural compounds like lipids or glycoproteins, which are part of the cell wall structure (Markou et al., 2013; Passos et al., 2014). The lipid content in microalgae is around 3%–20% (dry weight) (Huerlimann et al., 2010). Although lipids have higher theoretical methane yields than carbohydrates and proteins (Sialve et al., 2009), during their AD the intermediate products generated, mainly long-chain fatty acids and volatile fatty acids, may inhibit the process (Park and Li, 2012). Cirne et al. (2007) observed inhibition in the AD process for lipid concentrations of 31%, 40%, and 47% (w/w, COD basis). These authors added lipase to favor the enzymatic hydrolysis of the lipids, but the intermediates produced in the AD process caused inhibition. Sialve et al. (2009) also found better results for AD carried out with total lipid contents that were lower than 40%. In this respect, the removal of lipids from microalgae before AD and the use of the spent microalgae after lipid extraction may prevent inhibitory lipid concentrations (Cirne et al., 2007; Park and Li, 2012; Sialve et al., 2009). Proteins make up around 50% of the total microalgal biomass (Becker, 2007). The high content in protein causes low carbon/nitrogen (C/N) ratios and high ammonia production during the course of AD due to the degradation of amino acids (Rittmann and McCarty, 2001). Ammonia generation may lead to low methane yields by ammonia toxicity in some cases (Chen et al., 2008).

According to Briand and Morand (1997), "the nature of the polymers present in the substrates and their biochemical structure affect, to a greater or lesser degree, the substrate's accessibility to enzymes." In this sense, microalgae do not contain lignin (<2%), and cellulose and hemicellulose contents are 7.1% and 16.3%, respectively (Verveis et al., 2007). Low-lignin type compound concentration facilitates the hydrolytic-acidogenic bacteria enzymatic action and development of the first step of the AD.

Microalgal intracellular composition varies depending on the microalgae stage of growth. Harvesting at a precise time of growth could favor the collection of one or other composition of interest (González-Fernández et al., 2012a). In addition to the composition of microalgae, another point to contemplate when considering them as substrates for AD is the high moisture of grown microalgae. Harvesting and concentrating (or dewatering) microalgal biomass after growth represents an important challenge to make the production

of biogas or other biofuels viable (Ward et al., 2014). Although wet biomass can be used (Milledge and Heaven, 2014), the yields obtained can improve if a concentrating strategy is used.

Golueke et al. (1957) were the first authors to report the potential of using microalgae to produce biogas, although the yield they obtained was somewhat less than that obtained from sewage sludge. They focused their study on *Scenedesmus* spp. and *Chlorella* spp. coming from a wastewater treatment process. Golueke et al. (1957) reported that the low yields obtained, 0.17–0.32 L CH_4/g VS, could be due to the high ammonia content, to the ability of microalgae to survive in the anaerobic reactor, and to the resistant cell wall of microalgae. The main experimental methane yields reported for the AD of microalgae are in the range of 0.09–0.44 L CH_4/g of VS (González-Fernández et al., 2012a). The low methane yields obtained from some microalgae have been attributed to the composition of the microalgal cell wall by numerous authors (Mussgnug et al., 2010; Ras et al., 2010; Zamalloa et al., 2011). The best gas yields were found for microalgal species without cell wall or with protein-based cell walls without cellulose or hemicellulose (Mussgnug et al., 2010). It is well-known that microalgae present a wide diversity, and have different cell wall compositions. The biogas production and methane yields obtained from them are determined by the species, and it is possible to find very different behaviors during AD (Mussgnug et al., 2010). Regarding the ability of microalgae to survive in AD reactors, several authors found intact cells of microalgae after long periods under AD conditions. Mussgnug et al. (2010) found viable cells of the microalga *Scenedesmus* (under the microscope) after 6 months inside the anaerobic digester. Finally, in addition to the cell wall composition, the low C/N ratios, and the subsequent ammonia toxicity, along with the high humidity of microalgae, one of the main limitations of microalgal AD is salinity. Sodium concentration in the media can be an important inhibitor for the methanogenic archaea (Ward et al., 2014). Furthermore, in addition to the inherent limitations of microalgae, another aspect to take into account when evaluating the methane yields obtained from AD is AD bioreactor configurations (González-Fernández et al., 2015). Despite these limitations, the AD of microalgae is one of the most promising alternatives to energy production and climate change mitigation because, as already explained, they have high growth rates and they do not compete with food crops for arable land. Furthermore, microalgae can be easily integrated into the biorefinery approach (Chisti, 2007). Nowadays, it is accepted that the biorefinery approach may be a useful way to achieve economic viability for microalga exploitation, considering the advantage of the initial step for the extraction of valuable compounds (proteins, amino acids, and lipids for biofuel) and a second step or AD step for biogas production.

To this approach, different complementary ideas can be attached in an attempt to close the loop: growth of new microalgae in effluents coming the AD or digestates, use of CO_2 contained in the biogas for the growth of new microalgae, etc.

12.3 Bacteria and archaea in the AD of microalgae

AD is a well-established process; nevertheless, most of the time the control of the AD process is based on engineering and macroscopic parameters such as organic loading rates, hydraulic retention times, etc., while microbial communities are not given much attention, and very often do not have much weight. However, "understanding the involvement of specific microorganisms would be useful to control performance, maximum substrate decomposition and methane production" (Rincón et al., 2008). Two factors must be taken into account when treating the microbial populations participating in the AD of microalgae. First, microalgae in a substrate for AD were first studied in the 1950s by Golueke et al. (1957), and second, the determination of this microbial consortium, in the case of the AD of microalgae, is complex, owing to the difficulties in the separation of the nucleic acids of eukaryotic and prokaryotic microorganisms (Bakke et al., 2011). There are several studies. Lakaniemi et al. (2011) studied the generation of hydrogen and methane from microalgal biomass. They studied both freshwater (*Chlorella vulgaris*) and marine species (*Dunaliella tertiolecta*) using a municipal anaerobic reactor sludge at mesophilic temperature and found *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi* to be the main phyla. However, the inocula they used became enriched during the experiments and the bacteria found for both microalgae were very different. Cho et al. (2015) studied the influence of the temperature on the microbial community during the AD of microalgae and found mainly *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, regardless of the temperature. On the other hand, *Bacillus* sp. was more notable at thermophilic temperatures. González-Fernández et al. (2018) carried out an interesting study comparing the use of different microbial inocula during the AD of microalgae. The results of this study concluded that AD of microalgae was independent of the inocula source. However, an acclimatization of the inocula to the microalgae treated gave better methane yields. These authors suggested the use of specific inocula as an alternative to the use of costly pretreatments to break the microalgae cell wall (González-Fernández et al., 2018). The knowledge concerning the microorganisms involved in the AD of microalgae is important because it will allow us to know in depth how the anaerobic system works and prevent possible failures of the anaerobic digesters. Microbial communities must be thoroughly investigated, and more research is needed.

12.4 Main drawbacks affecting AD of microalgae

12.4.1 Complex cell walls

The low methane yields obtained from some microalgae are attributed to the composition and the lack of digestibility of the microalgal cell wall (Golueke et al., 1957; Mussgnug et al., 2010; Ras et al., 2010; Zamalloa et al., 2011). As reported in Section 12.1, microalgae present a wide

diversity, and it is possible to find different cell wall compositions. In this regard, the methane yields obtained from microalgae are dependent on the chosen strain; for this reason biogas production from microalgae cannot be forecast and must be assessed on a case-by-case basis (Mussgnug et al., 2010). Cell wall disruption is critical to release the cell content and to maximize biogas yields. In this sense, the main target of pretreatments is to break the microalgal cell wall, releasing the intracellular material contained inside the cytoplasm and solubilize the microalgal particulate biomass. This process gives the hydrolytic-acidogenic bacteria easier access to the intracellular organic matter. Pretreatments can be classified as: (a) mechanical: size reduction, ultrasound, sonication, microwave-assisted extraction; (b) thermal: heating, freezing, dying, steam explosion; (c) chemical: acids, alkalis, oxidative chemicals; and (d) biological or treatment with enzymes: cellulases, alcalases, proteases, etc. Among the mechanical pretreatments, size reduction (cutting and blending), sonication processes, ultrasound (>50 kHz), or microwave can be applied.

Conversion efficiency in biogas depends on the level of cell wall deterioration (Mussgnug et al., 2010), and results are dependent on the pretreatment conditions. For instance, methane yield increased up to 80%–90% for a specific ultrasound energy application of 100–200 MJ/kg TS (total solids) while it was not more than 20% for a specific energy application of 75 MJ/kg TS (González-Fernández et al., 2012b). The efficiency of pretreatments is highly dependent on the kind of microalgae studied, the macromolecular composition, and the structure of the cell wall (Passos et al., 2014). Species like *Dunaliella* sp. without a cell wall or species like *Chlamydomonas* sp. without cellulose or hemicellulose in its cell wall showed better results in terms of methane yield than those with a carbohydrate-based cell wall like *Chlorella* sp. or *Scenedesmus* sp. (Mussgnug et al., 2010). When thermal pretreatments are applied, temperature and exposure time are the two main factors to consider, although temperature seems to be the most influential factor (Chen and Oswald, 1988; Mendez et al., 2014; Passos et al., 2014). Thermal pretreatments include both heating and freezing/thawing operations, although Kinnunen et al. (2014) showed that methane productions after freezing were lower than those produced after heat application. Low temperature pretreatments (<100°C) have the advantage of being less energy demanding and cheaper, but are not so effective as pretreatments at high temperatures (>100°C). At low temperatures, a 12% increase in methane yield can be achieved versus a 60%–220% increase obtained from pretreatments at 80–100°C (González-Fernández et al., 2012b, c; Passos et al., 2013). Hydrothermal pretreatments are carried out at temperatures above 100°C with gradual pressure release and shorter times of application than thermal pretreatments. For instance, an increase in methane yield by 60% was achieved, working for 3–10 hours at 80–100°C (González-Fernández et al., 2012c; Passos et al., 2013). Steam explosion or thermal hydrolysis has also been used for microalgal cell wall disruption. During steam explosion, the application of high temperatures (≥150–160°C) is combined with the application of pressure (around 4–6 bars) for a few minutes. After this short time, the pressure in the samples is lowered quickly and the samples are swiftly cooled down.

The decompression produced generates the breaking of the cell wall. Lorente et al. (2017) obtained a complete cell wall rupture working with *Nannochloropsis gaditana* at 150°C and at 4.7 bar of saturated steam (5 min). Chemical pretreatments are associated with the use of oxidative chemicals, alkalis, or acids. The effect of using these chemicals on the biomass may be important but chemical pretreatment entails, as the main problem associated, the toxicity of the by-products generated and the costs of the chemicals consumed (González-Fernández et al., 2015). These kinds of pretreatments are not used very often as single pretreatments, and are more often found in combination with thermal pretreatments. In fact, the study carried out by Cho et al. (2013) showed clear results after an alkali pretreatment at a pH = 13 the AD process suffered the consequences of high pH values in the methanogenic populations even after neutralizing the pH to 7 prior to the AD process. The energy intake linked to the pretreatment of microalgae biomass has been found in some cases to be equal to or higher than the power obtained (Mendez et al., 2014).

Biological pretreatments are promising alternatives to traditional energy-consuming pretreatments (Bohutskyi and Bouwer, 2013; González-Fernández et al., 2015; Passos et al., 2014). They are based on the use of hydrolytic enzymes. These enzymes carry out the cell wall disruption and transform complex compounds of the microalgal cell wall to simple lower molecular weight compounds, thus favoring biomass solubilization. Another advantage of biological pretreatments is the low generation of toxic compounds. The main factors to consider when applying a biological pretreatment are enzyme concentration together with temperature and exposure time (Passos et al., 2014). The most frequent studies have been carried out using cellulases or an enzymatic complex with cellulases (Ehimen et al., 2013; Fu et al., 2010; Yang et al., 2014). A recent study carried out on *Chlorella sorokiniana* comparing different pretreatments combined with different enzyme/substrate ratios, times, and pH (Córdova et al., 2019) showed the boundary of this kind of pretreatment, reaching an increase over 21% in biogas in all cases. Biodegradability increased from 22% to 86% over the raw microalga. The best results were obtained by applying a 2% enzyme/substrate ratio and a pH of 4.8 (Córdova et al., 2019).

12.4.2 Low C/N ratios

The low methane yields obtained after the AD of microalgae have also been related to the high concentration of protein in microalgae (low C/N ratios) (Chen et al., 2008). The optimal C/N ratio for AD is established to be between 20 and 30 (Habiba et al., 2009; Yen and Brune, 2007). For values lower than these, the requirements of carbon and nitrogen of the bacterial consortia are imbalanced (Sialve et al., 2009). C/N ratios around 10 or even lower are found for microalgae (Geider and La Roche, 2002; Yen and Brune, 2007; Ward et al., 2014). The high concentrations of proteins in microalgae (Park and Li, 2012), between 6% and 71% of dry

matter depending on the microalgae (Becker, 2007), can produce an accumulation of nitrogen in the form of ammonia (ammonium ion and free ammonia) during the AD process. Toxic ammonia thresholds are between 1.7 and 14 g NH_4^+-N/L, but total ammonia content over 3 g NH_4^+-N/L and free ammonia content over 0.15 g NH_3-N/L are toxic for methanogenesis, whatever the pH and the temperature (Chen et al., 2008). Ammonia is inhibitory for methanogenic archaea, which stops their activity, causing an accumulation of volatile fatty acids within a digester (Sialve et al., 2009). In the face of this situation, several strategies have been adopted by different authors. The most common has been the co-digestion of microalgae with carbon-rich substrates (Fernández-Rodríguez et al., 2014, 2019; Hassan et al., 2016) but there are also studies using ammonia-adapted inoculum (Mahdy et al., 2017), bio-augmentation (Fotidis et al., 2014), etc. The digestibility of a substrate with an unbalanced C/N ratio can be improved by co-digestion processes mixing it with other substrates with complementary compositions (Hassan et al., 2016; Fernández-Rodríguez et al., 2014). Co-digestion allows for both balancing the C/N ratio and diluting complex or inhibitor compounds (Hartmann and Ahring, 2005). The co-digestion of microalgae with carbon-rich substrates seems to have synergistic effects and a positive influence on the methane yields obtained (González-Fernández et al., 2015; Li et al., 2017; Ramos-Suárez and Carreras, 2014). In some cases, co-digestion with high carbon content substrates can lead to methane yields even twice as high as those with a low nitrogen substrate alone (Ward et al., 2014).

However co-digestion does not always work. The anaerobic co-digestion of microalgae, Chlorophyta and cyanobacteria, with carbon-rich substrates has been widely studied (Li et al., 2017; Rincón et al., 2018; Rusten and Sahu, 2011). Li et al. (2017) co-digested a Chlorophyta such as *Chlorella* sp. with chicken manure, improving the methane production by 14.20% and 76.86% compared with the single digestion of chicken manure and *Chlorella* sp. However, Rusten and Sahu (2011) co-digested *Chlorella* sp. with wastewater sludge, but methane production did not increase when compared to the single wastewater sludge (Rusten and Sahu, 2011). Schwede et al. (2013) co-digested *Nannochloropsis salina* with corn silage, obtaining the best results for the C/N ratio of 21.2 and an increase in the methane yield of 9% over the yield obtained from corn silage as a mono-substrate. The *Scenedesmus* genus was studied by Ramos-Suárez and Carreras (2014). These authors co-digested *Scenedesmus* sp. and *Opuntia maxima* cladodes, obtaining improved methane yields and kinetics. The best C/N ratio they found was 15.6. For this ratio, the methane yield was 233.6 ± 16.4 mL CH_4/g VS, 66.4% and 63.9% higher than those obtained from *Scenedesmus* sp. and *O. maxima* cladodes digested alone (Ramos-Suárez and Carreras, 2014). Astals et al. (2015) also found an improvement in the methane yield obtained from *Scenedesmus* sp. when co-digesting with pig manure, increasing the methane yield from 163 to 245 mL CH_4/g VS (Astals et al., 2015). The *Micractinium* genus was studied using co-digestion mixtures with waste-activated sludge, obtaining improvements in the biodegradability and volatile solid removal, and in the biogas yield, reaching a value of 209 mL/g VS (Wang and Park, 2015). Fernández-Rodríguez et al. (2014) found an improvement when *Dunaliella salina* was co-digested with olive mill solid

waste (OMSW), a carbon-rich substrate. The co-digestion mixture 50% OMSW-50% *D. salina* showed the highest biodegradability, but the best C/N ratio was found to be 26.7. At this ratio (75% OMSW-25% *D. salina*), the maximum methane production was obtained: 330 mL CH_4/g VS. In addition to co-digestion, other alternative strategies have been proven in an attempt of overcome the ammonia toxicity. Currently, most of the inoculums that are used come from wastewater treatment plants or from animal manures, and they are not capable of holding high ammonia concentrations (Santos-Ballardo et al., 2016). The acclimatization of inocula to growing concentrations of ammonia may allow for feeding the bioreactors with higher ammonia concentrations than those typically found. Mahdy et al. (2017) studied the influence of an ammonia-acclimatized inoculum in the anaerobic biodegradation of the microalga *C. vulgaris* in co-digestion with cattle manure. They found that the highest methane yield obtained in batch mode was 431 mL CH_4/g VS, and this was obtained from the co-digestion mixture 80% *C. vulgaris*–20% manure (on VS basis). Using this co-digestion mixture and an ammonia-tolerant inoculum, the process worked in spite of ammonia concentrations between 3.7 and 4.2 NH_4^+-N/L (working in continuous mode). The results showed by Mahdy et al. (2017) concluded that the use of this kind of inoculum is a promising key to protein-rich microalgal AD.

12.4.3 Humidity of microalgal biomass

The low VS loading rates of microalgae were reported in 1957 by Golueke et al. (1957). These low VS loading rates are given by the low concentration of microalgal biomass in large volumes of water with moistures ranging between 78% and 90%. De Schamphelaire and Verstraete (2009) worked with a nondefined culture of freshwater microalgae mixed with *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*. They obtained 0.49 Nm^3 biogas/kg VS, but they could only reach an organic loading rate of 10 mg/$L_{reactor}$ day working in continuous mode at mesophilic temperature. These results led to the need to search for appropriate microalgal concentrations for a continuous operation mode in large-scale AD reactors. Furthermore, the use of microalgae that are not concentrated leads to the loss and washing off of microorganisms contained in the anaerobic reactor. In this sense, different strategies have been tested to avoid the loss of the AD inoculum: the use of gravity-settling tanks before dewatering and concentration by centrifugation (Collet et al., 2011); improving the AD reactor configurations (Inglesby and Fisher, 2012; Zamalloa et al., 2012); use of belt filter systems (Sandip et al., 2015), etc. The harvesting and concentration (dewatering) of the microalgal biomass may be expensive; it may account for 3%–15% of the production costs of microalgae (Fasaei et al., 2018).

12.4.4 Salinity

Sodium concentrations over 10 g/L in anaerobic digesters are strongly inhibitory for AD (Kugelman and McCarty, 1965). High salt concentrations increase osmotic pressure and inhibit methanogenic archaea. Diverse studies have been developed with marine microalgae by

Lakaniemi et al. (2011), Mottet et al. (2014), and Srinuanpan et al. (2017), and different sodium concentrations have been reported as optimal for methanogenic archaea. Chen et al. (2008) reported optimal values of around 0.230 g Na^+/L, but optimal values around 0.350 g Na^+/L have also been reported by Patel and Roth (1977). On the other hand, inhibitory concentrations for acetoclastic methanogens have been found to be 5 g Na^+/L (10% inhibition), 10 g Na^+/L (50% inhibition), and 14 g Na^+/L (100% inhibition) in neutral pH and mesophilic conditions (Rinzema et al., 1988), although Chen et al. (2003) found deterioration in the methanogenic activity at higher salt concentrations of 16 g Na^+/L, under thermophilic conditions. In light of these values, anaerobic microorganisms are very versatile, and after a period of acclimatization, where the microorganisms are progressively exposed to growing salt concentrations, they can bear high salt concentrations (Feijoo et al., 1995; Lefebvre and Moletta, 2006). Lefebvre and Moletta (2006) found the methanogenic activity reduced by half for concentrations $\geq$20 g Na^+/L after acclimation. Salt inhibition may also occur for other cations like potassium (Chen et al., 2008).

12.5 AD of spent microalgae

Microalgae are a sustainable source of high-value compounds. Pigments like astaxanthin or lutein, fatty acids like docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) or omega-3, and antioxidants like β-carotene are the main compounds to be exploited (Vigani et al., 2015). They are also a source of enzyme polymer, natural dye, peptide, etc., which are exploited for industrial purposes (Moreno-García et al., 2017). In most cases, after the extraction of intracellular compounds, the wastes, or spent microalgae generated, still have a high quantity of cell material that can be used as organic matter to feed AD processes (Ramos-Suárez and Carreras, 2014). In addition, the intracellular metabolite extraction could be considered as a kind of pretreatment for the microalgal cell wall. Some works have been developed in this respect. Ramos-Suárez and Carreras (2014) carried out a comparative study of the biogas production obtained from the raw microalga *Scenedesmus* and from the spent *Scenedesmus*, recovered after obtaining amino acids and lipids, respectively. These authors also studied the co-digestion of both raw and spent microalgae with carbon-rich substrates such as paper sludge, *O. maxima* cladodes, and glycerine. The studies concluded that both methane yield and the rate of methane production were improved in the AD of spent *Scenedesmus* as a consequence of the disruption of the cell wall and the solubilization of organic matter. The methane yield obtained after the extraction of amino acids from *Scenedesmus* was 272.8 L CH_4/kg VS, and it was 212.3 L CH_4/kg VS after extracting lipids from *Scenedesmus*. For the raw microalga *Scenedesmus*, the yield was 140.3 L CH_4/kg VS. Mussgnug et al. (2010) evaluated the potential of the microalga *C. reinhardtii* to generate biogas after hydrogen production via sulfur deprivation under anaerobic conditions.

The induction of H_2 production increased the content in starch and lipids within the cells, and this resulted in a biogas yield increase to 123% in the spent microalga in contrast to the raw microalga. Hernández et al. (2014) compared the methane yields obtained from four different microalgae after lipid extraction by supercritical CO_2 extraction; they found better results for all the extracted microalgae: *Isochrysis* T-ISO, *N. gaditana*, *Scenedesmus almeriensis*, and *Tetraselmis* sp. The best methane yield, 236 mL CH_4/g VS_{added}, was found for the microalga *Tetraselmis* sp. On the other hand, Astals et al. (2015) found an increase in methane yields from 0.163 m^3/kg VS to 0.223 m^3/kg VS after a pretreatment to extract protein and lipids from the microalga *Scenedesmus* sp. In the same way as Ramos-Suárez and Carreras (2014), they obtained better results when digesting spent microalgae than nonlipid-extracted microalgae. However, this trend is not always the same, as the results obtained appear, once again, to depend on the strains studied (Mussgnug et al., 2010). Authors like Quinn et al. (2014b) reported worse methane yields for spent microalgae than for raw microalgae. Quinn et al. (2014b) reported results three times lower for the lipid-extracted microalgae than the raw microalgae: 140 mL CH_4/g VS versus 430 mL CH_4/g VS.

12.6 Nutrient and CO_2 recycling: Toward a sustainable closed loop

Autotrophic microalgae need CO_2 as a carbon source to grow. The use of CO_2 contained in the biogas coming from AD processes for microalgal growth is an advantage worth taking into account. This operation is two-fold: first, it allows for reduced costs in microalgae growth because extra CO_2 is not necessary, and second, it allows for biogas upgrading. The idea of biogas purification using intensive microalgal cultures was studied and reported a long time ago (Conde et al., 1993), and could be an interesting alternative to other more expensive, conventional biogas upgrading systems. Most of the methods employed to upgrade biogas such as membrane separation, water scrubbing, etc. entail substantial costs like energy consumption, chemicals, etc. (Yan et al., 2014). In this sense, Thiansathit et al. (2015) studied the efficacy of using biogas for growing *Scenedesmus obliquus*. They utilized biogas coming from an anaerobic digester treating animal wastes and achieved a maximum *S. obliquus* productivity of 0.145 g/L d, and a maximum specific growth rate of 0.56 d^{-1}. Furthermore, they found that the ammonia or H_2S contained in the biogas did not negatively affect the growth of the microalgae (Thiansathit et al., 2015). On the other hand, Srinuanpan et al. (2017) used *Scenedesmus* sp. and marine *Chlorella* sp., among others, to improve the methane content in biogas by exploiting the capacity of oleaginous microalgae to generate and accumulate lipids at the same time they mitigate CO_2. These authors obtained a methane increase from 60% to 90% in biogas at a CO_2 removal rate of 5.097 g CO_2/day $L_{microalgal\ culture}$ and a lipid productivity of 88.57 mg/day $L_{microalgal\ culture}$ (Srinuanpan et al., 2017). The use of oleaginous microalgae is an important alternative because it allows CO_2 removal from the biogas at the same time that lipids and new biomass are produced. Srinuanpan et al. (2017)

compared different oleaginous marine (*Chlorella* sp.) and oleaginous freshwater microalgae (*Chlorella* sp., *Botryococcus* sp., *Nannochloropsis*, and *Scenedesmus* sp.). They analyzed the effects of important parameters such as initial cell concentration, gas flow rate, KNO_3 concentration as nitrogen source, and light intensity. The microalga *Scenedesmus* gave the most satisfactory CO_2 removal (95%–98%) among the tested microalgae (Srinuanpan et al., 2017). Regarding lipid production, the marine *Chlorella* sp. and *Scenedesmus* sp. were the microalgae that produced the highest concentrations at 300–320 mg/L. The predominant fatty acids found were C16–C18 (Srinuanpan et al., 2017). These lipids may be extracted and converted to fatty acid methyl esters (FAME). Different authors have employed this strategy using specific microalgae, all of them obtaining CO_2 removals above 90% (Yan et al., 2014; Yan and Zheng, 2013).

Other strategies that could make microalgal AD more sustainable and less expensive are microalgae growth in wastewater or even in effluents produced after AD processes or "digestates," taking advantage of the carbon and nutrients still remaining in them. The culture medium composition in microalgal cultivation affects the production of compounds of interest and biomass. However, reducing the cost of microalgal cultivation is decisive to the economic viability of compounds of interest (Nayak et al., 2016). The growth of microalgae in wastewaters provides a tertiary treatment for these wastewaters, taking the advantage of the nutrients remaining in the residual streams, and being accepted as a viable option to produce new microalgal biomass (Rahman et al., 2015). Furthermore, AD digestates are most frequently used as crop fertilizers as they are rich in nutrients, but these nutrients can also be utilized for the growth of microalgae. The employment of digestates allows for reducing the use of extra nitrogen and phosphorous (Björnsson et al., 2013) and reduces the possible organic load remaining in the digestates. Reduction in COD between 44% and 85% can be achieved. Nitrogen/phosphorous ratios around 15, at phosphorous concentrations around 5 mg/L, have been found to be sufficient (De la Noüe and Bassères, 1989).

Prajapati et al. (2014) worked with *Chroococcus* sp. and digested this microalga anaerobically, obtaining methane yields of 317.31 ± 1.9 mL CH_4/g VS added, and recently they used the diluted obtained digestate to grow new microalgae. Digestate was diluted to avoid the limitations by light availability owing to the dark color of digestates coming from AD. They found that a content of 30% in digestate gave the best microalgae productivity 0.79 ± 0.06 g/L (Prajapati et al., 2014). González-González et al. (2018a) also found the viability of growing *Scenedesmus dimorphus* in digestates coupled to biogas production. On the other hand, the influence on the growth of the inoculum to substrate ratio has also been evaluated. In this sense, Uggetti et al. (2014) found that increasing the initial concentrations of digestate and microalgae improved the biomass production, although initially the growth rates were negatively affected.

12.7 Biorefinery concept

The use of the biorefinery concept as the production of high-value bio-products such as foods, nutraceuticals, chemicals, etc. coupled with energy production from biomass (Taylor, 2008) is a fascinating option when treating with microalgae. The application of the biorefinery concept to microalgae has revealed a possible way in which to accomplish an economically feasible process (Chisti, 2007; Stephens et al., 2010). The co-production of high-value compounds and bioenergy appear to be more economically viable than the generation of a biofuel alone (Jonker and Faaij, 2013). Authors like González-González et al. (2018b) advocated an integrated biodiesel and biogas production from microalgae. Others like Mussgnug et al. (2010) supported a two-step biorefinery concept coupling the combination of a first step for hydrogen production from microalga *S. obliquus* with a second step for biogas production using the spent microalgae (the biogas yield increased to 123% in the spent microalgae after hydrogen production). Collet et al. (2011) evaluated the benefits and drawbacks of producing biogas from the microalga *C. vulgaris* through a life-cycle assessment study. They also compared the results to other technologies such as algal biodiesel and first-generation biodiesel, concluding that the best option may be the combination of both lipid recovery and biogas production.

12.8 Conclusions

Biogas production through AD from certain microalgae may be a viable alternative to other substrates currently used. The central point of research efforts in the future must be focused on comprehending the role of the microalgal cell walls and the mechanisms associated to the pretreatments necessary to break them, as well as co-digestion strategies to improve the C/N ratio. Recycling nutrients remaining in wastewaters and digestates after AD processes to growth new microalgae and recycling the CO_2 coming from biogas lines must be integrated, enabling a reduction in costs by biogas purification and by CO_2 consumption (for improving biomass productivity) in an attempt to close the loop. The use of CO_2 from biogas has not been researched to a great extent, and more studies are required. The integration of valuable compound production such as lipids, nutraceuticals, chemicals, etc. plus AD of spent microalgae must be thoroughly investigated, and more research is needed at pilot scale to obtain scalability data.

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CHAPTER 13

Biodiesel from microalgae

Zheting Bi[a], **Brian He**[b]

[a]*Zoetis, Lincoln, NE, United States* [b]*Department of Biological Engineering, University of Idaho, Moscow, ID, United States*

Chapter outline

13.1 Introduction 329
13.2 Strains of microalgae for biodiesel production 331
13.2.1 Lipid-rich microalgae as a biodiesel feedstock 331
13.2.2 Cultivation of high lipid bearing microalgae 332
13.2.3 Process parameters affecting microalgae cultivation 335
13.2.4 Practical techniques for stimulating lipid production in microalgae 338
13.2.5 Genetically engineered strains of microalgae for biodiesel production 342
13.3 Microalgae preparation for biodiesel production 343
13.3.1 Harvesting microalgae 343
13.3.2 Drying microalgae 345
13.3.3 Lipid extraction 348
13.4 Microalgal biodiesel production 350
13.4.1 Transesterification of microalgal lipids 350
13.4.2 Supercritical alcohol transesterification for algal biodiesel production 352
13.4.3 Process parameters of in situ supercritical transesterification of microalgal lipids 354
13.5 Summary and perspectives 358
References 361

13.1 Introduction

The negative effect of burning petroleum fuel and the potential for a petroleum shortage have dramatically concerned the world. Developing a sustainable yet environmentally friendly energy resource is the right solution (Kröger and Müller-Langer, 2012). The International Energy Agency (IEA) has investigated the current energy consumption status in 2009 and claimed that the United States will strive to achieve 6% of total transportation fuels as biofuels by 2030 (Barry et al., 2016). The United States has been working on exploring renewable energy sources since the 1970s and has already become a top producer of biodiesel and

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bioethanol in the world. For the most part, vegetable oil is the major feedstock for biodiesel production in the United States and the rest of the world. However, converting food-based resources extensively for biodiesel production threatens the world food supply. Discovering other resources that do not compete directly with the food supply is vital for biofuel production.

Microalgae exhibit several important attributes for use as a resource for renewable energy. With advantages including simplicity to culture, faster growth rate as compared to terrestrial energy crops, high biomass productivity, attractive biochemical profiles and good energy content, microalgae are strong candidates as a bioenergy resource. Biodiesel production from microalgae has a high lipid productivity per unit area with marginal land, which is more beneficial in contrast to terrestrial energy crops which require high-quality farming lands (Chisti, 2007; Tang et al., 2010). In addition, microalgae utilize carbon dioxide (CO_2) from power plants, with sunlight and other readily available nutrients (Tang et al., 2010), helping recycling the carbon from fossil resources and positively impacting the environment.

Microalgae, including species of *Chlorella*, *Spirulina*, *Dunaliella salina*, and *Hoematococcus pluvislis*, have been commercially cultivated for more than 30 years. Initial utilization of microalgae was in the food and pharmaceutical industries (Borowitzka, 1999) for their high concentrations of omega-3 oils and chlorophyll (Harun et al., 2010). Microalgae have tremendous potential for use as an energy resource too. Algal biomass can be used directly to generate heat, steam, and electricity, or converted to gaseous and liquid biofuels (Chisti, 2007). Meanwhile, some specific strains of microalgae are able to produce a high yield of lipids which are favorable for conversion into biodiesel (Chisti, 2007; Wahlen et al., 2011). Neutral lipids of triacylglycerides (TAGs; also called triglycerides or triacyclglycerols) are the dominant components in vegetable oils and a large fraction in microalgal lipids. In addition, microalgal lipids contain polar components, mainly from the membrane structure, such as phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine, and glycolipids (Gong and Jiang, 2011). The presence of such polar lipids in microalgal lipids brings challenges in converting them to biodiesel.

Under various stress conditions, TAG accumulation in microalgae can be regulated and modified (Khozingoldberg and Cohen, 2006). Biologists have found various feasible ways to genetically modify microalgae into special strains that are capable of producing useable lipids (Radakovits et al., 2010). Under the Aquatic Species Program funded by the US Department of Energy's Office of Fuels Development, the National Renewable Energy Laboratory (NREL) has conducted a thorough investigation into the potential of microalgae as a resource for producing renewable transportation fuels from 1978 to 1996 (Sheehan et al., 1998). In this research, approximately 300 species of microalgae, mostly green microalgae and diatoms, have been identified as capable of producing lipids. It was concluded that the open-pond systems are the practical and economical way of microalgae cultivation, with a yield of 50 g/m^3 per day (Sheehan et al., 1998). In recent years, microalgae have been seriously

studied as a potential feedstock for commercial biofuel production, and a microalgae-based biofuel industry is currently under development (Tang et al., 2010). According to market analysis and economic modeling, the main obstacle to the microalgal biodiesel commercialization is the significantly higher operational costs, mainly in cultivation and harvesting (Borowitzka, 1992). Therefore, increasing production efficiency to reduce the unit cost is the key for developing a viable microalgae-based biofuel production.

13.2 Strains of microalgae for biodiesel production

13.2.1 Lipid-rich microalgae as a biodiesel feedstock

Microalgae are unicellular microscopic, heterotrophic-autotrophic photosynthesizing organisms that grow in freshwater or marine systems (Borowitzka, 1999; Brennan and Owende, 2010; Milledge, 2010). There are nearly 200,000–800,000 genera in existence, of which about 35,000 species have been studied (Tang et al., 2010). Currently, microalgal biology are frequently studied, with the focus on the high lipid content and rapid growth rate for biodiesel production.

Algae, among the oldest aqueous organisms and the first residents on the earth, can be categorized into microalgae and macroalgae. Microalgae are tiny, unicellular, and normally grow in suspension within a body of water. In contrast, macroalgae are the large, multicellular algae and often grow in a pond with roots in the soil (Muller-Feuga et al., 2003). Both microalgae and macroalgae are capable of converting CO_2 to organic matters. Algae are classified in multiple major groups of cyanobacteria (*Cyanophyceae*), green algae (*Chlorophyceae*), diatoms (*Bacillariophyceae*), yellow-green algae (*Xanthophyceae*), golden algae (*Chrysophyceae*), red algae (*Rhodophyceae*), brown algae (*Phaeophyceae*), dinoflagellates (*Dinophyceae*), and "pico-plankton" (*Prasinophyceae* and *Eustigmatophyceae*) (Huang et al., 2010). Lipid-rich microalgae include mainly green, red, and brown algae, and some diatoms and cyanobacteria (Huang et al., 2010), which can photosynthesize light and CO_2 into lipids of approximately 20%–50%wt of their dry biomass weight (Brennan and Owende, 2010; Huang et al., 2010). Lipid contents and productivities in strains of algae vary largely from species to species (Chen et al., 2011; Gouveia and Oliveira, 2009; Mata et al., 2010).

Microalgae require less freshwater than terrestrial plants to grow, and are able to survive in harsh environments (Harun et al., 2010; Rawat et al., 2013). In a thorough review on biodiesel production from algae, the US Department of Energy concluded that the worldwide fuel demand can be met by growing microalgae using fewer than 6 million hectares of land, which is less than 0.4% of the arable land for oilseed crops (Barry et al., 2016). The rapid growth rate and high lipid content make microalgae much more competitive than terrestrial crops in terms of oil/lipid production. Certain strains of microalgae can accumulate large amounts of lipid bodies under adverse conditions, such as nutrient deprivation (Hu et al., 2008; Sharma et al., 2012).

The lipid content of microalgae is dependent on not only species but also factors of cultivation methods (e.g., reactor types of open pond, close photobioreactors, and auto-photobioreactors), nutrients in medium, salinity, light intensity and sources, pH, temperature, and dissolved oxygen levels. Different microalgae can grow under cultivation conditions with varied lipid content and biomass productivity (Table 13.1).

13.2.2 Cultivation of high lipid bearing microalgae

The lipid content and productivity of microalgae vary from species to species. Among the most commonly researched species, *Botryococcus braunii* and *Chlorella vulgaris* have the highest lipid contents (% on dry matter basis), and *Chlorella sorokiniana* and *Nannochloropsis oculata* have the highest lipid productivity (mass per volume per unit time) (Table 13.2). Additionally, the lipid content is also dependent on other factors, such as types of reactors for cultivation (e.g., open pound, and closed photobioreactors), nutrient availability in the medium, salinity, light sources and intensity, pH, temperature, dissolved oxygen levels, etc. (Abomohra et al., 2012; Chen et al., 2011; Gong and Jiang, 2011; Verma et al., 2010). Microalgae can be cultivated under photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic conditions. Different strains of microalgae can grow under one or more cultivation conditions with varied lipid content and biomass productivity. Each of the cultivation conditions has advantages and disadvantages.

Autotrophic cultivation is the basic type of cultivation for most microalgae species. It refers to the condition of CO_2 being the carbon source for cell growth and lipid production (Chen et al., 2011). Solar or artificial light provides the energy for photosynthesis, which has been studied extensively on characterizing the susceptible light sources for individual lipid-rich microalgal strains (Fork, 1979; Kendirlioğlu Şimşek and Cetin, 2017; Napolitano, 1994;

Table 13.1 Lipid content and biomass yield of various biodiesel feedstocks.

Plant	Lipid content (%wt)	Biomass yield (kg/ha/year)	References
Rapeseed	35.0	600–1000	Issariyakul and Dalai (2014)
Soybean	21.0	300–450	
Sunflower seed	44.0–51.0	280–700	
Palm	40.0	2500–4000	
Coconut	63.0	600–1500	
Microalgae	**Lipid content (%wt)**	**Biomass yield (kg/L/year)**	**References**
Chlorella protothecoides CCAP 211/8D	50.3–57.8	0.8–2.7	Xiong et al. (2007)
Chlorella sp. F&M-M48	32.0–34.0	0.14–0.19	Chiu et al. (2009)

Table 13.2 Lipid contents and productivities of various microalgae species under different cultivation conditions.

Species	Cultivation condition	Lipid content (%wt d.b.)	Lipid productivity (mg/L/day)	Biomass productivity (g/L/day)	References
Chlorophyta/green algae					
Botryococcus braunii UTEX 572	Phototrophic[a]	25.0–75.0	5.5	0.03	Yoo et al. (2010b)
Chlorella emersonii CCAP 211/11N	Phototrophic[b]	25.0–34.0	10.3–12.2	0.04	Scragg and Bonnett (2002)
	Phototrophic[a]	29.0–63.0	8.1–49.9	0.03–0.05	Illman et al. (2000)
Chlorella sp. F&M-M48	Phototrophic[a]	18.7	42.1	0.23	Huang et al. (2010)
	Phototrophic[a]	32.0–34.0	121.3–178.8	0.37–0.53	Chiu et al. (2009)
Chlorella protothecoides CCAP 211/8D	Phototrophic[a]	11.0–23.0	0.2–5.4	0.002–0.02	Illman et al. (2000)
	Heterotrophic[f]	43.0–46.0	1881.3–1840.0	4.0–4.4	Yun Cheng (2009)
	Heterotrophic[c]	50.3–57.8	1209.6–3701.1	2.2–7.4	Xiong et al. (2007)
	Heterotrophic[c,g]	46.1	932.0	2.0	Huang et al. (2010)
	Heterotrophic[c]	43.0–48.7	732.7–932.0	1.7–2.0	Huang et al. (2010)
Chlorella vulgaris KCTC AG	Phototrophic[a]	6.6	6.9	0.1	Yoo et al. (2010a)
	Phototrophic[a]	33.0–38.0	4.0	0.01	Huang et al. (2010)
	Heterotrophic[c,d]	23.0–36.0	27.0–35.0	0.08–0.15	Huang et al. (2010)
	Mixotrophic[c,e]	21.0–34.0	22.0–54.0	0.09–0.25	
	Phototrophic[b]	5.1	7.4	0.18	Gouveia and Oliveira (2009)
Dunaliella salina	–	6.0–25.0	116.0	0.22–0.34	Mata et al. (2010)
Neochloris oleoabundans UTEX 1185	Phototrophic[a,b]	15.9–56.0	10.7–38.8	0.03–0.15	Gouveia and Oliveira (2009)
	Phototrophic[b]	29.0	26.1	0.09	
	Phototrophic[a]	7.0–40.3	38.0–133.0	0.31–0.63	Li et al. (2008)
Nannochloropsis oculate NCTU-3	Phototrophic[a]	22.7–29.7	84.0–142.0	0.37–0.48	Chiu et al. (2009)
Phaeophyta/brown algae					
Pavlova lutheri CS 182	Phototrophic[a]	35.5	50.2	0.14	Huang et al. (2010)
Pavlova salina CS 49	Phototrophic[a]	30.9	49.4	0.16	

Continued

Table 13.2 Lipid contents and productivities of various microalgae species under different cultivation conditions—cont'd

Species	Cultivation condition	Lipid content (%wt d.b.)	Lipid productivity (mg/L/day)	Biomass productivity (g/L/day)	References
Isochrysis sp. F&M-M37	Phototrophic[a]	27.4	37.8	0.14	
Isochrysis sp. *(T-ISO)* CS 177	Phototrophic[a]	22.4	37.7	0.17	
Rhodophyta/red algae					
Porphyridium cruentum	Phototrophic[a]	9.5	34.8	0.37	Huang et al. (2010)
Bacillariophyceae/diatoms					
Phaeodactylum tricornutum	Phototrophic[a]	18.7	44.8	0.24	Huang et al. (2010)
Thalassiosira pseudonana	Phototrophic[a]	20.6	17.4	0.08	

[a]CO_2.
[b]Air.
[c]Glucose.
[d]Acetate.
[e]Glycerol.
[f]Jerusalem artichoke hydrolysate (JAH).
[g]Corn powder hydrolysate (CPH).

Orcutt and Patterson, 1974; Ra et al., 2018; Sung et al., 2018). Compared to other types of cultivation, autotrophic condition is more robust and has less severe contamination problems. In autotrophic cultivation of *Chlorella* sp., the highest lipid productivity can reach approximately 179 mg/L/day, sequestrating approximately 2% of CO_2 at 0.25 vvm of air flow (Chiu et al., 2008, 2009).

Heterotrophic cultivation uses organic carbon as both energy and carbon sources for microalgal growth. In heterotrophic culture, cell growth is significantly influenced by nutrient concentrations in the medium and process factors (Huang et al., 2010). A high lipid productivity of 1209.6–3701.1 mg/L/day was reported in *Chlorella protothecoides* cultivation under heterotrophic conditions (Xiong et al., 2007). Even under limited light supply, microalgae still can grow with a high yield of biomass as long as favorable carbon sources are provided (Yoo et al., 2010a). Heterotrophic cultivation could avoid the problems associated with limited light supply in phototrophic cultivation in large-scale photobioreactors. A 40% increase in lipid content was gained in *Chlorella protothecoides* cultivation by just changing the condition from phototrophic to heterotrophic (Chen et al., 2011).

Mixotrophic cultivation refers to the condition where microalgae undergo photosynthesis using both organic and inorganic carbon compounds as the carbon source for growth, under either phototrophic or heterotrophic conditions, or both. Compared to phototrophic and heterotrophic, mixotrophic cultivation is rarely used for lipid production because of the high cost of organic carbon and inadequate yield (Mata et al., 2010).

Photoheterotrophic cultivation is the condition where the microalgae require light as the energy source and organic compounds as the carbon source, while mixotrophic cultivation can use organic compounds to serve this purpose. Hence, photoheterotrophic cultivation needs external supply of both sugars and light at the same time (Chojnacka and Marquez-Rocha, 2004). Although frequently used for enhanced production of some light-regulated metabolites (Ogbonna et al., 2002), photoheterotrophic cultivation is rarely used for producing microalgal lipids.

Among these four types of cultivation, heterotrophic is capable of producing a much higher lipid yield than other cultivation conditions. However, heterotrophic culture is less resistant to bacterial contamination, especially in open-pond systems, in addition to its requirement of organic carbon as the energy source. Phototrophic cultivation can uptake CO_2 in flue gases and is commonly used in lab-scale cultivation. Compared to the high operation cost in heterotrophic cultivation, phototrophic cultivation is relatively inexpensive (Brennan and Owende, 2010; Demirbas, 2011), but its lipid productivity is typically low due to the low cell growth rate. Mixotrophic and photoheterotrophic cultivations are also used for lipid production; however, they are restricted by their high contamination risk and special light requirements, which result in higher operating and processing costs (Amaro et al., 2011; Satyanarayana et al., 2011).

13.2.3 Process parameters affecting microalgae cultivation

Practically, microalgae are commonly cultivated in two types of systems: open ponds (including raceway systems) and enclosed photobioreactors (PBRs). Open-pond systems are the simplest and least expensive systems for cultivating microalgae, and more suitable for large operations. Major downsides of open-pond systems include significant water loss due to evaporation and the inadequate controls of the operating conditions, especially temperature fluctuations and potential contaminations by foreign organisms. In addition, open ponds do not provide the optimal conditions for efficient CO_2 utilization, and essentially eliminate the artificial application of CO_2 for optimal biomass production (Chisti, 2007).

PBRs provide better controls on the cultivation conditions and overcome the problems of water evaporation and contamination in open-pond systems. Major BPR configurations include flat-plate, tubular (horizontal/vertical), bubble column/airlift, and stirred tank bioreactors (Carvalho et al., 2006; Mirón et al., 1999). PBRs offer space savings and better control of CO_2 supply and utilization; they are also more suitable for some strains of microalgae that are susceptible to

contamination by other microbes. Meanwhile, enclosed PBRs require precise controls of all process parameters and involve higher initial investment, thus leading to higher operating and capital costs. Therefore, the choice for large-scale operations needs to be based on careful studies of economic feasibility (Amin, 2009; Brennan and Owende, 2010; Singh and Singh, 2010).

Healthy and productive growth of microalgae depends on correctly controlled parameters, including light source and intensity, carbon resources, and nutrients. For a biodiesel feedstock purpose, the process parameters must be optimized for the highest possible lipid content in microalgae and highest biomass productivity.

Cell density of the microalgal culture is an important factor that ensures adequate light exposure and uniform nutrient in the medium for healthy growth while an optimal biomass productivity is targeted (Cagliari et al., 2011; Richmond, 2007). Too high a cell density would block the light penetration into the culture body, causing an energy deficiency; too low a cell density would lead to less biomass productivity. Optimal cell density is also dependent on the types of microalgal strains regarding light requirements and the uptake rate of nutrients.

Light supply is vital for adequate yet efficient and productive photosynthesis. The type of light (e.g., solar vs artificial), method of light supply (e.g., sunlight from surfaces vs artificial light from both surface and internal culture), intensity of light, and range of wavelengths in the case of artificial light all affect the success of microalgae cultivation. Meanwhile, the needs for optimal light intensity depend on the strain of microalgae. Generally, the productivity of microalgal biomass is proportional to the sufficient supply of light. However, excessive light supply, or too high a local light intensity, may cause photoinhibition in photoautotrophic cultures. Heterotrophic cultivation may be considered if light inhabitation is a major concern (Hsieh and Wu, 2009).

Carbon sources significantly affect the content of algal lipids, especially in heterotrophic cultivation of microalgal species. In general, microalgae can grow on a diverse range of carbon sources, such as carbon dioxide, methanol, acetate, glycose, or other organic compounds. Some species of microalgae can utilize inorganic carbon sources in photoautotrophic cultivation, while others use directly organic carbon in the presence or absence of a light supply in heterotrophic cultivation (Yen et al., 2019). However, the most commonly used carbon source for microalgae as a resource for biofuels is still carbon dioxide or bicarbonates. This is because most microalgae have much higher CO_2 fixation rates for cell growth than terrestrial plants (10–50 times higher), which demonstrates the advantage of direct conversion of photoautotrophic growth of microalgae. Meanwhile, organic carbon sources would be too expensive for producing low-priced products such as biofuels. In addition, the goal of producing microalgae for biofuels is to recycle and/or sequester carbon from CO_2 in the air, thus CO_2 is the most logic and meaningful carbon source.

Carbon nitrogen ratio (C/N) is another significant factor in lipid biosynthesis in microalgae. The extent and rate of algal lipid accumulation depend on the carbon sources, and carbon is simultaneously involved in nitrogen assimilation. In particular, the carbon concentration might also influence microalgae growth and lipid accumulation, which require the critical nutrient of nitrogen (El-Sheekh et al., 2013; Kim et al., 2014). A high initial C/N ratio could enhance lipid productivity (Hu, 2007; Richmond, 2007; Skulberg, 2007) and the critical C/N ratio for lipid accumulation depends on the individual strains of microalgae. For example, a high initial C/N ratio greater than 86 was found to be beneficial for lipid accumulation in mixotrophic culture of *Chlorella vulgaris* (Li et al., 2016). It was found that lipid content was at 7.6%–11.3% when the initial C/N ratio was less than 50 but increased significantly to 23.9% at an initial C/N ratio of 92.7. It was also found that the C/N ratio affects the fatty acid composition of heterotrophic *Chlorella sorokiniana*, and the critical C/N ratio at which the transition from carbon to nitrogen limitation of growth occurred was found to be in the range of 20–25 (Chen and Johns, 1991).

Other nutrients, although in small quantities, are also important, especially nitrogen and phosphorus (Singh and Singh, 2010). It is generally agreed that a combined source of nitrogen and phosphorus is better than just a nitrogen source, as in the case of treated and untreated industrial wastewater as a nutrient source (Chinnasamy et al., 2010). One must note that nitrogen limitation is often used in the late stage of microalgal growth as a technique for enhancing lipid accumulation (Chisti, 2007; Demirbas, 2011; Gouveia et al., 2009; Hsieh and Wu, 2009; Kong et al., 2009; Wang et al., 2010).

Temperature of 20–30°C is optimal for many microalgae species (Chisti, 2007), although a higher temperature (50°C or higher) can be used for certain strains of thermophilic microalgae in PBRs (Chen et al., 2011). Water evaporation in open-pond systems usually keeps the cultivation medium cooled, limiting the upper temperature to 40°C under warm climate conditions. Temperature is a principal factor in the photocatalytic reactions (Xing et al., 2013) and affects the chemical equilibrium, gas solubility, and pH levels in the systems (Solimeno et al., 2015). A considerable variation in temperature may be experienced in commercial cultivation systems due to diurnal and seasonal behaviors. However, the temperature in a PBR may be 10–30°C higher than the ambient temperature without specific temperature controlling systems (Wang et al., 2012), which may not affect adversely the cultivation of microalgae, although a cost-effective and reliable temperature-control system is essential in the PBRs in order to keep the culture temperature within a favorable range.

Dissolved oxygen (DO) affects microalgal cultivation. The photosynthesis of microalgae makes use of light and CO_2 for growth and yields O_2 as the by-product. Efficient DO helps microalgae convert carbon sources to biomass. However, too high a level of DO may undesirably lead ribulose-1,5-bisphosphate carboxylase oxygenase, the primary carboxylation enzyme that delivers CO_2 for the Calvin cycle, to consume O_2 and produce CO_2 for photorespiration (Wang et al., 2012).

13.2.4 Practical techniques for stimulating lipid production in microalgae

Successful operation of microalgae cultivation systems involves many considerations of process controls, in addition to the process parameters alone, including types of reactors (e.g., open ponds vs PBRs), operation mode (e.g., batch vs continuous flow), hydrodynamics of the culture (paddled raceways vs gas-lift circulation PBRs), and nutrient supply and control (e.g., balanced vs nutrient limited) (Camacho et al., 2011; Michels et al., 2016; Tredici, 2010).

In batch systems, microalgae grow exponentially in the early growth stage to increase their biomass before lipid induction. In the late maturing stage, microalgae start to accumulate lipids as an energy storage, usually by nutrient starvation or other techniques (Sharma et al., 2012). In some cases, 5%–20% of dry mass weight is constituted of microalgal lipids. Essentially, microalgal biomass (e.g., cellulosics and carbohydrates) competes with lipids for photosynthesis. Under stress conditions, many strains of microalgae alter their biosynthetic pathways toward the formation and accumulation of neutral lipids including TAGs (20%–50%) (Sharma et al., 2012). Synthesis and accumulation of TAGs can also occur when chemical and/or physical environmental stimuli are imposed (Hu et al., 2008).

There has been a wide range of studies on the techniques for lipid induction, including nutrient starvation (e.g., nitrogen and/or phosphorus), light irradiation (e.g., light and dark cycles), pH regulation (e.g., buffer chemical addition), temperature control (e.g., in enclosed PBRs), and other chemical application (e.g., trace metals).

13.2.4.1 Nutrient starvation

Nutrient limitation invariably causes a steadily declining rate of cell division, which in turn causes a decreasing algal growth, thus eliminating the requirement of ample nutrients for the synthesis of new membrane compounds. As a result, the cells instead divert but deposit fatty acids as TAGs as a protective mechanism.

Nitrogen is the most critical nutrient affecting lipid metabolism in microalgae. For example, cultivation of *Scenedesmus* sp. under nitrogen and phosphorus limitations showed an increase in lipids as high as 30% and 53%, respectively (Xin et al., 2010). Lipid content in *Chlorella vulgaris* was significantly increased from 18% to 40% while protein content was reduced from 29% to 7% in the medium of low nitrogen concentration ($NaNO_3$ 37.5 mg/L) (Illman et al., 2000). Moreover, it was found that nitrogen depletion gradually leads to the change in *Chlorella vulgaris* cultivation from free fatty acid-rich to TAG-rich lipids (Widjaja et al., 2009).

It was reported that phosphorus limitation resulted in an increased lipid content, primarily as TAGs, in the cultivations of *Phaeodactylum tricornutum*, *Chaetoceros* sp., *Isochrysis galbana*, and *Pavlova lutheri*, but a decreased lipid content in the cultivations of *Nannochloris atomus* and *Tetraselmis* sp. Due to phosphorus deprivation, the production of C16:0 and C18:1 increased, but

the production of C18:4ω3, C20:5ω3, and C22:6ω3 decreased (Reitan et al., 1994). In contrast, phospholipids of phosphatidylcholine (PC), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), and sulfoquinovosyldiacylglycerol (SQDG) were found in escalatory levels in the phosphorus-starved *Chlorella kessleri* culture. It was also found that the overall yield of TAGs increased from 6.5% up to 39.3% under phosphorus starvation. Phosphorus depletion was also found to have a compensatory mechanism with sulfur depletion in green microalgal culture of *Chlamydomonas reinhardtii*, in which sulfur depletion led to the decrease in SQDG but a twofold increase in PG content (Sato et al., 2000). When *Chlamydomonas reinhardtii* was grown in media with limited phosphorus, there was a 40% decrease in PG and a stimulated increase in the SQDG content. Thus, compensatory mechanisms that keep the total sum of SQDG and PG contents constant under both phosphorus and sulfur limiting conditions occurred. Other studies also showed that sulfur deprivation led to an increase in total lipid content in the cultivation of *Chlorella* sp. and *Chlamydomonas reinhardtii* (Timmins et al., 2009).

It is clear that nitrogen starvation is the most widely studied approach for boosting lipid production in almost all the microalgal species that can be considered suitable for biofuel production (Sharma et al., 2012). Nitrogen is the most limiting growth factor for eukaryotic microalgae and the first nutrient to be applied for controlled nitrogen stress. However, high lipid production under nitrogen stress may take 2–5 days to occur and is complemented with a slow growth rate and low cell counts, thus leading to decreased total biomass and lipid productivities per unit time (Widjaja et al., 2009).

13.2.4.2 Temperature stress

Temperature has been found to have a major effect on the fatty acid compositions in microalgal lipids. A general trend of increased unsaturation of fatty acids with decreasing temperature has been observed in many strains of microalgae and cyanobacteria (Lynch and Thompson, 1982; Murata, 1975; Sato and Murata, 1980). This increase in unsaturated fatty acids causes the fluidity of membranes to change. When the cultivation temperature was shifted from 30°C to 12°C, the level of unsaturated lipids in *Dunaliella salina* significantly increased by 20% (Thompson, 1996). This change in fatty acid composition, however, is not always a one-way street. For example, a culture temperature decrease from 25°C to 10°C led to an elevation of oleates (single double bond) but a decrease in linoleates (two double bonds) in the cultivation of *Selenastrum capricornutum* (McLarnon-Riches et al., 1998). Therefore, temperature effect is dependent on the strains of microalgae also.

Studies on the effect of low temperature cultivation with some higher taxonomic status microalgae have shown increased unsaturated fatty acids accumulation. Higher content of polyunsaturated fatty acid eicosapentaenoic acid (C20:5) was reported to be increased from 20.3% to 30.3% and docosahexaenoic acid (C22:6) from 8.1% to 16.8% in the cultivation of marine microalgae *Pavlova lutheri* at 25°C versus that at 15°C (Tatsuzawa and Takizawa,

1995). Similar behaviors were shown in the culture of thermophilic cyanobacterium *Synechococcus lividus*, where the amount of stearic acid (C18:0) decreased while the unsaturated palmitoleic acid (C16:1) and oleic acid (C18:1) increased when the cultivating temperature was lowered from 55°C to 38°C (Fork, 1979). It is generally concluded that lowering the cultivation temperature will lead to an increase of unsaturated fatty acids in the microalgal lipids.

13.2.4.3 Salinity induction

Salinity of the cultivation medium can affect the physiological and biochemical properties of microalgae. When microalgal cells are exposed to a saline environment, the recovery of turgor pressure, adjustment of absorption and release of ions through cell membranes, and accumulation of osmosis-resisting matters are triggered. Such a salinity stress inside the cells results in the increment of lipid content (Alkayal et al., 2010; Talebi et al., 2013). Many strains of microalgae have been found to respond to the salinity stress insensitively. Species of *Dunaliella* are the best examples of microalgae that can tolerate high salt concentration (Azachi et al., 2002; Takagi and Yoshida, 2006; Xu and Beardall, 1997). A significantly higher ratio of C18 to C16 fatty acids was present in a 3.5 M NaCl culture compared to a 0.5 M NaCl culture (Azachi et al., 2002). Meanwhile, an increase of the initial NaCl concentration from 0.5 to 1.0 M followed by further addition of NaCl to 2.0 M resulted in a 67% increase in total lipid content (Takagi and Yoshida, 2006). An even larger increase in salinity from 0.4 to 4 M increased the total content of saturated and monounsaturated fatty acids, but decreased the polyunsaturated fatty acids in *Dunaliella* sp. cultures (Xu and Beardall, 1997). In cultivating three marine heterotrophic strains of microalgae, *Crypthecodinium cohnii* ATCC 30556 had the highest docosahexaenoic acid (C22:6) and total lipid content with a NaCl concentration of 9 g/L. In contrast, *Crypthecodinium cohnii* RJH had the highest content of docosahexaenoic acid (C22:6) at 5 g/L NaCl (Jiang and Chen, 1999).

Cultivating microalgae under salinity stress can also have the advantage of limiting potential contaminations from invasive organisms and competing microorganisms. However, too high a salinity, depending on the strains of microalgae, has the potential to inhibit the cell growth significantly and change the structure of microalgal cells due to osmosis pressure between media and cells. Thus, an optimal range of salinity must be determined specifically for the strains of microalgae to be cultivated.

13.2.4.4 Stress of pH

Fluctuation of the medium pH has been found to alter the composition of microalgal lipids (Sharma et al., 2012). Morphological observations revealed that alkaline pH inhibited the growth of microalgae and led to diverting the energy to form TAGs in the culture of *Chlorella* CHLOR1 (Guckert and Cooksey, 1990). In the culture of *Chlamydomonas* sp., TAGs

accumulated in large amounts as the storage lipid at pH 1 (Tatsuzawa et al., 1996). It was also shown that the increase in saturated fatty acids in membrane lipids at pH 1 represents an adaptive reaction of *Chlamydomonas* cells, which causes the decrease in fluidity of membrane lipids (Sharma et al., 2012; Soeder, 1980).

13.2.4.5 Stress of heavy metals

Heavy metals such as cadmium, iron, copper, and zinc have also been reported to increase the lipid content in some microalgae (Einicker-Lamas et al., 2002). Cadmium caused an increase in the total lipid content in all autotrophic, heterotrophic (in the dark), and mixotrophic (with light and an organic carbon source) cultures (Einicker-Lamas et al., 2002). Among the membrane lipids, sterol content was lower in cadmium-treated cells cultivated under illumination, with no significant changes in the total phospholipids. Although there was an increase in PG content, *Euglena gracilis* has been shown to have different sensitivities to copper and zinc (Einicker-Lamas et al., 2002). The effect of heavy metal irons on growth and lipid accumulation in *Chlorella vulgaris* was revealed by the increased total lipid content of up to 56.6% in the late exponential growth phase (Liu et al., 2008).

13.2.4.6 Light irradiation stress

Light is essential for photosynthesis in autotrophic cultivation. Microalgae grown under various light intensities exhibit remarkable changes in chemical composition of lipids and photosynthetic activity (Richardson et al., 1983). The metabolism for lipid formation and accumulation changes at different light intensities and wavelengths, leading to differences in lipid profiles in microalgae. Changes of intensity of light irradiation can be used as a stimulant for increasing TAG accumulation in many species. High light intensity beyond certain levels may cause oxidative damage to polyunsaturated fatty acids, but is required for the synthesis of monounsaturated fatty acids, especially C16:1, in microalgae (Skulberg, 2007). Typically, low light intensity induces the formation of polar lipids, particularly the membrane lipids associated with the chloroplast, whereas high light intensity enhances the increase of neutral storage lipids, mainly TAGs (Brown et al., 1996; Khotimchenko and Yakovleva, 2005; Napolitano, 1994; Orcutt and Patterson, 1974). In other cases, application of high light intensity increases the formation of nonpolar lipids, including TAGs, but decreases phospholipids in the filamentous green microalgae *Cladophora* sp. (Napolitano, 1994). This observation was also confirmed by other researchers (Khotimchenko and Yakovleva, 2005). In cultivating *Nannochloropsis* sp., the content of unsaturated fatty acids was lowered with an increased irradiance. A significant decrease in omega-3 fatty acids, mainly C20:5, from 29% to 8% of total fatty acids was revealed (Fábregas et al., 2004). Therefore, optimal light intensity is a species-dependent factor and needs to be determined individually.

Light/dark cycles at different microalgal growth phases also have a significant effect on the lipid compositions. Strong and continuous light or under 12/12 h light/dark conditions led to a

higher level of TAGs in the stationary phase (Mock and Kroon, 2002), in which the saturated and monounsaturated fatty acids increased while the polyunsaturated fatty acids decreased in the stationary phase (Brown et al., 1996).

It should be noted that light irradiation can only be controllable in PBR systems or in laboratory-scale cultures. The lightening cycles and/or the light intensity in open-pond/raceway systems are largely dependent on the location, climate condition, season, and depth/volume of the cultivation media, and are therefore hardly controllable. Controlled light supply adds cost to operation cost and thus the overall production cost of microalgal biofuels. Approaches of energy efficient LEDs and/or diverted sunlight have been investigated for use in large-scale PBRs as an effort to reduce the cost (Ra et al., 2018; Severes et al., 2017).

13.2.4.7 UV irradiance

A specific spectrum of light, UV irradiance, can also affect the growth and metabolism of microalgae. Current research into utilizing UV irradiance is mainly focused on its impact on algal growth, morphology, physiology, and oxidative stress, with a special emphasis on photosynthesis (Bhandari and Sharma, 2011; Fouqueray et al., 2007; He and Häder, 2002; Holzinger and Lütz, 2006; Wiley, 2009; Xue et al., 2005). A study has shown that a UV-A (wavelength of 315–400 nm) treatment significantly increased the chlorophyll-specific lipids; UV-A radiation combined with nutrients starvation had a synergistic effect on lipid accumulation in *Nannochloropsis oculate* (Srinivas and Ochs, 2012). When exposed to constant UV-A and varying UV-B (wavelength of 280–315 nm) radiation, the storage lipids (e.g. TAGs and saturated fatty acids) were reduced and structural lipids (e.g., phospholipids) increased in *Phaeocystis antarctica* (an Antarctic marine phytoplankton species). Increasing the level of UV-B irradiance to *Phaeocystis antarctica* led to increases in the contents of total lipids, TAGs, and free fatty acids (Skerratt et al., 1998). Adding UV-B radiation to the culture of *Tetraselmis* sp. (a green algal genus) showed an overall increase in saturated and monounsaturated fatty acids with a significantly lower (by 50%) content of polyunsaturated fatty acids (Goes et al., 1995). Therefore, UV irradiance can be used as a tool in regulating the fatty acid compositions and the level of saturations, if needed.

13.2.5 Genetically engineered strains of microalgae for biodiesel production

Research efforts have been made on metabolic engineering toward higher capacities of TAGs and/or omega-3 fatty acid accumulation in microalgae. Lipid overproduction has been achieved by disrupting the metabolic equilibrium within microalgae, such as the model species of *Chlamydomonas reinhardtii*, on molecular, genetic, and physiological aspects (Boyle et al., 2012; Courchesne et al., 2009; Jain et al., 2007; Molnar et al., 2009; Shrager et al., 2003; Siaut et al., 2011). Significant progress has been made on the expressed sequence tag databases, transcriptomes and nuclear, mitochondrial, and chloroplastidial genomes from several microalgae (Courchesne et al., 2009). Several nuclear genome sequencing projects have now

been completed, including those on *Chlamydomonas reinhardtii* (Merchant et al., 2007; Shrager et al., 2003), *Pharodactylum tricornutum* (Bowler et al., 2008), *Thalassiosira pseudonana* (Armbrust et al., 2004), *Cyanidioschyzon merolae* (Matsuzaki et al., 2004), *Ostreococcus lucimarinus* (Palenik et al., 2007), *Ostreococcus tauri* (Derelle et al., 2006), and *Micromonas pusilla* (Worden et al., 2009). The ultimate purpose of all the research is to explore an effective means of genetic modification of microalgal strains for optimized lipid production.

Researchers have proposed that activities of acetyl-coA carboxylase and fatty acid synthetase help determine the regulation of lipid synthesis, hence, efforts have been made to increase the expression of such enzymes (Donaldson, 1979). Another strategy to induce lipid accumulation is to inhibit the lipid catabolism, therefore shifting the equilibrium toward lipid synthesis (Trentacoste et al., 2013). Genes involved in the activation of both TAGs and free fatty acids, as well as genes directly involved in β-oxidation of fatty acids, can be inactivated, sometimes resulting in an increased cellular lipid content (Courchesne et al., 2009). The third strategy was a systemic approach of genetic engineering transcription (Zhang et al., 2014). In many cases, genetic transformation in terms of favoring the lipid synthesis resulted in the stable expression of transgenes, from either the nucleus or the plastid, but in some cases only a transient expression was observed. For example, *Chlorella ellipsoidea* was transformed to produce a transcription factor from soybean, which granted the microalgae an enhanced production of 52% of total lipids, an increase of 2.5-fold (Zhang et al., 2014). For the purpose of increasing lipids content in microalgae, a variety of transformation methods have been used to transfer DNA into microalgal cells, electroporation (Chow and Tung, 1999; Shimogawara et al., 1998), biolistic microparticle bombardment, and *Agrobacterium tumefaciens*-mediated gene transfer (Kumar et al., 2004).

Various methodologies achieved better results by affecting not only a certain step but the complete metabolism by giving it enough substrate to initiate lipase functions without consuming the finished lipid product and triggering a genetic switch to turn on the complete pathway. However, it must be pointed out that genetically engineered strains of microalgae can only be used at present in fundamental studies and are very strictly regulated. Thorough investigations and related regulations are still needed to examine the potential and feasibility of genetically modified strains of microalgae for practical applications in biofuel production. Therefore, genetically engineered strains of microalgae for biodiesel production still have a long way to go.

13.3 Microalgae preparation for biodiesel production

13.3.1 Harvesting microalgae

Microalgal culture is a low density slurry of small cells, typically of 3–30 μm (Demirbas, 2010; Shelef et al., 2008). Therefore, harvest of microalgal cells by separating them from the culture is the first step. The goal is to increase the effective particle sizes for easy sedimentation,

centrifugation, and filtration (Mata et al., 2010). The methods to achieve large particle aggregation can be either chemical or physical processes, including chemical flocculation, biological flocculation, ultrasonic aggregation, etc.

13.3.1.1 Chemical flocculation

Chemical flocculation can be achieved by adjusting the pH and/or by applying chemical flocculants (e.g., $FeCl_3$), before physical separation via centrifugation, filtration, microfiltration, and ultrafiltration (Knuckey et al., 2006; Medina et al., 1998).

Naturally, microalgal cells carry negative charges, which cause electric repelling among cells and prevent them from self-aggregating. The negative charges can be countered by adding cationic chemicals or flocculants (Medina et al., 1998), which combine with the negative charges and coagulate the microalgal cells. Examples of inorganic flocculants include $Al_2(SO_4)_3$, $FeCl_3$, and $Fe_2(SO_4)_3$. Another type of flocculants is polyelectrolytes or organic flocculants, the cationic polymers which physically link the cells together to form flocculates. The key characteristic of polymer flocculants is the charge. The higher the cationic charge of the polymers, the higher their binding capabilities. Other properties include the molecular weights and polymer structures. The advantages of organic flocculants includes less sensitivity to pH change, a wider range of applications, and a lower requirement of application dosage (Shelef et al., 2008).

Flocculation of microalgal cells can also be achieved with the addition of inorganic salts, which regulate the culture pH. For example, when calcium phosphate was used, the positive calcium ions attract and link with the negatively charged microalgal cells to become larger aggregates (Shelef et al., 2008). When the culture pH was regulated at 10.8 with NaOH, KOH, and $Ca(OH)_2$, Vandamme et al. (2012) observed a 98% algal biomass recovery, whereas at pH 9.7 regulated by $Mg(OH)_2$, a biomass recovery greater than 95% was achieved (Perez et al., 2014). Pezzolesi et al. (2015) also observed that at higher pH, the flocculated cells were less compact than those at lower pH and were easily resuspended in the culture medium. Inorganic flocculation is less costly than organic flocculation, but requires a high level of technology support to monitor the pH and sedimentation progress accurately (Kim et al., 2013). Thus, it is not yet proved for feasible use in separating microalgal cells in large-scale operations.

13.3.1.2 Physical separation

Centrifugation is the most preferred method for microalgae harvesting. Centrifugation relies on the centrifugal force to accelerate the movement and separation of microalgal cells from the medium. The biomass content at the discharge is dependent on the feed properties, e.g., particle sizes and particle-fluid density difference, and can be controlled in certain ranges by altering the feed flow rate and centrifugal force. The maximum solid content at discharge in continuous centrifugation processes is typically in the range of 10%–20%, although higher solid contents can be achieved with newer designs of centrifuges (Pahl et al., 2013). Centrifugation separations are compatible with most of the cultivation systems (e.g., batch or continuous, open-pond or enclosed PBRs) and thus are widely used in large-scale operations.

Filtration is a simple method that can be used for harvesting microalgal biomass. It is easy to use, has various configurations for different systems, and is a mature technology that has been used widely in various industrial applications. For example, using diaphragm filters with a harvest of small size (30 μm) microalga l cells of *Scenedesmus*, an efficiency of 100% was achieved (Borowitzka, 1999). It is also very effective for cell sizes larger than 70 μm, such as *Coelastrum* and *Spirulina*. However, the filter membrane becomes fouled and clogged easily due to the small size of the microalgal cells (smaller than 30 μm) such as *Scenedesmus*, *Dunaliella*, and *Chlorella* (Mohn, 1980), requiring frequent cleaning and replacement of the filter membrane and adding extra cost to the harvesting process.

Electroflocculation used for algae harvesting has the advantages of being nonspecies specific, easy to control, low chemical usage, no retentive anions (e.g., Cl and S), low power consumption compared to centrifugation, etc. (Chen et al., 2015; Lee et al., 2013; Vandamme et al., 2012). Electroflocculation uses a bipolar chamber with aluminum electrodes to attract microalgal cells into high density cakes. The downside of this technology, because of the metal irons in the cultivation medium, is the contamination of metal irons that may be carried into lipids and eventually affect the biodiesel quality.

13.3.1.3 Ultrasonic sedimentation

Ultrasonic sedimentation is an emerging technique which concentrates microalgal cells at ultrasonic wave knots due to acoustic forces, and uses the force of gravity to drag microalgal cells down to the collection zone (Bosma et al., 2003). When the ultrasonic field is applied, it creates areas of a maximum potential energy (bellies) and a minimum potential energy (nodes). The cells move almost instantly from the bellies to the nodes and stay there until the field is relieved. The primary ultrasonic field is further scattered by individual microalgal cells, and creates an attractive force between those cells, driving them together into the knots of the ultrasonic wave. Subsequently, aggregation of the cells at the knots sediment rapidly from the medium when the ultrasonic field is removed (Bosma et al., 2003). The advantages of this technique include the lack of shear force or damages to the cells, absence of fouling inside the machine, no moving parts, and feasibility for development for continuous operations, as evidenced by its application in insect cell separation (Bosma et al., 2003; Lee et al., 2010). This technique is especially applicable in separating cultures with very low cell density, up to 20 times lower than those required in other systems. However, the high-power requirement is a major drawback of ultrasound sedimentation.

13.3.2 Drying microalgae

Before microalgal biomass is processed further for biodiesel production, the harvested biomass slurry needs to be dried to a moisture content of 12%–15% (Shelef et al., 2008). By drying, the microalgal biomass becomes a stable storable product and is favorable for various downstream processes. Drying of high moisture sludges is an energy- and cost-intensive process. Thus,

drying wet microalgae (up to 95% of moisture content) to a low level of moisture content (such as 12%–15%) poses a major economic problem which may contribute to 70%–75% of the overall processing cost (Mohn, 1980). The selection of drying methods depends on the scale of operation and the types of microalgal biomass. Waste or low-quality heat from industrial processes, such as power stations, is typically considered but often requires expensive infrastructure upfront. Solar drying is then a logical choice for drying microalgae.

Solar drying is one of the oldest methods for food preservation and is still used today. It is inexpensive and very accessible, and can be accomplished either by direct solar radiation or by solar water heating. In the example of solar drying of microalga *Spirulina*, a solar dryer constructed with a wooden chamber covered with glass panels was able to dehydrate the final algal biomass to 4%–8% of moisture content in 5–6 h at 60–65°C (Becker and Venkataraman, 1982). In solar water heating systems, solar thermal energy is derived by solar panels to heat up the water, which is used to dry microalgal biomass in noncontacting drying facilities. Solar drying facilities used in other industries can be adapted directly to microalgae drying, although the operating parameters need to be altered accordingly to justify the characteristics of microalgal biomass. With proper system designs, the efficiency of solar drying can be regulated, and the cost can be minimized. However, solar radiation is generally uncontrollable and unpredictable, and highly dependent on the weather conditions. It is noted that direct solar drying often causes overheating and disintegration of algal chlorophyll, leading to a possibly low-quality biomass.

Rotary dryers are commonly used facilities for drying in general. They use a sloped rotating cylinder to move the material from one end to the other and regenerate the contact surface to particles. Rotary dryers stand out for their flexibility in handling a wider range of materials than other types of dryers and for their high processing capacity. When a pilot electric drum-dryer was used for drying microalgae *Scenedesmus* with a surface area of 2.5 m^2, the algal slurry could be thickened up to 25% dry solid at 120°C in 10 s with a power consumption of 52 kW h. If the energy source is replaced by steam, the processing cost would be lowered by 6.8 times (Becker and Venkataraman, 1982). In an assessment on energy requirement, 15.7 Mcal (65.7 MJ) of energy is required to evaporate 18.2 kg of water out of wet microalgae to yield 1 kg of dry biomass (Shelef, 1980), which is approximately 60% higher than that of evaporating pure water. Therefore, the energy requirement depends largely on the water content to be driven off the microalgal biomass. It is proposed that an initial solar drying is used to remove free water from the freshly harvested microalgal biomass to an acceptable water content before being dried further via other drying means to lower the energy cost.

Flash drying is a way of rapid moisture removal by spraying or injecting a material of fine particles into a stream of hot gas or superheated steam (Shelef et al., 1984). In many cases, flash dryers are much more compact than rotary dryers and can be used for most biomass drying (Amos, 1999). However, microalgal biomass harvested as flocculated aggregates must be

broken into fine particles so that it can be suspended and remain in good contact with the hot gas to accomplish heat and mass transfers adequately between the wet sludge and the hot carrier gas (Chen et al., 2015). In case of inadequate drying after one pass, some material can be recycled back and mixed with the incoming wet material to achieve further moisture removal. With the short retention time in flash dryers, biomass can be dried more uniformly and less destructively. The emissions in the exhaust can be much lower those that of rotary dryers (Amos, 1999), making flash drying a low fire risk facility. The cost of flash drying and the final dried product quality are greatly influenced by the hot gas source and the operation controls. Comparing with rotary dryers, flash dryers are energy efficient, but may involve high electricity costs due to the use of powerful blowers.

Spray-drying is a fast-drying process and preferred for drying materials that are temperature sensitive. It atomizes water droplets and spray downward into a chamber in contact with a hot gas stream from the opposite direction. The dried products are removed quickly from the bottom of the chamber. Therefore, the temperature stress on the materials to be dried is minimized. Spray-drying has been used for producing microalgae for human consumption (Soeder, 1980). Unlike freeze-drying, spray-drying is a fast process, is highly applicable to large-scale operations, and the resulting product is much less hydroscopic (Orset et al., 1999). Spray-drying poses a low risk of degradation to the dried microalgae. Research on morphological changes of *Chlorella* and *Spirulina* under spray-drying has shown that the microalgal particles of a few thousand cells are in the form of individual spheres with a void space in the center; in contrast, the freeze-dried microalgae are in the form of sheets of cells that adhere together in a linear fashion (Lin, 1985). Due to the setup and the method of its operation, spray-drying involves a high operating cost. Meanwhile, spray-drying could also rupture the cells due to its high-pressure atomization process, leading to unacceptable degradation of microalgal cells, thus causing the product quality to suffer (Lin, 1985).

Freeze-drying is a low temperature dehydration process that consists of four operations: freezing, vacuum, sublimation, and condensing. It is especially applicable to materials that are very sensitive to medium-high temperatures. Different from most conventional drying methods through evaporative water removal by heat, freeze-drying removes moisture from the materials by sublimation, where water as ice directly becomes vapor without undergoing the liquid stage. Freeze-drying is typically conducted under a vacuum to help mass transfer during sublimation. Due to the absence of liquid water and the low temperature requirement (typically −50°C to −80°C), most of the structure deterioration and microbiological reactions are stopped or dramatically slowed. Therefore, the chemical properties of materials are largely maintained, making it the top choice of moisture removal in food and/or pharmaceutical industries. Freeze-drying for moisture removal from microalgae has been evaluated by many researchers. For example, with wet *Spirulina* biomass at −50°C to −65°C and 0.06 atm pressure, a dried biomass of 4% water content was achieved (Becker and Venkataraman, 1982). Despite of its many advantages, freeze-drying has always been acknowledged as the most

expensive process for dehydration (Ratti, 2001). It would be the best choice if the product value justified the cost. As a material in bulk quantity with a high water content, microalgae can hardly justify the cost, and thus freeze-drying is not suitable for large commercial applications.

13.3.3 Lipid extraction

In traditional microalgal biodiesel production, lipid extraction is necessarily the first step. Generally, the extraction techniques can be sorted into mechanical methods and chemical methods. Numerous methods for oil extraction from oil seeds and nuts have been applied to lipid extraction from microalgae, such as oil expeller/press extraction, solvent extraction, and supercritical fluid extraction, and can be enhanced by ultrasonication and/or microwave (Brennan and Owende, 2010; Cooney et al., 2009; Halim et al., 2011; Lee et al., 2010).

The common equipment for mechanical extraction is the oil expeller/press. The cellular structure of oil seeds or microalgae is disintegrated by applying the mechanical forces through solid shear by hydraulic and/or screw presses. Elevated temperature of the oil seeds, e.g., 50–70°C, helps improve the efficiency of oil pressing and power requirement. Moisture content in the seeds plays also an important role in reducing the shear and lubricating the rollers or pressing plates. Hence, an appropriate moisture content (e.g., 10%–12%) of the oil seeds/microalgae must be maintained through proper dewatering and drying for a more effective mechanical extraction process. However, mechanical extraction is less efficient overall compared to solvent extraction. The oil yield is typically in the ranges of 60%–80%, losing quite a yield to the processed meal. This low oil efficiency may not be as critical in oilseed pressing, due to the high value of the meal, but is critical for lipid extraction from microalgae, which needs the highest lipid yield possible. Using a screw expeller press for lipid extraction from filamentous green microalgae, Topare et al. (2011) reported a 75% of total lipid yield, which is clearly lower than 98% by Soxhlet solvent extraction from microalgal cells. Some researchers suggest that the screw pressing method is too expensive and is slow for use in large-scale processes (Boldor et al., 2010; Mubarak et al., 2015).

Solvent extraction is nowadays the most commonly used method in industry to extract lipids from microalgae (Medina et al., 1998). In solvent extraction, sometimes at elevated temperatures (e.g., 100°C), TGAs and fatty acids are extracted into the solvent phase, which is then separated from the solvent in a separate process (Bosma et al., 2003; Halim et al., 2011, 2012; Medina et al., 1998). Organic solvents, such as benzene, cyclohexane, hexane, ethanol, acetone, and chloroform, can be used and the choice depends on the class of lipids to be extracted (Lee et al., 2010; Mubarak et al., 2015; Ranjith Kumar et al., 2015). Hexane is the most efficient solvent based on its high extraction capability and low cost (Cooney et al., 2009; Halim et al., 2011). It was also reported that using a combined solvent of ethanol and hexane can improve the lipid extraction efficiency in a two-step process quite effectively (Cerutti et al., 2012; Cooney et al., 2009).

In direct lipid extraction from wet microalgal sludge, strong solvents are preferred because they can also dissolve microalgal cell walls and separate the lipids from the aqueous medium while leaving the other components alone, such as sugar, amino acids, salts, and hydrophobic proteins (Mata et al., 2010). However, the selection of a "perfect" organic solvent can be an issue when implemented for larger-scale systems.

To enhance the effectiveness of solvent extraction, assistance by ultrasonication and microwave can be applied. Ultrasonication-assisted extraction process has the advantages of simple set-up and being easy to work with, imparting higher purity to the product, and being economical. The technique is highly effective and reproducible. This technology can enhance lipid extraction from microalgae by 50%–500% and with 10-fold reduced extraction time (Suali and Sarbatly, 2012). In an ultrasound-assisted extraction of docosahexaenoic acid (C22:6) from cultivated marine green microalgae, the tough algal cell wall was disrupted by ultrasound and the extraction yield was considerably improved to 25.9%, compared to 4.8% by Soxhlet method (Cravotto et al., 2008).

Microwave-assisted extraction uses a noncontact heat source which can penetrate into the biomaterials, interact with polar molecules like water in the biomass, and heat the whole sample uniformly. Higher oil yield, superior product quality, and reduced extraction time are the main advantages of microwave-assisted extraction (Mubarak et al., 2015). Martinez-Guerra et al. compared the lipid extraction by the conventional Bligh & Dyer method with the microwave-assisted hexane extraction from oleaginous microalgae *Chlorella* sp. and achieved a maximum lipid yield of 20.1%, much higher than 13.9% by the conventional Bligh & Dyer method (Martinez-Guerra et al., 2014). It was also reported that microwave-assisted extraction can achieve higher yields than other methods including the ultrasonication-assisted method (Lee et al., 2010).

Electroporation is another enhancement method of extraction being explored. Electroporation involves an increase in the electrical conductivity and permeability of the algal cells and cytoplasmic membrane, when an externally electrical field is applied. It was claimed that a much improved extraction yield of 92% was achieved after a single electroporation treatment, while only 62% of the total lipids were extracted without electroporation treatment (e.g., sonication-assisted extraction, expeller press, solvent extraction) (Ranjith Kumar et al., 2015).

Supercritical fluid extraction (SFE) makes use of high pressures and temperatures of a chosen fluid to rupture the cells without additional chemical reagents. This method has been proved to be extremely time-efficient with high yields (Cooney et al., 2009). Higher temperature and pressure, combined with the supercritical solvent effect, break down the cell walls and facilitate the diffusion of the solvent into the cell matrices at a much higher degree of efficiency that that of conventional and or other enhanced extraction methods. The major drawback of the SFE systems is the higher capital needed and operating cost due to the high temperatures and pressures requirement.

However, each enhanced extraction method has its limitations. For example, if a prolonged ultrasonication is applied, the production of free radicals is possible, which may be detrimental to the quality of the lipids that are being extracted (Mason et al., 1994). Microwave treatment is unsuitable for materials of volatile compounds due to the heat generation, and is applicable only with the use of polar solvents, which is not common in microalgal lipid extraction (Patel et al., 2018). Moreover, the formation of free radicals can also occur during microwave treatment, in addition to the increased temperature, making it a less favorable choice for microalgal lipid extraction (Zheng et al., 2011).

13.4 Microalgal biodiesel production

Microalgae have great potential to be used as a raw material for biodiesel production. Critical appraisal of the viability of microalgae projects revealed that microalgae-based biofuels deserve strong attention for commercialization. Like other feedstocks for biodiesel production, such as vegetable oils and animal fats, microalgal lipids are highly viscous and thus not suitable for direct use in modern diesel engines. After the extraction out of microalgal cells, the lipids must undergo further conversion processes to make biodiesel. Transesterification is the chemical conversion of oils/fats or lipids into biodiesel that has acceptable fuel properties for use in diesel engines.

Microalgae are recognized for their high productivity of total lipids and mostly neutral lipids with lower degrees of unsaturation (Mata et al., 2010), which affect the fuel properties of biodiesel produced. Generally, the fatty acid profiles of microalgal lipids tend to be at both extremes, namely high content of saturated fatty acids and high content of polyunsaturated fatty acids (Knothe, 2011a), which relate directly to the poor cold flow property and oxidative stability of microalgal biodiesel. Due to the high level of FFAs in microalgal lipids, traditional base-catalyzed transesterification is not applicable. The most commonly used method is the two-step conversion process. The FFA is esterified into fatty acid methyl esters (FAMEs), if methanol is used, in the first step using a strong acid (commonly sulfuric acid) as the catalyst, and the rest of the lipids (mainly triglycerides) are then transesterified into FAMEs using a base catalyst (Canakci and Van Gerpen, 1999; Gerpen, 2005).

13.4.1 Transesterification of microalgal lipids

Transesterification of oils or fats (chemically triglycerides) is a stepwise reversible chemical reaction. The chemical reaction rate is very slow at ambient temperature without the presence of a catalyst. To increase the reaction rate, elevated temperature and catalysts are commonly used. If a very high operating temperature is applied, such as in the case of supercritical methanolysis in which the temperature is commonly 230–250°C, the application of a catalyst may not be necessary (Bi et al., 2015). If catalysts are used, the

transesterification can be further categorized as homogeneous alkali/acid catalyzed, heterogeneous alkali/acid catalyzed, and enzymatic.

Use of either homogeneous or heterogeneous catalysts can both achieve significant success in converting oils/fats into biodiesel. In industrial applications, the commonly used homogeneous base catalysts include hydroxides, such as sodium hydroxide (NaOH) and potassium hydroxide (KOH), and methoxides (also called methylates), such as sodium methoxide ($NaOCH_3$) and potassium methoxide ($KOCH_3$) (Dalai et al., 2006; Felizardo et al., 2006; Kulkarni et al., 2006). When hydroxides are used, the catalyst solutions are typically preprepared by reacting the hydroxides with methanol to form methoxides. It is the methoxides that are the active species for catalysis.

The mechanism of base-catalyzed transesterification of vegetable oils has been proposed and discussed by many researchers (Demirbas, 2005; Noureddini and Zhu, 1997). Generally, transesterification of triglycerides involves cleaving the ester bonds between glycerol (a triol) and fatty acids by another alcohol, in the presence of a catalyst, in three steps (Fig. 13.1). Each step involves an intermediate activated by the catalyst used. For example, in the first step of triglyceride being transesterified into diglyceride, the carbonyl carbon atom of the triglyceride molecule is attached by the methoxide ion to form a tetrahedral intermediate. The intermediate reacts with an alcohol (methanol) to regenerate the methoxide ion in the second step, resulting in the formation of a fatty acid ester and a diglyceride (Eq. 13.1a).

Generally, the base-catalyzed transesterification proceeds faster than the acid-catalyzed reaction. However, the use of base catalysts is limited only for oils with low FFA levels, typically less than approximately 3%wt of FFA (He, 2019). Therefore, base catalysts are not suitable for transesterifying high FFA feedstocks, such as microalgal lipids. The major issue is the soap formation between the base and FFA. Soap formation is highly undesirable because it not only can drastically reduce the yield and inhibit the subsequent purification of biodiesel, but also is highly likely to emulsify the reaction mixture and make biodiesel separation from the by-product glycerol impossible (Kulkarni and Dalai, 2006). Therefore, acid-catalyzed transesterification is typically used for feedstocks with high FFA content, like the case of microalgal lipids, prior to the base-catalyzed transesterification of triglycerides.

To date, the most commonly used acid catalysts are sulfuric acid (H_2SO_4) and hydrochloric acid (HCl). As an significant advantage, strong acids can catalyze both esterification of FFAs and

$$\text{Triglycerides} + \text{ROH} \rightleftharpoons \text{Diglycerides} + \text{RCOOR}_1 \quad (13.1a)$$

$$\text{Diglycerides} + \text{ROH} \rightleftharpoons \text{Monoglycerides} + \text{RCOOR}_2 \quad (13.1b)$$

$$\text{Monoglyceride} + \text{ROH} \rightleftharpoons \text{Glycerol} + \text{RCOOR}_3 \quad (13.1c)$$

Fig. 13.1
Transesterification of triglycerides in stepwise reactions.

transesterification of triglycerides simultaneously (Jacobson et al., 2008), and are more efficient when the FFA level exceeds 1%wt (Zhang et al., 2003). However, an acid-catalyzed system is not always a good choice for commercial applications due to requirements of a longer reaction time, a higher reaction temperature, a high molar ratio of alcohol to oil, and serious environmental and corrosion-related problems (Jacobson et al., 2008; Wang et al., 2006).

Because both base-catalyzed and acid-catalyzed systems have their drawbacks and limitations, heterogeneous catalysts are proposed to resolve the conflict. Similar to homogeneous catalysis, heterogeneous catalysis is also subcategorized into base catalysts and acid catalysts. Heterogeneous base catalysts have the advantages of ease in separating from the reaction mixture, a high possibility to reuse, and ease in incorporation into continuous-flow systems (Lam et al., 2010; Lotero et al., 2005). Unfortunately, heterogeneous base catalysts also have their drawbacks, such as frequent catalyst poisoning when exposed to air, leaching of catalyst active sites, sensitivity to FFA content, and decreased yield of FAMEs when soap is formed (Chisti, 2007; Kouzu et al., 2008). In application to biodiesel production from microalgal lipids, a sulfonated Amberlyst-15 resin catalyst was claimed to be promising (Fu et al., 2013).

Lipase-catalyzed transesterification has drawn researchers' attention in the last decade to overcome the downstream processing problems posed by base/acid-catalyzed transesterification. Enzymatic catalysis has the advantages of less or zero by-products, easy recovery of products, mild reaction conditions, insensitivity to high FFA, catalyst reusability, and eco-friendliness (Kulkarni and Dalai, 2006), thus appearing to be a promising alternative. Meanwhile, there are still constraints especially when implemented in industrial scales, including the high cost of enzymes, enzyme deactivation, and a slow reaction rate (Bajaj et al., 2010). For example, the enzymatic lipase *Candida antarctica* could be used for more than 50 cycles (Shimada et al., 1999). Another example is that high FAME yields of 98%, 91%, and 97% were obtained when three lipases of *Candia* sp., *Penicilium expansum*, and *Burkholeria* sp., respectively, were used in the methanolysis of microalgal lipids from *Chlorella* (Hama and Kondo, 2013), showing the positive potential of enzymatic transesterification.

13.4.2 Supercritical alcohol transesterification for algal biodiesel production

Although catalysts play a vital role in reducing the time for transesterification, their presence promotes complications of final products, resulting in increased process cost and a hazard to the environment. Therefore, to avert the drawbacks of catalyzed transesterification, in situ transesterification without external catalyst supply is considered and investigated. In situ transesterification originally refers to a biodiesel production process from crushed oilseeds, with or without catalyst application. It was first demonstrated with sunflower seeds, resulting in

a 20% higher biodiesel yield than that from conventional methods (Harrington and D'Arcy-Evans, 1985a,b). The most superior advantage of in situ transesterification is the ability to produce biodiesel from oil seeds without oil extraction first. This concept of in situ transesterification was also applied to producing biodiesel directly from dry or wet microalgae without the necessity of extracting the lipids first so that the limitations of catalytic transesterification, including sensitivity to high water and FFA contents, can be overcome by noncatalytic transesterification (Pinnarat and Savage, 2008; Warabi et al., 2004).

Transesterification in supercritical fluids has several notable advantages over the conventional processes. In the noncatalytic transesterification of TAGs via SCF, the problems of high contents of FFA and water, and insolubility of oils in alcohol, do not exist anymore (Tan et al., 2010). In the supercritical state, the density of substance fluid is generally in a range between 20% and 50% of that in the liquid state, and the viscosity is close to that in the gaseous state (Wen et al., 2009), which leads the molecules in the supercritical fluids to have high kinetic energy like a gas and high density like a liquid. Therefore, the chemical reactivity can be enhanced in this state. At the critical temperature—up to 400°C depending on the alcohol used—the FFA in the lipids performs a very effective catalyst role. A high yield of FAMEs can be obtained in the absence of an externally supplied catalyst (Bi and He, 2016). When supercritical methanol (SCM; critical temperature of 240°C) is used, the reaction time can be cut to 20 min, compared to 60 min or more in conventional catalyzed conversion of palm oil to biodiesel (Tan and Lee, 2011). Under supercritical ethanol (SCE; critical temperature of 243°C) without catalyst application, a nearly complete conversion was achieved in only 2–4 min (Madras et al., 2004), which agrees with similar findings by others (Kusdiana and Saka, 2001).

Without base catalyst application, soap formation due to the FFAs reacting with a catalyst, a major concern in the base-catalyzed process, is no longer a concern (Chisti, 2007; Pinnarat and Savage, 2008). The noncatalytic SCF process also has the environmental advantage of generating no waste because it does not need the downstream processing of the impurities caused by the catalyst, such as minerals and soap, in the final product.

SCF transesterification can also work on wet microalgae biomass, which overcome the barrier of microalgae cell walls and directly reacts with microalgal oils generating microalgal biodiesel. In the first step, wet algal biomass reacts in subcritical water to hydrolyze intracellular lipids, conglomerate cells into an easily filterable solid that retains the lipids, and produce a sterile, nutrient-rich aqueous phase. In the second step, the wet fatty acid-rich solids undergo supercritical transesterification with ethanol to produce biodiesel in the form of fatty acid ethyl esters (FAEE) (Levine et al., 2013).

Evidentially, SCF transesterification also has many disadvantages. Operated under supercritical conditions, SCF transesterification requires higher operating temperature, thus higher energy input are needed, which require waste energy recovery and reuse to justify the energy cost. SCF transesterification involves high operating pressure, which increases the

capital costs for the whole reactor system (Carrapiso and García, 2000; Pinnarat and Savage, 2008). Naturally, there has been a heated debate on the process efficiency in terms of energy utilization and safety due to the elevated temperature (210–350°C) and pressure (7–20 MPa) employed in the SCF processing. Hence, these challenges and issues need to be addressed and properly justified before the SCF technology can be technically and economically feasible in large-scale applications for commercial biodiesel production. Despite the high temperature requirement, the SCM process may still be energy-efficient if considering the total energy required in the drying of wet microalgae, and extraction and conversion of lipids in traditional processes (Kasim et al., 2010; Kusdiana and Saka, 2004). Therefore, SCF transesterification has the advantage over the traditional processes in terms of overall process efficiency and productivity, which are highly desirable in large production facilities.

13.4.3 Process parameters of in situ supercritical transesterification of microalgal lipids

13.4.3.1 Chemical composition of microalgal lipids

Traditional feedstocks for biodiesel production, such as virgin and/or rapeseed and soybean oils, have relatively consistent and uniform fatty acid profiles leading to consistent processing parameters and product quality. Generally, these feedstocks contain low water content, low FFA content, low phospholipids, and fewer impurities.

Microalgae, as a promising biodiesel feedstock, have many superior advantages including high productivity, high lipid content, and potentially industrialized productions. Additionally, the microalgal biomass may be just dewatered to a certain moisture content (e.g., 80%–90%), without drying, and used directly in processing such as in in situ SCF transesterification processes, which greatly reduces the energy input to the system.

Commercial production of microalgae has occurred for more than 30 years as a resource for food, health, and pharmaceutical industries (Borowitzka, 1999). The commonly cultivated species of microalgae are *Chlorella*, *Spirulina*, *Dunaliella salina*, and *Haematococcus pluvialis*. For use as a feedstock for biofuels, microalgae that are capable of producing lipids are of significance. Many classes of lipids can be found in microalgal cells. Based on chemical structures and polarity, they are classified into polar and neutral lipids. The polar lipids include those components in the membrane structure (such as monoglycosyldiacylglycerol, diglycosyldiacylglycerol, trimethyl-beta-alaninediacylglycerol, and sulphaquinovosyldiacylglycerol), and glycolipids (such as monogalacto-sylduacylglycerol and digalactosyldiacylglycerol). The neutral lipids are triacylglycerols (TAGs, also called triglycerides), waxes, and isoprenoid-type lipids (Gong and Jiang, 2011). Among these lipids, only TAGs are easily transesterified into fatty acid esters (or biodiesel) by traditional methods. Depending on the strains, cultivation conditions, ages at harvest, etc., microalgal lipids may contain largely varied fatty acid profiles. Microalgal lipids are often composed of both extremes, namely high content of saturated fatty acids and high content of polyunsaturated fatty acids with numerous algal

species enriched in both saturated and polyunsaturated fatty acids, whereas common fatty acids found in vegetable oil are palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids (Knothe, 2011b). Overall, palmitic acid appears to be the one most commonly occurring in the fatty acid profile of microalgal oils. Myristic acid (C14:0) is also very common, and possibly the second most common saturated fatty acid in microalgal lipids. The content of palmitic acid and/or other saturated fatty acids in many cases is comparable to or greater than that of many vegetable oils. Generally, the C18 fatty acids are less prominently represented in microalgal oils than in vegetable oils. The polyunsaturated fatty acids in microalgal oils vary, with C16:4, C18:3, C18:4, C20:5, C22:5, and C22:6, but other polyunsaturated fatty acids also occur, influencing oxidative stability.

The chain length of fatty acids can affect biodiesel quality. Longer chain length of fatty acids results in biodiesel with a higher cetane number and reduced nitrogen oxide (NOx) emissions in engine exhausts (Knothe, 2009, 2011a). The oxidative stability of fish and algal oils with high amounts of polyunsaturated fatty acids is reduced significantly when natural antioxidants and carotenoids are removed (Frankel et al., 2002), which is very likely when producing biodiesel. In any case, with the emphasis on saturated fatty acids and polyunsaturated fatty acids in their profiles, many algal biodiesel fuels would likely exhibit poor cold flow and poor oxidative stability simultaneously. This is the result of saturated fatty esters affecting cold flow regardless of the nature of the unsaturated fatty esters present and the fact that unsaturated fatty compounds also affect oxidative stability more significantly than their amounts may indicate, as discussed above (Knothe, 2011b).

The lipid content varies between 20% and 50% in the commonly seen species belonging to the genera of *Porphyridium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Tetraselmis*, *Phaeodatylum*, *Chlorella*, and *Schizochytrium* (Bellou et al., 2014). To meet the expectation of high lipid yields, the high lipid content in microalgae can be achieved by varying the culture conditions, such as temperature, light irradiance, and most importantly nutrient availability. Microalgae belonging to different classes may have a particular fatty acid profile that is generally recognized as group specific. Diatoms are able to synthesize high amounts of palmitoleic acid (C16:0) and C20:5, whereas some mutants may also produce arachidonic acid (C20:4) (Schneider et al., 1995; Sriharan et al., 1991). Strains of golden microalgae *Chrysophyceae* produce a wide variety of fatty acids including palmitoleic (C16:1), eicosapentaenoic (C20:5), and docosahexaenoic (C22:6) acids. Green microalgae, *Chlorophyceae* sp., possess an active acyl-CoA desaturase system which producing C18 fatty acids (Regnault et al., 1995) and other long chain polyunsaturated fatty acids, such as α-linolenic (C18:3), eicosapentaenoic (C20:5), and docosahexaenoic (C22:6) acids (Bellou et al., 2014; Huang et al., 2013; Makri et al., 2011; Patil et al., 2007; Reitan et al., 1994).

A major issue in large-scale microalgal biodiesel production is the high levels of FFAs and phospholipids contents. FFA content can vary from 1% to 92% of the total lipids, depending on the strain, cultivation condition, and extraction method (Sarpal et al., 2016; Yao et al., 2015).

These free fatty acids are undesirable in the biodiesel process as they strongly influence the processing and the quality of the product. To convert algal lipids with high FFA content, acid catalyzed esterification was recommended first to convert FFA before alkaline catalytic transesterification. In addition, the SCF transesterification process is also capable of converting microalgal lipids with high FFA content to biodiesel without catalyst assistance (Alenezi et al., 2010).

13.4.3.2 Processing parameters

Temperature and pressure are the most important parameters for SCF transesterification. Research has shown that the conversion rate was only 50% at 177°C (subcritical methanol) in converting vegetable oils, but increased to over 95% at 250°C (supercritical methanol) (Demirbas, 2005). The same conversion rate elevation from 50% to 95% was also found in processing coconut oil, but the temperature was much higher, from 270°C to 350°C (Demirbas, 2005). Apart from temperature and pressure (which are essential to the successful conversion of algal lipids to biodiesel by supercritical transesterification), alcohol type, alcohol to biomass ratio, and co-solvent are also critical to SCF transesterification. SCF transesterification can work on both dry and wet microalgae biomass for biodiesel production. Jazzar et al. (2015) studied direct biodiesel synthesis with wet (~80 wt% moisture) and dry unwashed marine microalgae *Nannochloropsis gaditana* through SCF transesterification. The maximum biodiesel yields of 46% and 48% were reached from wet and dry unwashed algal biomass, respectively, at 255–265°C, 50 min reaction time, and using a methanol to dry algae ratio of 10:1 (vol./wt.) (Jazzar et al., 2015).

In situ transesterification or direct transesterification is another term that emphasizes a transesterification conversion process without oil extraction. Combined with supercritical fluid, in situ SCF transesterification can overcome the immiscibility and physical obstacle between microalgal lipids and alcohol. In their study, using a two-step process of hydrolysis and supercritical in situ transesterification, Levine et al. first hydrolyzed *Chlorella protothecoides* (UTEX #255) biomass and then transesterified microalgal lipids in supercritical ethanol to achieved a FAEE yield of 79% with a 5:1 ethanol:FA molar ratio in 150 min and a 89% FAEE yield with a 20:1 ethanol:FA molar ratio in 180 min at 275°C (Levine et al., 2013). A substantial thermal decomposition of unsaturated FAEE was observed when the reaction temperature was above 275°C. About 25%–50% of lipids were lost in the thermal carbonization, and the remaining portion of lipids could be converted completely in an in situ SCF transesterification stage. Noncatalytic in situ SCF transesterification has also been extensively studied for microalgal biodiesel production. Bi et al. (2015) proposed an in situ SCF transesterification process that achieved a FAME yield of 62.7 mol% at 250°C for 30 min with 1:75 lipid-to-methanol molar ratio wherein the microalgae biomass contain 55%wt of lipid and 16.6%wt of FFAs. They revealed that under supercritical state, FFA is an organic acid that can be considered as an acid catalyst. Especially when methanol was heated up to a higher

temperature (i.e. 250°C), the chemical polarity of methanol is enhanced, the chemical activity is boosted, and the acidity of FFA is increased. Therefore, the presence of FFA in the system of TAGs and methanol for the transesterification reaction could promote the overall reaction yield (Bi and He, 2016). They also revealed that the major caveat of in situ SCF transesterification on biomass is the lack of selectivity of conversion pathway, which means both lipids transesterification and carbohydrate hydrolysis, and carbonization and polymerization reactions are executed at the same time. This leads to an unselected product mixture with low FAME yield. Fortunately, these scientists found out, by controlling temperature and pressure, that the subcritical methanol state has achieved a higher FAME yield (64.5 mol%) with a purity of 33.1 wt% at 210°C, 120 min with lipid-to-methanol ratio of 1:75 (Bi et al., 2015).

To explain the effect of sub/supercritical fluids, Bi et al. (2015) examined the morphology changes of microalgae biomass pre and post in situ transesterification at various temperatures. Before processing, the microalgal cell structure was intact with visible pores on the surface. As the processing temperature increased from 170°C, the cell structure gradually collapsed, the pore sizes enlarged, and the spherical structure broke down. The particle size gradually decreased as the reaction temperature increased. When the temperature reached 210°C, the spherical structure began to deform, and a greater degree of destruction was obvious. At 290°C, the cell structure had been completely destroyed, the microalgal biomass no longer held its spherical structure, and it disintegrated into smaller pieces. This observation suggests that methanol at elevated temperatures is able not only to penetrate the cell wall and reach lipids, but also to break the cell structure, which leads to the stage of biomass liquefaction. Under extreme thermal conditions, therefore, methanol properties are enhanced to such a level that the biomass cell structure can physically collapse, and the lignocellulosic matters can thermally decompose. It is highly likely that the decomposed intermediates will react with each other and/or with methanol in a complicated manner.

Alcohol provides the alkyl group that substitutes the fatty fraction of TAGs. The selection of the alcohol is based on cost and performance consideration. The fuel qualities of alkyl esters have received varying evaluations in terms of alcohol used. Huber et al. (2006) and Saraf and Thomas (2007) commented that higher or branched alcohols can produce better fuel characteristics in biodiesel. In contrast, a report from the National Renewable Energy Laboratory states that methyl ester and ethyl ester are similar in heat content, but ethyl ester formed by transesterification reaction is slightly less viscous than methyl ester (Tyson and McCormick, 2006).

Stoichiometrically, transesterification of TAG requires three molecules of methanol. However, since the transesterification process is reversible, an enormous amount of methanol is needed to achieve a complete conversion. The effect of oil-to-methanol molar ratio was first studied by Kusdiana and Saka (2001) in the range of 1:3.5–1:42. It was reported that at the lower methanol molar ratio (6 or less), incomplete conversion to biodiesel was observed,

resulting in lower yield of biodiesel. However, at a higher methanol molar ratio of 1:42, a complete conversion was apparent with FAME yield of 95%.

A co-solvent has been proposed to reduce the severity of conditions needed for supercritical biodiesel production in methanol. The addition of a co-solvent can increase the mutual solubility between TAGs and methanol. Consequently, biodiesel can be produced under milder conditions. Co-solvents that have been considered include propane, carbon dioxide, ethane, *n*-butane, *n*-hexane, *n*-heptane, and tetrahydrofuran.

13.4.3.3 Effect of water and FFAs in feedstock

Microalgal lipids have a characteristic high level of FFAs. Fresh harvested microalgal biomass also contains a large amount of water—up to 98%. The presence of high water and FFAs in the reaction system causes saponification (Chisti, 2007), and leads to lower yield of methyl ester (Nakpong and Wootthikanokkhan, 2010) and increased difficulty in product separation (Chai et al., 2014) in traditional base-catalyzed transesterification process. However, it is not a problem at all when using in situ SCF transesterification.

SCF transesterification of TAGs has a great strength in converting wet microalgae in situ and high FFA content is not an issue, but rather a natural catalyst under SCF conditions (Bi and He, 2016). One obstacle of traditional transesterification of TAGs is the immiscibility of alcohol with oil/fat in the initial reaction stage. Under supercritical fluid conditions, alcohol and TAGs form a homogeneous mixture, leading to a much faster reaction due to the fact that alcohol dielectric constant decreases at the supercritical state (Gonzalez et al., 2013; Tan and Lee, 2011; Trentin et al., 2011). Studies demonstrated that the water in microalgae serves as an active co-solvent along with supercritical alcohol, which helps increase the lipid solubility in alcohol and acidity of the reaction mixture (Patil et al., 2011). Additionally, FFAs are organic acids. Under the supercritical methanol status, the acidity of FFAs is increased dramatically. Therefore, the presence of FFAs promotes the overall reaction rate by performing the catalytic role in the SCF system.

13.5 Summary and perspectives

Microalgae are a sustainable resource for biodiesel production with the advantages of high productivity, noncompetitiveness with agricultural lands, industrialized setting for production, and a great potential for CO_2 fixation. Biodiesel production from microalgae can provide not only a sustainable resource for biofuel production, but also opportunities for business development and job creation. Most importantly, utilization of biodiesel, the only advanced biofuel that is commercialized in the United States, can greatly reduce the consumption of fossil fuel diesel and thus the emissions of greenhouse gases and other pollutants, improve air quality and thus our society's well-being, and pave a solid foundation for sustainable development of technologies, economics, and environment protection for the next generations.

In the past decades, scientists, engineers, and industrial entrepreneurs have made tremendous effort in developing a microalgae-based biodiesel industry, from microalgal biology to engineering development of processing technologies. Scientists have screened and identified multiple strains of microalgae that are high in lipid content and suitable for large-scale cultivation. Cultivation methods, i.e., open-pond systems vs enclosed photobioreactor (PBR) systems, were explored exclusively to identify their pros and cons in adopting various strains of microalgae, parameters of cultivation, adaptation of location and climate conditions, availability of solar radiation and water supply, and last but not least, the production cost. R&D activities have been conducted in laboratory, pilot, and precommercial production scales on various processing aspects, including means of cultivation, nutrient supply, crop protection, harvesting, dewatering/drying, lipid extraction, conversion techniques, techno-economic assessment, and feasibility of commercialization. It is generally agreed that although there is still room for the technologies to improve, biodiesel production using microalgae as the feedstock is technologically feasible.

On its way to commercialization, the production and utilization of microalgal biodiesel still face challenges, as discussed from the following aspects.

Constant and adequate supply of carbon dioxide (CO_2) is essential for microalgae cultivation. The maximum efficiency of photosynthesis in plants utilizing a C3 photosynthesis pathway growing in the normal atmosphere was estimated to be 4.6% (Zhu et al., 2008). Microalgae growing in a carbon dioxide supplemented culture outdoors can achieve a possible maximum of 8.3% for wildtype species (Chisti, 2013a). This leads to a requirement of at least 1.83 tons of CO_2 for each ton of microalgal biomass (Chisti, 2007). Nearly all pilot-scale microalgal cultivation facilities have shown that nearly 50% of the total cost for producing the biomass comes from CO_2. Concentrated sources of CO_2 are mainly the flue gases in power plants. This implies that the microalgae cultivation facilities must be colocated with the power plants and/or dedicated pipelines with regulation mechanism must be constructed, inducing tremendous upfront capital investment. Meanwhile, the CO_2 from power plants contains harmful impurities and needs additional effort in cleaning and detoxification, adding to the operating cost.

Sufficient nutrient supply of nutrients, especially nitrogen and phosphorous, is essential for microalgae to grow healthily and productively, and is another major contributor to the overall production cost. Although nitrogen can be fixed from air by biological fixation of cyanobacteria and other bacteria (Bergman et al., 1997; Brill, 1980; Zehr, 2011), the rate of nitrogen fixation is by no mean adequate for microalgal growth. Attempts are now being made to engineer a nitrogen fixing capability in some nonlegume crops (Santi et al., 2013). However, the practical application in microalgae cultivation still has a long way to go. The phosphorous supply can be enhanced through recovery from animal fecal matters that are rich in phosphorous, but there are serious concerns of potential contamination from such a phosphorus resource.

Water supply, either wastewater or seawater, is often the very first consideration for microalgal cultivation, especially in open-pond/raceway systems in which water loss due to evaporation is

significant. Seawater, which is plentiful on our planet, can be used for microalgal cultivation. Unfortunately, using seawater does not eliminate the need of freshwater to compensate for evaporative losses in seawater cultivation. Freshwater is also needed for washing the salt off the microalgal biomass prior to lipid extraction and/or conversion. Therefore, closed production facilities with water recycling strategies are preferred in the case of limited water supply (Pate et al., 2011).

Limitations of the algae biomass production technology need to be overcome. Production of microalgal biodiesel requires a vast quantity of microalgal biomass. The most commonly used raceway ponds in microalgal biomass cultivation have exceedingly low productivity compared to what microalgal biology allows. A practical upper limit to microalgal oil productivity of conventional raceways is about 37,000 L/ha/year (Chisti, 2013b). Photobioreactors are more productive and can achieve a much higher concentration of microalgal biomass (e.g., 5 kg/m^3) than raceways (e.g., 0.5–1.5 kg/m^3), but are more expensive and require a higher level of energy to operate (Carvalho et al., 2006; Molina Grima et al., 2003; Wang et al., 2012), which is of course unacceptable for producing biodiesel, a bulk commodity product (Sompech et al., 2012; Wongluang et al., 2013).

Light source and supply also have some major technical hurdles to overcome. Sunlight is an inexpensive and sustainable source of energy for microalgae to grow. Novel biomass production facilities rely on sunlight to achieve high productivity in the media. Given a sufficiency of all nutrients, a high density of microalgal biomass is attainable only at high light intensities and only within a shallow depth (Zijffers et al., 2010). Therefore, technologies of solar light collection and supply to a deeper culture of microalgae cultivators, especially PBR systems, are critical for improving the cultivation efficiency and productivity.

Cost of production is the ultimate hurdle that waiting to overcome. Despite its advantages, microalgal biodiesel can never be a reality if its price is uncompetitive with fossil diesel or unaffordable for consumers. It is suggested that a microalgal biomass with a 40% lipid content must be produced at a cost of no more than \$0.25/kg to compete with petroleum at the current price of \$629/m^3 (Chisti, 2013a).

All existing technologies for large microalgal biomass production are quite expensive (Richardson et al., 2012; Williams and Laurens, 2010). The estimates of cost of production of microalgal biodiesel have ranged from \$1.68 to \$75/L (Nagarajan et al., 2013), or in the range of \$0.42–0.97/L in a raceway pond system (Haas et al., 2006). Compared to the cost of \$0.53/L in conventional biodiesel production using soybean oil, the feedstock cost is estimated as \$0.52/kg, indicating that the current production cost of microalgal biodiesel is far too high to be economically viable.

It is likely that technologies will advance further to make the commercial production of biodiesel from microalgae a reality in the next decade or so. The industry and the research community have agreed upon the new strategy of combining the production of microalgal biodiesel with value-added coproducts from microalgae. Further effort of research and

development activities in biology and engineering will collectively improve the efficiencies of energy and productivity and thus reduce the overall cost to such a level that microalgal biodiesel is as affordable a fuel as fossil diesel.

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CHAPTER 14

Bioethanol production from microalgae

Didem Özçimen, Anıl Tevfik Koçer, Benan İnan, Tugba Özer

Yıldız Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul, Turkey

Chapter outline

14.1 Introduction 373
14.2 Microalgae 374
14.3 Bioethanol 376
14.4 Bioethanol production from microalgal biomass 379
14.4.1 Microalgae cultivation 379
14.4.2 Harvesting and drying 381
14.4.3 Pretreatments and saccharification 383
14.4.4 Fermentation and distillation 384
14.5 Conclusion 385
References 386

14.1 Introduction

Nowadays, fossil-based production is being replaced with a more sustainable production based on biomass due to political issues, reviving countries' economies, employment creation, and global warming. In many fields, industrial materials made from fossil resources are being substituted by bio-based products. Production of bioethanol, which is a bio-based product, was about 16.1 and 7.9 billion gallons in the USA and Brazil, respectively, in 2018 (Sydney et al., 2019).

Bioethanol is an environmentally friendly biofuel that has similar properties to gasoline and can be obtained from biomass types that contain high amounts of cellulose and starch via a fermentation process. Today, bioethanol production is carried out from first-generation raw materials such as wheat, sugar beet, and corn, and second-generation bioethanol raw materials such as lignocellulosic forest residues. Although high yields can be obtained from first-generation raw materials, there is a debate because they are also food sources (Koçer et al., 2019). In order for second-generation raw materials in bioethanol production to represent an alternative to first-generation ones, efficient and expensive pretreatments are required. Therefore, recent

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technological developments and alternative raw materials are being investigated in order to facilitate the process. Initially, algae, which represent a third-generation biofuel raw material, were only evaluated for biodiesel production; however, they have recently been used for producing bioethanol due to their cellulosic structure (Özçimen and İnan, 2015).

Microalgae are prokaryotic (lacking a cell membrane and nucleus, blue-green algae) or eukaryotic (cell membrane, one or several nucleated, green and red algae) microorganisms that can grow rapidly and live under severe conditions because of their single-cell or simple multi-cell structure. Microalgae, having a size range between a few microns and 100 μm, can grow in any places where water exists (Mata et al., 2010). Microalgae have high content of lipids and carbohydrates so that they can be used as raw materials in industrial applications including food, cosmetics, pharmaceutics, and biofuels. Microalgae can be used for both biodiesel and bioethanol production with the biorefinery approach, which has become common in recent years (Rashid et al., 2019).

In this chapter, applicability of bioethanol production from microalgae is investigated and bioethanol yield is compared with other bioethanol feedstocks. Pretreatment methods for microalgae are identified, and the advantages and disadvantages of the process are discussed. In addition, the potential of microalgal bioethanol production is assessed, from microalgae production to the fermentation process.

14.2 Microalgae

Microalgae, which are simple microorganisms, contain chlorophyll and are responsible for more than 50% of the world's oxygen production. Microalgae have the ability to grow autotrophically or heterotrophically, and can survive in various and extreme conditions such as freshwater, saline water, high pressure, and high temperature (Mata et al., 2010; Özçimen et al., 2015). There are several micron-sized species of microalgae as well as 100 μm-sized species. It has been estimated that there are more than 50,000 species of microalgae, but the biochemistry and ecophysiology of only very few species have been discovered (Özçimen et al., 2018). Since microalgae can grow quickly and easily in different environments and conditions, microalgae growth medium varies according to the type of microalgae and desired product (Özçimen et al., 2012, 2016). For example, in order to produce biodiesel, algae species with high lipid content should be selected. In addition, the environmental conditions need to be optimized to increase the microalgae lipid content. Moreover, stress factors should be applied by lowering the temperature and boosting/limiting the nitrogen concentration in the medium (Markou and Nerantzis, 2013). This idea is the same for bioethanol production. Furthermore, genetically modified microalgae with increased carbohydrate content can be used for bioethanol production (Radakovits et al., 2010).

Two systems—open and closed systems—are used in microalgae cultivation. Open systems, which are extensively chosen in industrial applications, can be classified as natural systems such as lakes and lagoons or artificial systems such as raceway ponds and circular ponds, and are the simplest and oldest systems for algae production. Since open systems have low investment and operating costs, they are preferred in the industry. However, the system has disadvantages due to the difficulty of controlling the production conditions and the high risk of contamination. Open pools are one of the cheapest systems used in large-scale production (Gouveia, 2011). The other system used in microalgae cultivation is closed system photobioreactors. Photobioreactors have many advantages compared to open pools, such as the continuous control of the ambient conditions, minimum risk of contamination, occupying less space, being robust to environmental conditions, and obtaining higher yields of biomass (Pulz, 2001).

Microalgae contain crucial components having high commercial potential, but it is difficult to commercialize their production due to various problems and lack of knowledge. Under ordinary conditions, these valuable components may not be synthesized by microalgae or may be synthesized in very limited amounts. However, these products can be produced by microalgae under stress conditions such as high or low temperature, high salinity, deprivation of nutrients such as nitrogen or phosphate, and short-term ultraviolet exposure (Khan et al., 2018). Chemical composition of microalgae can change according to the microalgae species, cultivation type, and cultivation conditions (Özçimen and İnan, 2015). For example, high temperature, high nitrogen, and high phosphate values increase biomass production, while low

Table 14.1: The biochemical contents of some microalgae species.

Carbohydrates (%)		Lipid (%)	Protein (%)	References
Chlorella vulgaris	25.30	19.61	33.64	Koçer et al. (2019)
Botryococcus braunii	58.34	20.3	16.35	Blifernez-Klassen et al. (2018)
Tetraselmis sp.	14.5	9.4	21.7	Arkronrat et al. (2016)
Dunaliella salina	85.58	11.47	8.46	Pirwitz et al. (2016)
Arthrospira platensis	14.2	8.8	32.1	Markou et al. (2015)
Chlamydomonas reinhardtii	22.60	12.60	64.70	Mahdy et al. (2016)
Scenedesmus obliquus	13.41	30.38	4.66	Chen et al. (2014)
Spirulina platensis	30.21	48.36	13.30	Jena et al. (2011)
Dunaliella tertiolecta	21.69	61.32	2.87	Shuping et al. (2010)
Microcystis aeruginosa	11.60	12.50	30.80	Miao et al. (2004)

light and low salinity values increase biomass production (Sun et al., 2018). The biochemical contents of some microalgae species are presented in Table 14.1.

Recently, use of microalgae as a source of lipid and carotenoid has attracted considerable attention. Microalgae have become recognized as suitable sources for biodiesel production since the lipid content of microalgae is between 20% and 50% of dry weight, and this can be increased by changing cultivation conditions and genetic modifications. Microalgal lipids can be used in different fields according to their carbon numbers. While fatty acids with 14–20 carbons are generally used for biodiesel production, unsaturated fatty acids with more than 20 carbons are used as health food supplements (Sun et al., 2018). The lipid or carotenoid content of microalgae can be enhanced with high density of light and nutrient limitation (Singh et al., 2016). As can be seen in Table 14.1, some microalgae species can store enormous amounts of carbohydrates in the cell and cell wall (Chen et al., 2013). These microalgae species are suitable raw materials for bioethanol production. Microalgal carbohydrates are produced by carbon fixation metabolism during photosynthesis. These carbohydrates are accumulated in the cell as storage material or stored in the cell wall. The carbon content of microalgae can be improved by applying cultivation strategies such as nutrient depletion, irradiance, temperature variation, and CO_2 supplement. Carvalho et al. (2009) and Fernandes et al. (2010) reported that carbohydrate accumulation in microalgae is affected by increased light intensity. Ho et al. (2011) suggested that nitrogen limitation is an effective method for increasing the accumulation of carbohydrates in microalgae. In the literature, it is observed that a single parameter does not necessarily have a direct effect on the carbohydrate content. Many researchers agree that carbohydrate accumulation can increase with the effect of multiple environmental factors at the same time (Chen et al., 2013).

14.3 Bioethanol

Ethanol (CH_3–CH_2–OH or EtOH) is produced from ethylene chemically or via fermentation from various biomass sources. Ethanol, which is a colorless clear liquid, is used as a solvent in medicine, cosmetics, cleaning supplies, and chemistry, and as a fuel in vehicles with the synthesis of different organic chemicals (Bruhn et al., 2011). Bioethanol produced from biomass is an alternative to that produced from fossil fuels due to being bio-based and renewable. It can be used directly or mixed with gasoline in the engine. Bioethanol has a high octane number and combustion rate, meaning that it has a higher evaporation temperature than gasoline. It is added to gasoline to raise the octane number. Bioethanol has advantages such as high compression ratio, short combustion time, and oil-free combustion, which provides superiority to gasoline in terms of theoretical efficiency (Agarwal, 2007). It has physical and chemical features as follows: specific gravity of 0.79 kg/dm^3, vapor pressure of 50 mm Hg, boiling temperature of 78.5°C, and a molecular weight of 46.1 g/mol. It has only 66% of energy

compared to the same amount of gasoline. Bioethanol can be used in mixtures called E-10 containing 10% bioethanol-90% gasoline, or E-85 containing 85% bioethanol-15% gasoline (Koçtürk and Avcıoğlu, 2013).

The production of bioethanol provides employment for people living in agricultural areas (Balat et al., 2008). The emissions of sulfur oxides (SOx), which are carcinogens and one of the main components of acid rain, can be reduced by mixing ethanol with gasoline (Nigam and Singh, 2011). The disadvantages of bioethanol are that it has less energy density than gasoline, a corrosive effect, low flame brightness, low vapor pressure, and water miscibility. While oxygen in bioethanol increases combustion, it reduces emissions of carbon monoxide, hydrocarbon, and other particles. However, these fuels tend to increase nitrogen oxide emissions (Agarwal, 2007). The combustion of ethanol-gasoline mixtures containing 85% bioethanol obtained from corn results in increasing the emission of five main pollutants (CO, VOC (volatile organic compound), PM10, Sox, and NOx) (Balat, 2011). In addition, ethanol production from some biomass such as corn causes soil erosion and requires more nitrogen fertilizer for production than other raw materials. These problems can also be seen in the production of bioethanol from sugarcane (Koçtürk and Avcıoğlu, 2013).

Bioethanol is produced from biomass with high sugar content. These raw materials are classified as first-generation, second-generation, and third-generation biomass sources. First-generation biomass such as wheat, barley, raw beet, and sugarcane includes high content of glucose and starch. Bioethanol can be produced with high efficiency and low cost from these raw materials. Twenty-five gallons of bioethanol can be obtained from one ton of sugar beet (Sarkar et al., 2012). However, the use of these biomasses directly for energy generation instead of food application leads to very serious ethical debates. For this reason, researchers tend to utilize waste biomass with lignocellulosic sugar content including agricultural waste, grass, and forest and wood residues, known as second-generation biomass (Koçer et al., 2019; Terzioğlu et al., 2013). Lignocellulosic materials are composed of cellulose, hemicellulose, and lignin. The use of such biomasses in bioethanol production has prevented many ethical debates. On the other hand, the algal biomass known as third-generation biomass is becoming popular due to the low yield of second-generation biomass and the costs of pretreatments in the production process (Alam et al., 2015). Bioethanol yields of some kinds of biomass in the literature are given in Table 14.2.

Ethanol production depends on the raw material that is used and consists of three main stages: the hydrolysis of the carbohydrates of the raw material to fermentable sugars (pretreatments); the fermentation of sugars into ethanol; and separation and purification of ethanol, respectively (Lam and Lee, 2015). The most challenging step in bioethanol production processes is the pretreatment step of biomass, in which polysaccharides such as starch and cellulose are broken down to their monomers to make them fermentable. The techniques are classified into four different groups: physical, chemical, biological, and physicochemical pretreatments

Table 14.2: Bioethanol yields of different feedstocks.

Feedstock	Bioethanol yield	References
Rice straw	45%–50%	Abbi et al. (1996)
Sugarcane bagasse	140 mg/L	Boopathy and Dawson (2008)
Corn stover	90%–98%	Mohagheghi et al. (2004)
Switchgrass	39.1%–59.6%	Tao et al. (2011)
Sweet sorghum	42%	Laopaiboon et al. (2007)
Macroalgae	~25%	İnan and Özçimen (2019)
Wheat straw	23% and 29%	Saha and Cotta (2006)
Fruit pulps	35.86%	Arumugam and Manikandan (2011)
Orange peels	~25%	Oberoi et al. (2010)

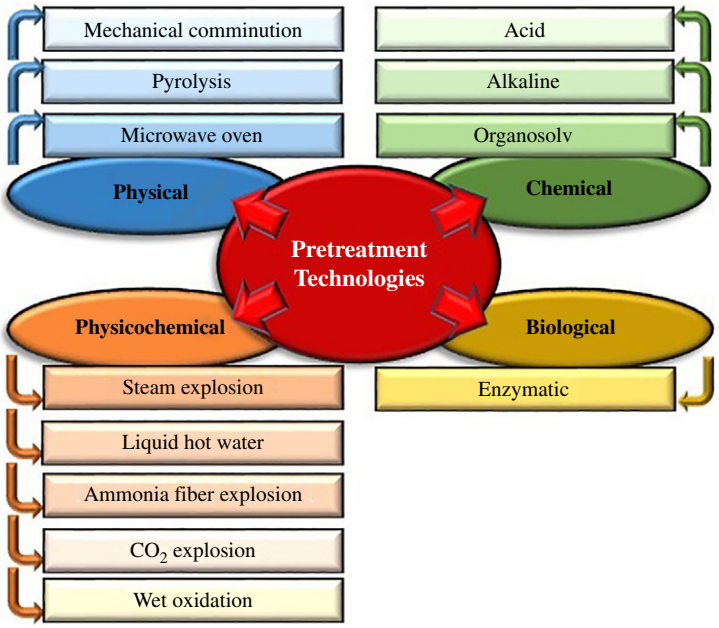

Fig. 14.1
Pretreatment methods for bioethanol production.

(Hill et al., 2006). Currently, these methods are not efficient enough, so, research and development studies are being carried out in order to improve the cost and performance of pretreatment (Sarkar et al., 2012). Pretreatments applied to biomasses are summarized in Fig. 14.1.

Another important step in bioethanol production is the fermentation process, in which 6-carbon sugars like glucose are converted to ethanol by a microorganism such as bacteria or yeast. *Saccharomyces cerevisiae*, which is one of the most commonly used microorganisms in this field, is able to tolerate high levels of bioethanol and inhibitory substances that may result from chemical pretreatments. An additional advantage of this microorganism is that it has high

osmotic resistance, activity at pH levels as low as 4, and high bioethanol production efficiency (Demirbaş, 2004). Another microorganism used in bioethanol production is *Zymomonas mobilis*, which provides fast and high-efficiency bioethanol production. Nevertheless, this microorganism is not able to tolerate the effects of various phenolic compounds released during the process (Doran-Peterson et al., 2008). Bioethanol yield of microorganisms depends on temperature, pH range, alcohol tolerance, osmotic tolerance, resistance to inhibitors, growth rate, and genetic stability (Demirbaş, 2004). The final stage of bioethanol production is the distillation process. Since the bioethanol produced by the fermentation process is actually a mixture consisting of water and ethanol, a distillation process should be applied in order to separate the bioethanol from the mixture (Özçimen and İnan, 2015).

14.4 Bioethanol production from microalgal biomass

Although bioethanol production from microalgae is very promising for preventing environmental problems such as climate change, and for producing biofuel, it still faces challenges such as large-scale, highly efficient production and commercialization.

The important topics for microalgae to become a competitive raw material in bioethanol production are: (i) microalgae selection and high biomass production with high carbohydrate content; (ii) harvesting; (iii) pretreatment; and (iv) an efficient fermentation process (Khan et al., 2018). The development of low-cost and highly efficient microalgal bioethanol production can be achieved by improving and optimizing each of the mentioned topics (Pulz and Gross, 2004).

In this section, all stages of bioethanol production from microalgae are summarized in the flow diagram drawn using the ChemCad design program to visualize the bioprocess (Fig. 14.2). As can be seen in this flow diagram, the production of bioethanol from microalgae consists of five main stages: microalgae cultivation, harvesting, hydrolysis, fermentation, and distillation.

14.4.1 Microalgae cultivation

The amount of microalgal biomass must be able to compete with other raw materials for sustainable bioethanol production. This can be achieved as a result of large-scale production and optimization of conditions. If microalgae production becomes cheaper and abundant, it could be preferable for bioethanol production (Khan et al., 2018). As shown in Fig. 14.2, the bioethanol production process starts with microalgae cultivation in a photobioreactor. For large-scale bioethanol production, open ponds can also be used instead of photobioreactors. After microalgae production takes place, microalgae biomass is transferred to harvesting and drying systems.

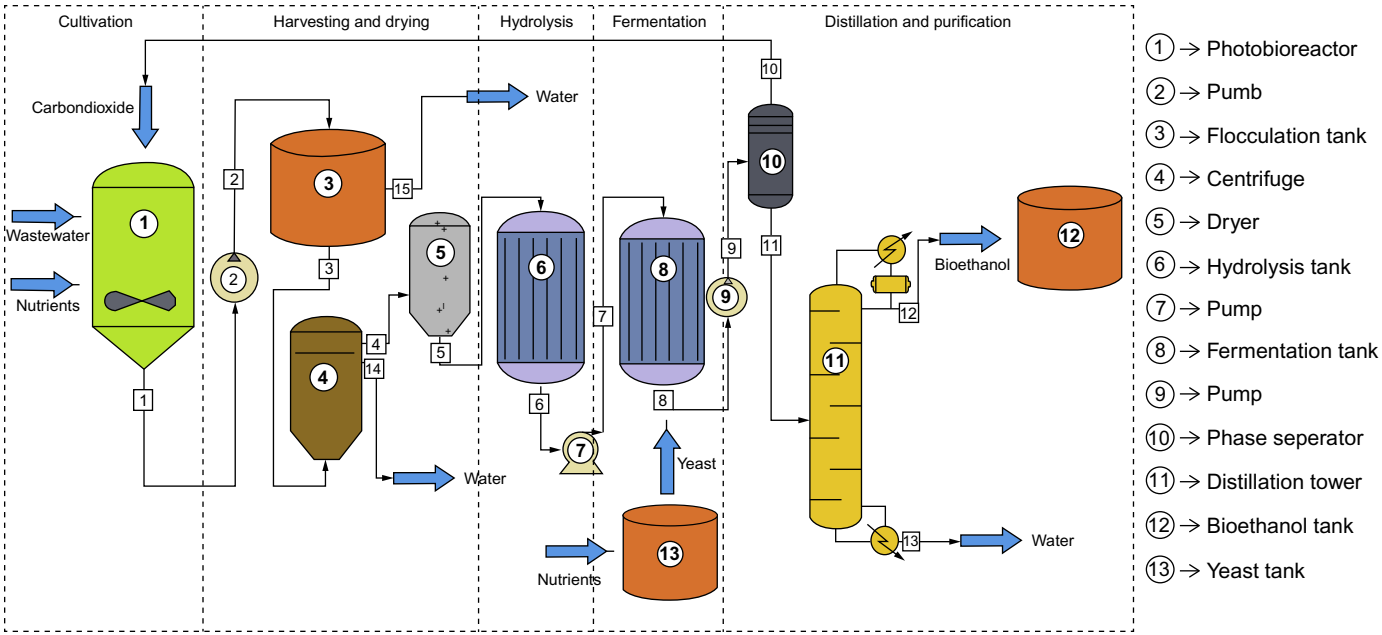

Fig. 14.2
Flow diagram of bioethanol production from microalgae.

It is crucial to choose microalgae species in microalgae production processes depending on the desired end product—for example, choosing *Dunaliella salina* and *Chlorella vulgaris* to obtain β-carotene (Özçimen et al., 2018) and *H. pluviaris* (Thomassen et al., 2016) to produce astaxanthin. While *Chlorella protothecoides* type microalgae with high lipid content can be selected for biodiesel production (Özçimen et al., 2017), microalgae species with high carbohydrate content should be preferred for bioethanol production. It has been reported that *Chlorella*, *Dunaliella*, *Chlamydomonas*, and *Scenedesmus* microalgae generate more than 50% of carbohydrates (Chen et al., 2013). Harun and Danquah (2011) carried out bioethanol production from *Chlorococcum humicola* species microalgae with a yield of 48%. It has been declared by Choi et al. (2010), Scholz et al. (2013), and Nguyen et al. (2009) that bioethanol production is achieved with 23.50%, 44%, and 29.10% yields by *Chlamydomonas reinhardtii* microalgae, respectively.

Microalgae can be grown in different conditions depending on their species. However, these organisms basically need light and CO_2 since they are photosynthetic microorganisms. In addition, they need nitrogen and phosphorus in order to provide growth and cellular activities, which constitute 10%–20% of the algal biomass. In addition, macronutrients such as Na, Ca, K, and Mg and micronutrients such as B, Co, Fe, and Zn are also important nutrient sources for microalgae cultivation (Khan et al., 2018). In this respect, many industrial wastewaters become very convenient sources for microalgae production. With the use of such wastewater in microalgae production, not only will industrial wastewater be recycled but also the cost of nutrients for microalgae production will be reduced (Özçimen et al., 2016).

The microalgae cultivation step for bioethanol production is a critical one because biomass with a high carbohydrate content is necessary for economically feasible bioethanol production. Thus, carbohydrate accumulation in microalgae can be increased by optimizing growth conditions and exposing microalgae to light and temperature stresses. In addition, microalgae can be subjected to genetic modifications to enhance carbohydrate production rates or starch accumulation (Markou and Nerantzis, 2013). Samiee-Zafarghandi et al. (2018) showed that phosphorus limitation significantly elevates carbohydrate accumulation in *Chlorella* sp. It has been revealed that the production of carbohydrates and biomass in microalgae can be enhanced with calcium and magnesium due to their importance for chlorophyll and various enzymes.

14.4.2 Harvesting and drying

Harvesting is an important step in microalgae cultivation which accounts for approximately 20%–30% of the total production cost (Grima et al., 2003). Determination of effective and economic harvesting methods is necessary for high-efficiency bioethanol production at low cost (Bibi et al., 2017). Microalgae that have high dry weight should be obtained by effective harvesting methods that are applicable for all microalgae species. Thus, one method or

combined methods can be applied for harvesting, and physical, chemical, and biological processes can also be used to ensure separation at the desired efficacy (Gouveia, 2011). Microalgae are harvested in two stages. The first step is to separate the microalgal biomass from the bulk suspension (bulk harvesting). Flocculation, flotation, or gravity sedimentation methods are usually used for this purpose. The second step is thickening the resulting algal slurry, in which centrifugation and filtration methods are generally used and the actual energy is applied (Bibi et al., 2017). The harvesting method varies according to microalgae properties such as size and density. For example, a microscreen harvesting method is suggested for *Spirulina* microalgae due to their long and spiral shape (Brennan and Owende, 2010). Harvesting yield can be reduced by cell density less than 0.3 g/L and microalgae cells smaller than 2 μm (Bibi et al., 2017).

As shown in Fig. 14.2, the produced microalgae are transferred to the holding reactor by a pump after reaching a certain concentration (>0.5 g/L). Here, microalgae are allowed to precipitate with the aid of flocculants and water is withdrawn from the system until the final microalgae concentration is up to 10–20 g/L. After microalgae concentration reaches the desired level, the microalgae are centrifuged with stream 3. Here, the microalgae is centrifuged to obtain a concentration of 100–200 g/L and purified from nutrient solutions. The resulting algal paste is then sent to a solar dryer with stream drying. Once the drying process is completed, the pretreatment and saccharification steps are started.

Flocculation is a very well-known technique used in bulk harvesting for large amounts of algae suspensions (Uduman et al., 2010). The microalgae are negatively charged in order to prevent self-agglomeration in the suspension. In the flocculation process, the negative charged microalgae cells become neutral to be precipitated by adding flocculants to the suspension. These flocculants should be inexpensive, user-friendly, renewable, and available in small doses. Flocculants can be inorganic or organic such as $Al_2(SO_4)_3$ (aluminum sulfate), $Fe_2(SO_4)_3$ (ferric sulfate), and polyacrylamide. Due to the use of inorganic flocculants like metal salts in harvesting, the obtained biomass includes a high concentration of inorganic material, which causes problems for the use of these biomasses in further stages. Natural polymeric materials such as chitosan are used as flocculants to avoid this disadvantage. However, the cost of flocculation increases in this case (Akış and Özçimen, 2019). Knuckey et al. (2006) have performed harvesting process with 80% yield by using Fe^{3+} flocculants. It was reported by Grima et al. (2003) that aluminum was utilized for harvesting *Scenedesmus* and *Chlorella* microalgae. Some microalgae species get flocculated in response to pH, dissolved oxygen level, and nitrogen stress; this is called autoflocculation and occurs as a result of precipitation of carbonate salts in algae cells at high pH (Sukenik and Shelef, 1984).

The centrifugation method is a very suitable method for microalgae having a relatively thick cell wall and a diameter greater than 5 μm. In the literature, it has been shown that the microalgae can be extensively recovered (up to 80%–90%) by centrifugation at 500–1000 g speed for 2–3 min. Although high-yield microalgae biomass can be acquired with this method,

it is time-consuming and expensive (Grima et al., 2003). Heasman et al. (2000) reported that microalgae can be harvested with a yield of 95%–100% by centrifugation at a rate of $13{,}000 \times g$, but this method is not suitable for large-scale systems due to the high energy consumption. It is suggested in the literature that centrifugation is not a suitable method for bulk harvesting, but it might be a useful method for thickening.

The drying process is necessary for using microalgal biomass efficiently in further processes. Harvested microalgal biomass contains approximately 70%–90% water. The most commonly used techniques are solar drying, spring freeze drying, and fluidized bed drying for removing the water from the biomass (Bibi et al., 2017). Solar drying is the most effective technique for large-scale production among others which are very expensive and often used in laboratory-scale systems (Prakash et al., 1997).

14.4.3 Pretreatments and saccharification

Physical, chemical, and biological pretreatments are applied to harvested and dried microalgae with the aim of breaking down cell membranes and releasing the carbohydrate (Hill et al., 2006). Although there are many studies on this subject in the literature, it is still challenging to identify the best technique (Özçimen and İnan, 2015).

As shown in Fig. 14.2, harvested and dried microalgae are transferred to a tank (6) with stream 5 for pretreatment and saccharification, respectively. In this tank, the cell membranes of microalgae are broken down in order to release the carbohydrates inside the cell. The microalgal carbohydrates are then broken into small fermentable sugars by saccharification and sent to the fermenter (8) with streams 6 and 7 for fermentation.

Chemical pretreatments, which are usually applied to the microalgal biomass with acids such as H_2SO_4, HCl, and HNO_3 and bases such as NaOH, KOH, and Na_2CO_3, are fast, cheap, and easily applicable. However, they have disadvantages in that they require extreme conditions such as high temperature, high pressure, and high acid concentration, which results in degradation of some carbohydrate structures and formation of toxic compounds. Since these degraded structures can reduce ethanol yield by inhibition of yeast metabolism during the fermentation stage (Bensah and Mensah, 2013), optimization of chemical pretreatment conditions is very important (Chen et al., 2013). Microalgae can be exposed to pretreatment and saccharification applications simultaneously due to their simple cell structure and lack of lignin content. *Chlamydomonas reinhardtii* and *Chlorella vulgaris* species were pretreated with 3% H_2SO_4 microalgae and bioethanol yields were calculated as 29.1% and 40%, respectively (Lee et al., 2011; Nguyen et al., 2009). In addition, *Chlorococcum infusionum* microalgae species were treated with NaOH by Harun et al. (2011).

Another method commonly used in pretreatment of microalgae is enzymatic pretreatment, in which hydrolase, cellulose, and starch with enzymes form reducing sugars containing glucose.

Enzymatic pretreatment is more advantageous than chemical methods in that it does not release toxic by-products and hydrolysis occurs with a higher conversion percentage. In addition, extreme conditions such as high temperature and high pressure are not necessary, which makes this method more advantageous in terms of cost (Cara et al., 2007). This method becomes convenient and easy and does not need special enzymes for breaking down these structures (Taherzadeh and Karimi, 2007). Kim et al. (2012) applied enzymatic pretreatment to microalgae biomass for bioethanol production from *Schizocytrium* sp. species.

Choi et al. (2010) investigated bioethanol production potential of *C. reinhardtii* microalgae by using amylase and glycoamylase in the pre-treatment stage. Furthermore, Harun and Danquah (2011) performed saccharification with a yield of approximately 64% by applying enzymatic pretreatment on *Chlorococum humicola* microalgae. Mahdy et al. (2016) revealed that approximately 84% of the total sugar content of *C. vulgaris* and *Scenedesmus* sp. microalgae are converted by using enzyme mixtures. In addition to enzymatic and chemical pretreatments, other techniques can be applied to microalgae such as ultrasonic, microwave, electric pulses, wet oxidation, and hydrothermal (Ali and Watson, 2016; Ferreira et al., 2016; Hu et al., 2011; Jeon et al., 2013; Parniakov et al., 2015; Pirwitz et al., 2016; Ruiz et al., 2015).

14.4.4 Fermentation and distillation

The fermentation step of microalgae-based sugars consists of two common methods: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In Fig. 14.2, the method in which the hydrolysis and fermentation steps are used separately is preferred for illustration. The sugar solution obtained in the previous steps is transferred to the fermenter with a pump (9). In addition to the glucose solution, *S. cerevisiae* is added to the fermenter in order to initiate fermentation. As a result of fermentation, the obtained product is moved to the phase separator and distillation tower with streams 8, 9, and 11. Next, 99% pure ethanol is achieved and sent to the storage tank by stream 12. As a by-product, released CO_2 gas is conveyed to the microalgae cultivation system with stream 10 to be utilized in microalgae cultivation. Sugars that are not able to transform into bioethanol and other wastes are stored in a tank for utilizing in different processes. The released process water is stored for reuse later.

In the last 20 years, many researches have been carried out with the SHF method for microalgal bioethanol production. Nguyen et al. (2009) produced bioethanol with a yield of 0.292 g-ethanol/g-biomass from microalgal biomass. Ho et al. (2013b) acquired bioethanol from *Scenedesmus obliquus* microalgae with a yield of 0.213 g-ethanol/g-biomass using *Zymomonas mobilis* sp. bacteria. Recent studies on bioethanol production from microalgal biomass in the literature are summarized in Table 14.3.

Table 14.3: Bioethanol production from different microalgae species.

Microalgae	Pretreatment	Fermentation conditions	Bioethanol yield (g/g)	References
C. reinhardtii	3% H_2SO_4, 110°C, 30 min	SHF, 24 h, S. cerevisiae S288C	0.291	Nguyen et al. (2009)
C. vulgaris	3% H_2SO_4, 110°C, 105 min	SHF, 24 h, *E. coli* SJL2526	0.4	Lee et al. (2011)
Chlorella sp.	35–55°C enzymatic hydrolysis and 0.1, 0.3, 0.5, 0.7, 1 N H_2SO_4, 121°C, 15 min acid hydrolysis	SHF and SSF, 30°C, 20 h, *S. cerevisiae*	0.4 and 0.16	Lee et al. (2015)
Chlorococum sp.	28–60°C, 72 h, enzymatic hydrolysis	SHF, 50 h, *S. cerevisiae*	0.48	Harun and Danquah (2011)
C. reinhardtii	12 N H_2SO_4	SHF, 48 h, *S. cerevisiae*	0.44	Scholz et al. (2013)
S. obliquus	2% H_2SO_4, 121°C, 20 min	SHF, 4 h, *Z. mobilis*	0.213	Ho et al. (2013b)
C. minutissima	1 N H_2SO_4, 100°C, 60 min	SHF, 40°C, 48 h, *S. cerevisia*	0.185	Sert et al. (2018)
C. infusionum	0.75% NaOH, 120° C, 30 min	30°C, 72 h, *S. cerevisiae*	0.26	Harun et al. (2011)
N. oculata	0.75% NaOH, 120° C, 30 min	SHF, 30°C, 48 h, *S. cerevisia*	0.004	Reyimu and Özçimen (2017)
T. suecica	0.75% NaOH, 120° C, 30 min	SHF, 30°C, 48 h, *S. cerevisia*	0.073	Reyimu and Özçimen (2017)
Chlorococum sp.	Supercritical CO_2 lipid extraction	SHF, 60 h, *S. bayanus*	0.383	Harun et al. (2010)
C. reinhardtii	55 and 90°C, enzymatic hydrolysis	SSF, 40 h, *S. cerevisiae*	0.235	Choi et al. (2010)
C. vulgaris	50C, 72 h enzymatic hydrolysis	48 h, 30°C, *S. cerevisiae*	0.07	Kim et al. (2014)
Schizochytrium sp.	Hydrothermal degradation and enzymatic hydrolysis	SSF, 72 h, *E. coli*	0.055	Kim et al. (2012)
C. vulgaris	enzymatic hydrolysis	SSF, 30°C, *Z. mobilis*	0.178–0.214	Ho et al. (2013a)
C. vulgaris	0.1%–5.0% H_2SO_4, 121°C 20 min	SHF, 30°C, *Z. mobilis*	0.233	Ho et al. (2013a)

14.5 Conclusion

Nowadays, the increasing need for renewable energy sources as a result of environmental and economic problems has led researchers to carry out researches on biofuel production. For this reason, cultivation and harvesting processes of microalgae, conversion of them to suitable biofuels, and optimization of these processes have come into prominence. In this chapter, each step of bioethanol production from microalgae (cultivation, harvesting, pretreatment,

hydrolysis, fermentation, and distillation) has been explained and studies in the literature have been summarized. Moreover, a flow diagram of the microalgal bioethanol production bioprocess was presented for visualizing all steps of the bioprocess. It is concluded that microalgae are appropriate raw material for bioethanol production, but further studies should be performed on cultivation and pretreatment stages. In order to make microalgal bioethanol production more economical and widespread, first, biodiesel is produced from microalgal oil and only microalgal waste after lipid extraction process can be used for bioethanol production instead of all microalgae containing lipid. In addition, by-product liquid after bioethanol production in a fermenter can be valorized for biogas or animal feed productions with a biorefinery approach, which is significant in terms of both bioeconomic and environmental sustainability.

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CHAPTER 15

Biohydrogen from microalgae

Harshita Singh[a], **Debabrata Das**[a,b]

[a]*Advance Technology Development Center, Indian Institute of Technology, Kharagpur, India* [b]*Department of Biotechnology, Indian Institute of Technology, Kharagpur, India*

Chapter outline

15.1 Introduction 391
15.2 Pathways of hydrogen production from microalgae 392
15.2.1 Biophotolysis 393
15.2.2 Biohydrogen production using microalgal biomass as a feedstock in dark fermentation 402
15.3 Challenges and future perspectives of biohydrogen production from microalgae 409
15.4 Conclusion 413
Acknowledgment 413
References 413
Further reading 418

15.1 Introduction

Currently, one of the major issues at the global level is to meet rising fuel demand along with reductions in nonrenewable source-based energy generation and CO_2 emissions. Hydrogen is widely acknowledged as a "carbon-neutral energy carrier" with high potentiality to replace fossil fuels (Das and Veziroglu, 2008). Hydrogen possesses several attractive attributes such as high energy content (142 kJ/g), generation of clean product (water vapor) on combustion, and direct applicability in a fuel cell to generate electricity (Das et al., 2014). Nevertheless, the main challenges to realize a hydrogen-based economy lie in efficient production strategies and safe storage and distribution systems (Nagarajan et al., 2017). Presently, at commercial scale, hydrogen is produced from methane reforming (48%), oil/naphtha reforming (30%), coal gasification (18%), and water electrolysis (3.9%) (Das et al., 2014). However, all these technologies are associated with various socioeconomic and environmental limitations (Holladay et al., 2009). Biological hydrogen production technology is considered as a promising alternative to these commercialized thermochemical and electrochemical processes, and can provide a sustainable energy supply (Das and Veziroglu, 2001; Khetkorn et al., 2017; Show et al., 2018). The biological process is an eco-friendly and less energy-intensive approach for hydrogen generation.

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There are several microorganisms such as microalgae (green algae and cyanobacteria), photosynthetic bacteria, and nonphotosynthetic bacteria that can produce hydrogen via different metabolic pathways. Among these microorganisms, microalgae present tremendous potentiality for hydrogen generation along with an efficient carbon capturing system, thus making the production process carbon-neutral. Both green microalgae and cyanobacteria are endowed with efficient photosynthetic machinery required for photobiological hydrogen production using the most abundant natural resources: sunlight and water. The key enzymes involved in this process are hydrogenase and nitrogenase (Eroglu and Melis, 2011). Biohydrogen production through biophotolysis was firstly reported in the green microalgae, *Scenedesmus obliquus*, in 1942 (Gaffron and Rubin, 1942). Since then, several microalgal species have been extensively explored for hydrogen production via a biophotolysis process, with considerable advances in this field being made in recent years (Torzillo et al., 2015; Show et al., 2018).

Besides biophotolysis, over the past few years microalgal biomass has emerged as a plausible feedstock in dark fermentation processes for sustainable hydrogen generation (Sambusiti et al., 2015; Wang and Yin, 2018). Microalgae can synthesize and accumulate high amounts of carbohydrate, which can be utilized by the hydrogen-producing bacteria (HPB) for hydrogen generation via anaerobic fermentation process. The lignin content in microalgal-based carbohydrate is negligible. Therefore, their saccharification is much easier compared to lignocellulosic biomass for biohydrogen production (Chen et al., 2013). However, milder pretreatment strategies (physical, chemical, and biological methods) are usually employed to facilitate the cell wall breakage and solubilization of complex carbohydrates into fermentable sugars (Sambusiti et al., 2015; Wang and Yin, 2018).

This chapter aims to describe the fundamentals of biohydrogen production from microalgae through biophotolysis as well as dark fermentation. The factors affecting microalgal hydrogen production have been discussed. In addition, the pretreatment techniques commonly used for microalgal biomass hydrolysis are included. The challenges and prospects for microalgal hydrogen production are also outlined.

15.2 Pathways of hydrogen production from microalgae

There are two main processes for biohydrogen production by exploiting microalgae: (i) light-dependent pathway and (ii) light-independent pathway. The light-dependent pathway for H_2 production carried out by green microalgae and cyanobacteria includes biophotolysis. This process can be differentiated into two distinct pathways: direct biophotolysis and indirect biophotolysis. The light-independent pathway for H_2 production includes dark fermentation, which is carried out by anaerobic bacteria from microalgal (carbohydrate-rich biomass) feedstock.

15.2.1 Biophotolysis

Biophotolysis involves the action of light energy on the photobiological (microalgal) system that causes the dissociation of the water molecule into H_2 and O_2. This process is closely related to photosynthesis. The photosynthetic machinery of both the eukaryotic green microalgae and prokaryotic blue-green microalgae (cyanobacteria) is homologous to that of vascular plants. The components of the photosynthetic apparatus are located in the thylakoid membranes present inside the chloroplast and cytoplasm of the green microalgae and cyanobacteria, respectively. Inside the thylakoid membranes, sunlight is captured by the light-harvesting complexes (LHCs) (consisting of chlorophyll *a*, antenna chlorophylls, carotenoid, and phycobiliproteins) associated with two photosystems (PS I and PS II). Photosystem I and photosystem II also contain special types of chlorophyll dimers, i.e., P700 and P680, respectively, which are known as reaction centers (RCs). In microalgae, these two RCs act in tandem to catalyze the light-driven movement of electrons from H_2O to $NADP^+$. The process begins with the trapping of photons by the LHC of PS II and subsequent transfer of energy to RC, P680. Upon energy transfer, P680 reaches its excited state. Excited P680 (P680*) transfers the electron to a neighboring molecule known as pheophytin. P680* is capable of splitting the water molecule into protons, electrons, and O_2.

Consecutively, the electron derived from water splitting is taken up by the oxidized P680 to return rapidly to its ground state. The reduced pheophytin drives the electron sequentially through different electron acceptors of PS II, i.e., plastoquinone (PQ), cytochrome (Cyt) b_6f complex, and plastocyanin (PC) to reduce the RC, P700. In a further reaction, the electron from excited P700 (P700*) is ferried through intermediary electron acceptors of PS I, i.e., special chlorophyll molecule (A_0), phylloquinone (A_1), and iron-sulfur protein (Fe-S) to reduce the ferredoxin (Fd). The reduced Fd is the key intermediate for channeling the electron either into CO_2 fixation or the H_2 evolution pathway. In the presence of light and O_2, reduced Fd donates the electron to a flavoprotein, ferredoxin: $NADP^+$ oxidoreductase (FNR). This enzyme transfers the electron to $NADP^+$ and generates NADPH. The NADPH provides reducing power for CO_2 assimilation into carbohydrates in the Calvin cycle. However, under favorable and anaerobic conditions, electrons from the reduced ferredoxin can be directed toward hydrogenase or nitrogenase enzyme for hydrogen generation. The two processes for H_2 production, i.e., direct and indirect biophotolysis, are elaborated in the subsequent sections.

15.2.1.1 Direct biophotolysis

In direct biophotolysis, the electrons generated due to water splitting are directly used for H_2 production. During this process, H_2 generation at the reducing side of the PS I is associated with the simultaneous evolution of O_2 at the oxidizing side of PS II (Fig. 15.1). The key enzyme

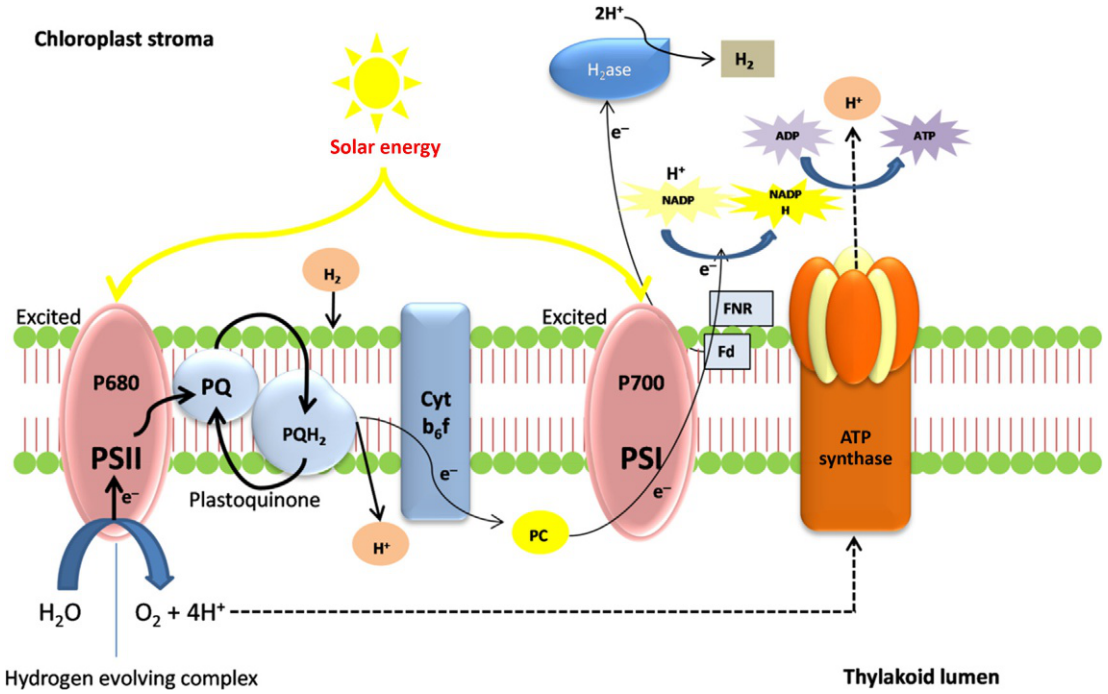

Fig. 15.1

Biohydrogen production by direct biophotolysis. *Modified from Kuppam, C., Pandit, S., Kadier, A., Dasagrandhi, C., Velpuri, J., 2017. Biohydrogen production: integrated approaches to improve the process efficiency. In: Microbial Applications. Springer, Cham, pp. 189–210.*

responsible to catalyze H_2 production is hydrogenase (Eroglu and Melis, 2011). Green microalgae are known to have highly active [Fe-Fe] hydrogenase enzymes with relatively high (12%–14%) solar-to-H_2 conversion efficiency (Melis, 2009).

Direct biophotolysis is advantageous as the process utilizes the most abundantly available resources, i.e., water and sunlight, for the production of zero carbon fuel, hydrogen. However, the major issue with direct biophotolysis is the inevitable generation of O_2 (at the PS II side), which is a powerful suppressor of hydrogenase enzymes. In this process, upon illumination, H_2 evolution occurs for a short time, before the O_2 inhibits and eventually degrades the hydrogenase (Eroglu and Melis, 2011). Various efforts have been made to overcome the problem of hydrogenase inactivation due to the accumulated O_2. For example, Melis et al. (2000) proposed a sulfur deprivation strategy for temporal separation of oxygenic photosynthesis and photobiological H_2 evolution. This is a two-stage biophotolysis process wherein the first stage involves photosynthetic O_2 evolution and biomass generation under suitable conditions. In the second stage, carbohydrate containing algal biomass is transferred

into a cultivation media deprived of sulfur, which leads to H_2 evolution. This happens due to the fact that sulfur is essentially required for the biosynthesis of PS II protein. Deprivation of sulfur results in the partial suppression of PS II, which results in reduction of O_2 evolution. The anaerobiosis condition created in the second phase thus facilitates the hydrogenase activity and leads to sustained H_2 production.

Under sulfur-deprived conditions, a large number of studies have been conducted on the green algae, *Chlamydomonas reinhardtii*. The challenges met when using such systems are formidable (Hallenbeck et al., 2012). For example, cultivation of *Chlamydomonas reinhardtii* under sulfur-deprived conditions in a tubular photobioreactor with optimized process parameters for 120 h resulted in a cumulative H_2 yield of 3 mL H_2/L of culture media only, which represents a very low H_2 production rate, i.e., 1.04 µmol H_2/mL/h (Tamburic et al., 2011). Seibert et al. (2001) sought random and site-directed mutagenesis to maximize the oxygen tolerance of hydrogenase enzyme in *Chlamydomonas reinhardtii*. The employed strategy resulted in development of a mutant *Chlamydomonas reinhardtii* strain with 10 times higher O_2-tolerance compared to the wild strain. To date, the rational attempt to genetically engineer microalgal [Fe-Fe]-hydrogenases for improved H_2 yield is limited due to the lack of a crystal structure in these enzymes. However, crystal structures of homologous hydrogenases of bacterial origin are well-established. These structures could serve as the basis of investigations into the mechanism associated with algal hydrogenase inhibition (Srirangan et al., 2011).

15.2.1.2 Indirect biophotolysis

In indirect biophotolysis, the electrons generated due to the water splitting are not directly used up for H_2 evolution. These electrons are firstly supplied to the CO_2 assimilation pathway for the synthesis of storage carbohydrates. Subsequently, the organic reserves (starch in green microalgae and glycogen in cyanobacteria) are catabolized to serve as electron donors for H_2 production (Fig. 15.2). Thus, unlike direct photolysis, in this process electrons are not directly gained from water for H_2 evolution.

The overall mechanism of indirect biophotolysis can be represented as follows (Eqs. 15.1, 15.2):

$$12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2 \tag{15.1}$$

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2 \tag{15.2}$$

In indirect biophotolysis, the H_2 evolution is partially separated from oxygenic photosynthesis via spatial or temporal separation mechanisms. Spatial separation is observed in some nitrogen-fixing cyanobacteria such as *Nostoc*, *Anabaena*, *Calothrix*, and *Oscillatoria* (Das and Veziroglu, 2008). Besides vegetative cells, these blue-green algae possess a

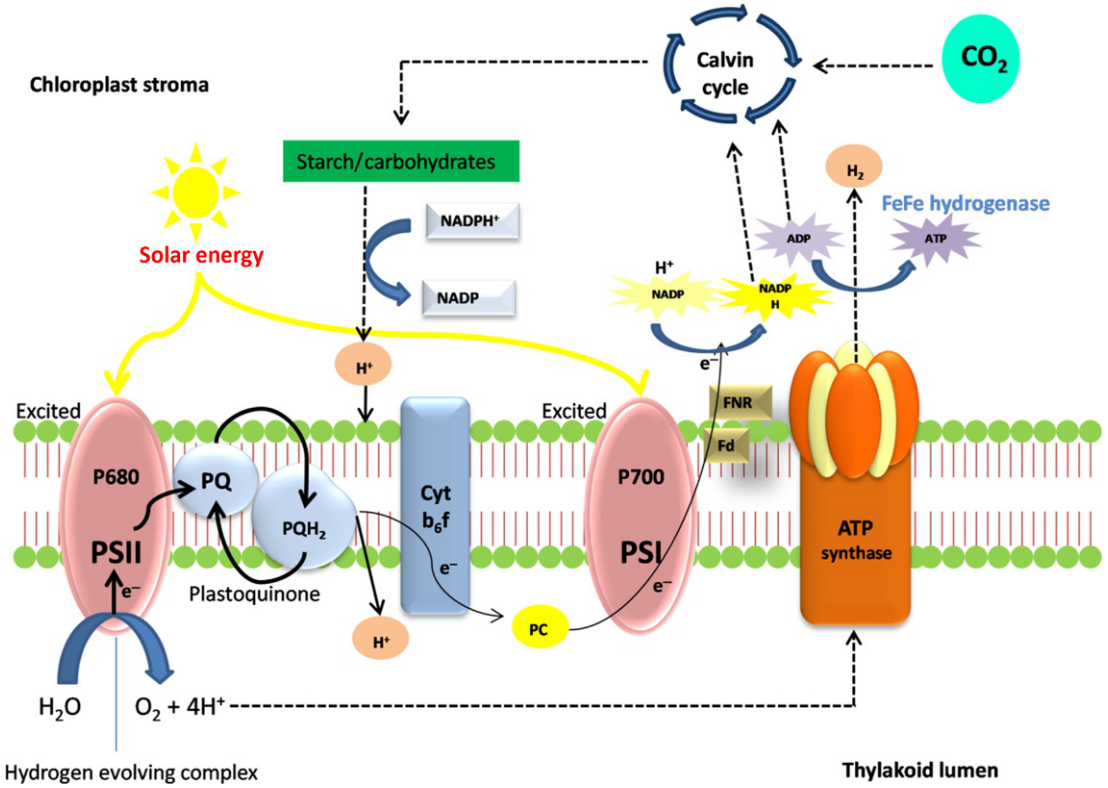

Fig. 15.2

Biohydrogen production by indirect biophotolysis. *Modified from Kuppam, C., Pandit, S., Kadier, A., Dasagrandhi, C., Velpuri, J., 2017. Biohydrogen production: integrated approaches to improve the process efficiency. In: Microbial Applications. Springer, Cham, pp. 189–210.*

specialized cell known as a heterocyst. This lacks the oxygen evolving PS II and thus promotes the anaerobiosis required for N_2 fixation. The anaerobic environment is also facilitated by the presence of an O_2-impermeable cell wall around the heterocyst that does not allow the diffusion of O_2 from the nearby vegetative cells. The N_2 fixation reaction is catalyzed by an O_2-sensitive enzyme, nitrogenase. During the N_2 fixation process, H_2 is also generated according to the following reaction (Eq. 15.3):

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (15.3)$$

The reducing equivalents for the above biochemical reaction are derived from the carbohydrates that are generated and stored in the vegetative cells through the Calvin cycle. The heterocyst-based H_2 evolution in cyanobacteria requires substantial amounts of energy (16 ATP per moles of H_2 produced). This ATP requirement is fulfilled by the cyclic

photo-phosphorylation that occurs in the thylakoid membrane of the heterocyst with the help of light energy. However, this results in low solar energy conversion efficiency into H_2 (Benemann, 2000).

In temporal separation, the naturally occurring diurnal variation of light intensity separates the O_2 and H_2 evolution. During daytime, microalgal metabolism follows the oxygenic photosynthesis and CO_2 fixation mechanism, while in the dark period, the fixed carbon (stored carbohydrates) is metabolized to create electrons for H_2 generation. Temporal separation is generally observed in nonnitrogen fixing cyanobacteria (*Synechocystis*, *Synechococcus*, *Gloeobacter*). These blue-green algae possess a bidirectional [NiFe] hydrogenase. This enzyme has the capability to catalyze both the synthesis and the oxidation of H_2. The bidirectional hydrogenase is advantageous over nitrogenase as it does not require ATP for H_2 generation. Moreover, the turnover number of this enzyme is exceptionally high compared to the nitrogenase (98/s vs 6.4/s) (Mathews and Wang, 2009). However, unlike nitrogenase, which can catalyze the H_2 production reaction even under high H_2 partial pressure, the bidirectional [NiFe] hydrogenase requires low H_2 partial pressure to operate efficiently (Mathews and Wang, 2009).

Several types of bioprocess and genetic engineering strategies that have been explored to improve the efficiency of photobiological H_2 production from microalgae are given in Table 15.1.

15.2.1.3 Enzymes involved in photobiological hydrogen production from microalgae

The major enzymes involved in photobiological hydrogen production from microalgae include: (i) [Fe-Fe] hydrogenase, (ii) [Ni-Fe] bidirectional hydrogeanase, (iii) [Ni-Fe] uptake hydrogenase, and (iv) nitrogenase.

15.2.1.3.1 [Fe-Fe] hydrogenase

[Fe-Fe] hydrogenase is abundantly present in fermentative anaerobic bacteria and is the only type of hydrogenase found in eukaryotic green microalgae (Das et al., 2014). In microalgae, this enzyme is encoded by the *hyd*A gene present in the nucleus and is located in the stroma of the chloroplast after its maturation (Khetkorn et al., 2017). The enzyme is usually monomeric with an average molecular weight of 45–50 kDa. [Fe-Fe] hydrogenase is linked to the photosynthetic electron transport chain via ferredoxin, where under hypoxic conditions it can catalyze the reduction of H^+ to produce H_2. This enzyme possess a unique catalytic center (H-cluster), which makes it about 1000-fold and 100-fold more efficient than nitrogenase and other hydrogenases, respectively (Mathews and Wang, 2009; Khetkorn et al., 2017). The H-cluster is composed of [4Fe-4S] cubane covalently bridged with a bimetallic Fe center. The Fe atoms of the metallic center are coordinated with nonprotein ligands, CN^- and CO groups (Das et al., 2014). [Fe-Fe] hydrogenase is considered to be the most efficient H_2-producing enzyme. However, this enzyme is O_2 intolerant and gets irreversibly inhibited by

Table 15.1 Photobiological hydrogen production from microalgae under various strategies.

Microalgae	Strategy	H_2 production	References
Anabaena variabilis AVM 13	Disruption of uptake hydrogenase genes (hupSL) by interposon insertion	68 µmol/mg chl/h	Happe et al. (2000)
Chlamydomonas reinhardtii cc124	Sulfur re-addition in TAP (-S) medium	5.9 µmol/mg chl/h	Kosourov et al. (2002)
Chlamydomonas reinhardtii C137	Brief exposure to high light intensity	6.0 mL/g dcw/h	Markov et al. (2006)
Nostoc sp. PCC 7422	Inactivation of uptake hydrogenase gene (hupL) through recombination	100 µmol/mg chl/h	Yoshino et al. (2007)
Chlamydomonas reinhardtii	Double amino acid substitution in the protein of PS II and sulfur deprivation	166 mL/mg chl/h	Torzillo et al. (2009)
Synechocystis sp. PCC 6803	Sulfur deprivation and supplementation of β-mercaptoethanol	14.3 µmol/mg chla/min	Baebprasert et al. (2010)
Cyanothece sp. ATCC 51142	Glycerol supplementation	467 µmol/mg chl/h	Bandyopadhyay et al. (2010)
Anabaena siamensis TISTR 8012	Fructose supplementation and optimization of light intensity	32 µmol/mg chla/h	Khetkorn et al. (2010)
Chlamydomonas reinhardtii cc849	Transformation of codon optimized genes (*hemH* and *lba*)	23.5 µmol/mg chl/h	Wu et al. (2011)
Aphanothece halophytica	Nitrogen deprivation	13.8 µmol/mg chl/h	Taikhao et al. (2013)
Lyngbya sp.	Supplementation of benzoate	17.05 µmol/g chla/h	Shi and Yu (2016)
Anabaena siamensis TISTR 8012	Cell immobilization, sulfur deprivation and supplementation of fructose with β-mercaptoethanol	3.1 mL/mg chla/h	Taikhao and Phunpruch (2017)

the presence of O_2. Recently, Rumpel et al. (2015) presented the structural insight of the ferredoxin-[Fe-Fe] hydrogenase complex in *Chlamydomonas reinhardtii*.

15.2.1.3.2 [Ni-Fe] bidirectional hydrogenase

The [Ni-Fe] bidirectional hydrogenase can be found in both nitrogen-fixing and nonnitrogen-fixing blue-green algae (Tamagnini et al., 2007). The enzyme has a complex molecular structure and is composed of hetero-pentameric subunits encoded by hoxEFUYH. The hoxYH constitutes the hydrogenase part of the enzyme, while hoxEFU constitutes the diaphorase part

of the enzyme (Das et al., 2014). The larger catalytic center of the hydrogenase encoded by *hoxH* gene consists of Ni and Fe atoms bounded with nonprotein ligands, CN^- and CO groups. The smaller hydrogenase subunit encoded by the *hoxY* gene consists of a [4Fe-4S] cluster. This smaller subunit is required to transfer the electrons to the larger catalytic subunit. The remaining three subunits encoded by *hoxE*, *hoxF*, and *hoxU* genes act as electron donors between NAD(P)H and the active site of hydrogenase (Azwar et al., 2014). The [Ni-Fe] bidirectional hydrogenase is cytoplasmic and has the ability to catalyze both the hydrogen production and hydrogen consumption reactions depending upon the cell's redox potential (Eroglu and Melis, 2011).

15.2.1.3.3 [Ni-Fe] uptake hydrogenase

The nitrogen-fixing cyanobacteria also have a [Ni-Fe] uptake hydrogenase. The enzyme possesses two subunits encoded by *hupSL* genes. The larger subunit encoded by *hupL* gene comprises of four cysteine residues in its active site. The smaller subunit encoded by *hupS* gene consists of three [4Fe-4S] clusters. This enzyme catalyzes the oxidation of H_2 to recover efficiently the electrons released during the nitrogen fixation reaction. The electrons are transferred by the *hupL* active site to the electron acceptor molecule through *hupS* [4Fe-4S] clusters. Thus, uptake hydrogenase provides the reductants to the tricarboxylic acid (TCA) cycle for CO_2 assimilation.

15.2.1.3.4 Nitrogenase

The heterocyst-based photobiological H_2 production in cyanobacteria is catalyzed by the enzyme nitrogenase. This enzyme is primarily responsible for reducing the atmospheric N_2 into ammonia. During the N_2 fixation reaction, H_2 is concomitantly generated as a by-product. Nitrogenase is a multiprotein enzyme complex comprised of two protein moieties: the larger dinitrogenase (Mo-Fe-S protein or protein I) and the smaller dinitrogenase reductase (Fe-S protein or protein II). The dinitrogenase part is a heterotetramer consisting of two α and two β subunits ($\alpha_2\beta_2$) encoded by *nif*D and *nif*K gene, respectively. The reductase part is a homodimer encoded by the *nif*H gene. This subunit transfers the electrons from the external electron donor to the dinitrogenase subunit. Subsequently, dinitrogenase catalyzes the reduction of N_2, which leads to the formation of two molecules of NH_3. Depending upon the type of metallic cofactor present at the catalytic site of the enzyme, nitrogenase is classified into three types: molybdenum, iron, and vanadium nitrogenases. All these variants of nitrogenases are reported to produce H_2 during the nitrogen fixation reaction, but with variable stoichiometry (Singh and Das, 2018). In the absence of N_2, nitrogenase may exclusively lead to H_2 generation by utilizing the protons and high-energy electrons. All the reactions (NH_3 and H_2 production) catalyzed by nitrogenase require high expenditure of energy (ATP). Therefore, H_2 production by nitrogenase seems to involve wastage of energy for the cell (Das et al., 2014). To minimize the loss of energy, uptake hydrogenase recycles the H_2

produced during the N_2 fixation process. Due to this consumption of H_2, the net H_2 evolution by cyanobacteria is barely observed, at least in aerobic conditions (Almon and Böger, 1988).

15.2.1.4 Factors influencing photobiological hydrogen production from microalgae

15.2.1.4.1 pH

The microalgal hydrogen production process is significantly affected by the pH of the media. pH not only influences the microalgal growth but is also crucial for the hydrogen-producing enzymes (hydrogenase and nitrogenase) (Rashid et al., 2013). The photobiological production of H_2 is usually observed at alkaline pH. The pH range of 6–10 has been reported as optimal for both microalgal growth and H_2 generation (Song et al., 2011; Alalayah et al., 2014). Both hydrogenase and nitrogenase are sensitive to pH. In microalgal systems, it has been observed that a low pH value (<5.0) inactivates the H_2-producing enzymes and leads to a low H_2 production rate (Rashid et al., 2013). Moreover, the variation in pH with time during sulfur deprivation might affect the H_2 productivity of the process. When microalgae are cultivated in an S-depleted medium, the final pH reaches different values with respect to time. This change in pH leads to the predomination of different intermediary metabolic by-products that might hinder H_2 productivity. For instance, Kosourov et al. (2007) found that the H_2 production rate of S-deprived *Chlamydomonas reinhardtii* cells was higher at pH 7.7, while it decreased at pH 6.5. The suitable pH value for photosynthesis and biophotolysis also depends upon the microalgal species. For example, marine microalgae require different pH compared to fresh microalgal species, due to low nitrate assimilation in the case of the former (Rashid et al., 2013). Antal and Lindblad (2005) explored the effect of medium pH on H_2 production in different cyanobacterial species. The pH-dependent optimization of microalgal H_2 production process could substantially improve the H_2 yield.

15.2.1.4.2 Temperature

Like pH, temperature also influences the microalgal growth and catalytic activity of the H_2-producing enzymes. Maintenance of suitable temperature may shift the microalgal metabolic pathway toward enhanced biomass and H_2 generation. The temperature ranges of 25–35°C and 30–40°C have been reported as optimal for the growth of most of the green microalgal and cyanobacterial species, respectively (Dasgupta et al., 2010; Tiwari and Pandey, 2012). Various studies investigated the optimal temperature required to achieve maximum H_2 production from microalgae (Dutta et al., 2000; Guan et al., 2004; Mona et al., 2011). For example, the suitable temperature for H_2 production using *Nostoc muscorum* was found to be 40°C (Dutta et al., 2000). Guan et al. (2004) reported that a higher temperature (55°C) is required for marine microalgae, *Tetraselmis subcordiformis*, to achieve the maximum H_2 production rate. On the other hand, the optimal temperature for *Nostoc linckia* was found to be 31°C (Mona et al., 2011). These results imply that selection of suitable temperature is essential to carry out the H_2 production from microalgae. During the cultivation of microalgae

in a photobioreactor, the temperature often elevates significantly. This rise in temperature occurs due to the dissipation of excess absorbed photons in the form of heat via a phenomenon known as nonphotochemical quenching (NPQ) (Das et al., 2014). Although a higher temperature is advantageous for lowering O_2 evolution, it can denature the functional proteins of the cell, thereby hampering microalgal growth and H_2 evolution. Thus, maintaining controlled temperature conditions inside the photobioreactor is necessary for obtaining higher H_2 production rates.

15.2.1.4.3 Light intensity

Light intensity significantly affects the metabolic activities of the photosynthetic organism. Microalgal cultivation under moderately low light intensity has been reported as optimal for H_2 evolution (Kaushik and Sharma, 2017). Dasgupta et al. (2010) identified that light intensity of 50–200 $\mu E/m^2/s$ is suitable for both microalgal biomass and H_2 production. Light intensities greater than 200 $\mu E/m^2/s$ can lead to photoinhibition. Moreover, O_2 evolution increases with increase in light intensities, which nullifies the anaerobicity required for sustained H_2 generation. However, light intensity of 110–20 $\mu E/m^2/s$ is advantageous at the initial stage of anaerobic condition (during sulfur deprivation). It has been observed that the anaerobic condition is maintained earlier in the presence of high light intensity than with low light intensity (Tsygankov et al., 2006; Rashid et al., 2013). Kim et al. (2006) investigated the effect of varying light intensities from 60 to 300 $\mu E/m^2/s$ on H_2 production from S-deprived *Chlamydomonas reinhardtii* cells. They found that H_2 generation increased with increment in light intensity up to 200 $\mu E/m^2/s$, while it was inhibited at 300 $\mu E/m^2/s$. This inhibition was associated with the photo-damage of PS II. Apart from the effect of light intensities, the influence of varying photoperiods (light/dark cycles) has also been tested for H_2 production in *Chlamydomonas reinhardtii* under S-deprived conditions. The H_2 contents of 47%, 86%, and 87% were achieved at 2, 3, and 4 h of alternate light and dark period, respectively (Vijayaraghavan et al., 2009). In some cyanobacterial species, absence of light (dark conditions) favors H_2 production (Tiwari and Pandey, 2012). In such organisms, stable day/night photoperiods are maintained so that the growth occurs in presence of optimal light intensity during the daytime, while at night, H_2 production takes place. This phenomenon is mostly seen in nonheterocystous blue-green algae such as *A. variabilis* SPU 003, which produces H_2 only in darkness (Tiwari and Pandey, 2012).

15.2.1.4.4 Nutrient media

The media composition plays a vital role in microalgal growth and H_2 production. The microalgae can be cultivated under autotrophic, mixotrophic, and heterotrophic conditions. During mixotrophic and heterotrophic cultivation, addition of a carbon source is essential. A wide variety of organic carbon sources can be supplemented in the media to achieve higher biomass and H_2 production. During the microalgal H_2 production process, the effect of carbon source on microalgal growth has not been well explored. However, several studies have

investigated the effect of carbon source supplementation on H_2 generation under anaerobic conditions. For instance, Chen et al. (2008) studied the influence of different organic carbon source supplementation on H_2 production from *Anabaena* species and found fructose and sucrose to be the preferred organic substrates. It was reported that in heterocystous cyanobacteria, addition of carbohydrates can stimulate H_2 production as the nitrogenase activity is enhanced due to electron donation by the simple organic compounds (Tiwari and Pandey, 2012). Apart from carbon, availability of nitrogen, sulfur, and phosphorous in the media significantly effects the H_2 yield.

A nutritional stress condition achieved by the deprivation of any of these nutrients is one of the major turning points in microalgal H_2 production technology. A sulfur deprivation strategy has been widely studied for sustained H_2 production by biophotolysis, and this method was discussed in Section 15.2.1.1. During sulfur stress, the presence of acetate in the medium has been found to be an important factor to achieve the anaerobic condition (Gonzalez-Ballester et al., 2015). Similar to sulfur stress, nitrogen and phosphorous stress conditions also inhibit PS II activity and induce H_2 evolution, but at a slower rate (Gonzalez-Ballester et al., 2015). Both in nitrogen-fixing and nonnitrogen-fixing cyanobacterial species, the nitrogen deprivation method is routinely applied to improve the H_2 yield. In *Synechocystis* sp., the transcription of *hox* genes (encoding [Ni-Fe] bidirectional hydrogenase) are regulated and induced under nitrogen-depleted conditions (Baebprasert et al., 2010). Baebprasert et al. (2010) found that in the case of *Synechocystis* sp., sulfur deprivation was also beneficial for H_2 production. However, for *Aphanothece halophytica* (a halotolerant cyanobacterium), unlike nitrogen depletion, sulfur depletion inhibits the microalgal growth and hydrogenase activity, and thus reduces H_2 evolution. In marine macroalgae, compared to sulfur depletion, phosphorous deprivation performs better for H_2 production because the marine environment or seawater is rich in sulfates. Therefore, it is difficult to achieve sulfur depletion conditions while using seawater media (Batyrova et al., 2015). These studies indicate that the suitable nutrient composition required for obtaining maximum H_2 production is different for different microalgae. Therefore, the effect of medium nutrients should be carefully examined to ensure their positive influence on microalgal growth and H_2 production.

15.2.2 Biohydrogen production using microalgal biomass as a feedstock in dark fermentation

15.2.2.1 Dark fermentation process

Dark fermentation (DF) or anaerobic fermentation involves the fermentative breakdown of organic substrates into soluble metabolites (volatile fatty acids and alcohols), hydrogen, and carbon dioxide. Unlike biophotolysis, where light energy is required for hydrogen generation, DF is a light-independent process of biohydrogen production. This process is contemplated as a promising biological route of H_2 production due to its higher rate of H_2 evolution and potentiality to utilize various kinds of feedstock including organic wastes. DF is manifested by

a diverse group of obligate and facultative anaerobic bacteria via different metabolic pathways. These hydrogen-producing bacteria (HPB) can be utilized as either pure or mixed cultures depending upon the type of substrate used for hydrogen production. A simple form of carbohydrate, mainly glucose, is the preferred substrate for the fermentation process. However, if the carbohydrate is in the form of a complex polymer, a hydrolysis step is required for its conversion into glucose. Under anaerobic conditions, HPB converts the glucose into pyruvate via the glycolytic pathway to regenerate the cell's energy currency, i.e., ATP. Subsequently, pyruvate may lead to the evolution of H_2 under different metabolic pathways. In principle, HPB carry out the H_2 production to maintain the cellular redox potential by disposing of the excess electrons in the form of reduced metabolic end product.

Generally, there are two metabolic pathways to accomplish H_2 production by dark fermentation. The NADH re-oxidation pathway involves the mixed acid pathway followed by obligate anaerobes, while the formate decomposition pathway involves the metabolism of facultative anaerobes.

The NADH reoxidation pathway mediated by obligate anaerobes such as *Clostridium* sp. can be represented by

$$NADH + H^+ \rightarrow H_2 + NAD^+ \tag{15.4}$$

This NADH is produced during the initial conversion of glucose into pyruvate by the following reaction of the glycolysis pathway:

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOOH + 2NADH + 2H^+ \tag{15.5}$$

In the subsequent reaction, pyruvate is oxidized to acetyl CoA, catalyzed by the enzyme pyruvate-ferredoxin oxidoreductase (PFOR). The oxidation of pyruvate requires the reduction of ferredoxin. The reduced ferredoxin is oxidized by [Fe-Fe] hydrogenase with concomitant release of H_2. The acetyl CoA can be metabolized into end products such as acetate, butyrate, or ethanol, with or without the consumption of NADH. Theoretically, 4 mol H_2/mol glucose consumed or 2 mol H_2/mol glucose consumed are formed when the microorganism follows the acetate or butyrate pathway, respectively (Eqs. 15.6, 15.7). However, no H_2 is generated when ethanol is the sole metabolic end product:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \tag{15.6}$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + CH_3CH_2CH_2COOH \tag{15.7}$$

The formate decomposition pathway carried out by facultative anaerobes such as *E. coli* involves the oxidation of pyruvate into formate and acetyl CoA. This reaction is catalyzed by the enzyme pyruvate formate lyase. Subsequently, formate hydrogen lyase catalyzes the cleavage of formate into H_2 and CO_2 (Eqs. 15.8, 15.9):

$$CH_3COCOOH + HCoA \rightarrow CH_3COCoA + HCOOH \tag{15.8}$$

$$HCOOH \rightarrow H_2 + CO_2 \tag{15.9}$$

Similarly to the butyrate pathway, 2 mol H_2/mol glucose consumed are formed when formate is the end product of the fermentation.

From the above reactions, it can be deduced that in dark fermentation, the final fate of pyruvate (into acetate, butyrate, or formate) determines the maximum theoretical hydrogen yield per mole of glucose consumed. However, in all cases, the actual hydrogen yield is lower than the theoretical due to the utilization of some amount of substrate for biomass generation. Moreover, depending upon the type of microorganism and culture conditions, substrate degradation might follow other biochemical pathways that lead to the formation of undesired by-products such as lactate, propionate, succinate, 2,3-butanediol, ethanol, butanol, and isopropanol. Generation of these metabolites limits H_2 production and lowers the overall yield of H_2.

15.2.2.2 Microalgal biomass as a feedstock

Dark fermentation has gained popularity primarily due to its potentiality to utilize a wide spectrum of feedstock. Pure carbohydrates, viz. monosaccharides, disaccharides, and polysaccharides, are the preferred substrate for HPB. However, to make the process economically feasible, biomass-based renewable sources of organic carbon should be explored. In this context, particularly, the production of second-generation biofuels from lignocellulosic substrates has gained tremendous attention due to advantages such as high carbohydrate content, abundant availability, and no competition with food crops. Although several studies have focused on lignocellulosic biomass, their native recalcitrant structure limits their applicability for biofuel production. This limitation encouraged the development of third-generation biofuel production from microalgal biomass. Microalgal biomass presents several potential benefits as a viable alternative to lignocellulosic feedstock for biofuel (biohydrogen) production; for example: (1) relatively simple cell walls with no lignin, therefore, mild pretreatment strategy required; (2) superior CO_2 mitigation ability; (3) higher productivity; (4) no requirement of arable land for mass cultivation; (5) can grow in brackish, saline, and wastewaters, thus reducing the freshwater footprint; (6) shorter harvesting period; and (7) compatibility to be utilized in a biorefinery approach (value-added product/lipid-extracted biomass still rich in carbohydrate can be utilized as feedstock for biohydrogen production), thus promoting commercial use (Sambusiti et al., 2015; Wang and Yin, 2018).

Microalgae have the potentiality to synthesize a wide range of metabolites including carbohydrates, lipids, proteins, and pigments. The concentrations of these metabolites are highly variable depending upon the species, cultivation, and environmental conditions (Sambusiti et al., 2015). The H_2 yield in DF depends primarily upon the carbohydrate content of the microalgal biomass because anaerobic breakdown of lipid and protein by the HPB is thermodynamically unfavorable (Xia et al., 2015). Therefore, selection of a suitable strain (having high carbohydrate content) and cultivation conditions are of paramount importance to obtain the microalgal feedstock with higher carbohydrate productivity for fermentative

biohydrogen production. Carbohydrates in microalgae are synthesized via photosynthesis and the CO_2 fixation metabolism. These carbohydrates are accumulated inside the microalgal cells mainly in two forms: as reserve material (e.g., starch or glycogen) and as structural components of the cell wall (e.g., cellulose, pectin, and sulfated polysaccharides). Both reserved and cell wall polysaccharides can be utilized for biohydrogen production upon their milder pretreatment and transformation into fermentable monosaccharides (Chen et al., 2013). Several species of microalgae such as *Chlorella*, *Scenedesmus*, and *Chlamydomonas* are known to accumulate large quantities of carbohydrate naturally and are appropriate feedstock for fermentation (Chen et al., 2013). However, employment of various nutritional deprivation strategies and operational conditions help to enhance the carbohydrate content of microalgae (Sambusiti et al., 2015; Wang et al., 2017). For instance, 4 days of nitrogen starvation triggered the carbohydrate accumulation in *Chlorella vulgaris* FSP-E to 51.3% w/w (Ho et al., 2013). Vitova et al. (2015) suggested sulfur deprivation as an effective approach for the enhancement of starch content.

It is evident from these studies that several nutritional starvation approaches can maximize the carbohydrate content of microalgae. However, these strategies result in low biomass production and thus have an adverse effect on overall carbohydrate productivity of microalgal species (Wang et al., 2017). In view of this, Pancha et al. (2015) followed a mixotrophic nutritional stress regime to enhance the carbohydrate accumulation in *Scenedesmus* sp. CCNM 1077 without any substantial reduction in biomass production. Moreover, to address the issue of biomass loss associated with nutrient stress conditions, a two-stage cultivation strategy has been developed. In this strategy, microalgae are first cultivated using a nutrient-rich medium to enhance biomass growth. After the consumption of about 90% of the medium's nutrients, the culture is switched to a nutrient-deficient medium to trigger carbohydrate accumulation. Using this approach, Wang et al. (2015) achieved carbohydrate accumulation of 71% w/w in *Chlamydomonas reinhardtii*.

15.2.2.3 Pretreatment of microalgal biomass for hydrogen production

The biohydrogen production from microalgal feedstock is limited by the presence of complex cell walls, which restricts the biodegradability of the carbohydrate component. HPB produces hydrolytic enzymes, but their concentrations are not sufficient to carry out the degradation of microalgal biomass. Thus, to achieve efficient cell wall disruption and release of microlagal carbohydrates, application of a pretreatment step is often recommended (Nagarajan et al., 2017). Pretreatment of microalgal biomass is a crucial step as it affects the biohydrogen yield and economy of the DF process significantly. Generally, an effective and sustainable pretreatment process must meet the following criteria: (1) improve the conversion of complex polysaccharides into fermentable monosaccharides; (2) avoid the formation of carbohydrate-derived inhibitory by-products; (3) be less energy intensive; and (4) be cost-effective (Sambusiti et al., 2015; Kumari and Singh, 2018). Literature studies

concerning the analysis of the hydrolysates revealed that the most common hexoses released after microalgal pretreatment are glucose and mannose. Galactose, rhamnose, fructose, and fucose are also liberated in lesser quantities. The common pentoses released include xylose, ribose, and arabinose (Monlau et al., 2014; Wang et al., 2017). HPB can utilize all these monosaccharides and transform them into pyruvate, which can be further funneled for biohydrogen production. The efficiency of a particular pretreatment method for cell disruption may not be the same for different microalgal species due to the difference in cell wall structure and age of the culture. Therefore, the optimal pretreatment process determined for one microalgal species cannot be employed for all the other species (Kadir et al., 2018). To date, the pretreatment techniques used for the hydrolysis of microalgal biomass fall under the following categories: physical, physicochemical, and biological pretreatment.

Various physical pretreatment methods such as milling, grinding, homogenization, sonication, microwave, and thermolysis are used to disintegrate the cell wall in a nonspecific manner. Compared to chemical pretreatment methods, these methods are less dependent on microalgal species. Milling and grinding are usually the first steps carried out to reduce the particle size and crystallinity of the biomass. These methods break the algal cell wall by mechanical compaction and shear stress generated due to the collision of biomass with beads. The studies revealed that these methods did not cause substantial cell disruption and resulted in poor carbohydrate and H_2 yield (Miranda et al., 2012; Cheng et al., 2012). A high-speed homogenizer configured with a rotor-stator assembly is also widely used for microalgal cell disintegration. Hydrodynamic cavitations and mechanical shear stress at the solid-liquid interface are a major mechanism behind the cell disruption. This method has the potential to process the biomass suspension with less water content. However, the yield of extracted sugars was found to be lower than 3% w/w, when biomass of *Scenedesmus obliquus* was homogenized at 24,000 rpm (Miranda et al., 2012).

Ultrasonication and microwave irradiation are other common methods for microalgal biomass pretreatment. The high-frequency acoustic waves in an ultrasonic disintegrator generates cycles of rapid compression and decompression. This results in the formation of microbubbles, which grow and implode, producing shock waves, highly reactive free radicals, high pressure, and heat, thereby damaging the cell wall. Pretreatment of *Chlamydomonas reinhardtii* biomass by sonication resulted in hydrogen production of 860 mL/L using hyperthermophilic bacterium *Thermotoga neapolitana* (Nguyen et al., 2010). Microwaves are electromagnetic energy with frequency varying from 0.3 to 300 GHz. Generally, microwaves of about 2.5 GHz frequency are known to be optimal for the breakdown of cell walls (Günerken et al., 2015). The rapid oscillation of electric field in a polar or dielectric medium causes chaotic movement of molecules and generation of heat energy due to frictional forces. This heat energy is capable of breaking hydrogen bonds. The effectiveness of this pretreatment method for cellular disruption depends largely upon the process temperature and the power applied. Kumar et al. (2016) observed negligible glucose release from *Chlorella vulgaris* biomass with microwave irradiation at lower reaction temperature (80°C). However, glucose yield of 20%

w/w was achieved when the temperature was increased to 100°C. A further increase in process temperature to 120°C did not improve the sugar yield (Kumar et al., 2016).

Application of acids, alkali, oxidizing agents, solvents, and detergents for the disintegration of microalgal cell walls comes under the category of chemical pretreatment. These methods have the advantage of lower energy requirement compared to mechanical methods. Among the chemicals, different acids (HCl, H_2SO_4, H_3PO_4) and bases (NaOH, KOH, $Ca(OH)_2$) have been widely used. Acids and alkali carry out the cell wall disruption through altering the electric charge of the cell membrane and solubilizing the ester bond, respectively (Wang and Yin, 2018). Although acid and base treatments at room temperature are less energy-intensive, they require a long exposure time for biomass hydrolysis and carbohydrate saccharification. It is believed that use of chemicals in combination with heat treatment is a more efficient method of biomass solubilization and sugar recovery for H_2 production (Xia et al., 2013). Besides the conversion of complex polysaccharides into fermentable sugars, acid-heat pretreatment might generate inhibitory by-products (e.g., furfural, 5-HMF) that can have a detrimental effect on the growth of HPB (Monlau et al., 2014). An ozonolysis process has also been exploited to enhance the fermentability of the microalgal biomass. This method for biomass pretreatment does not lead to the formation of inhibitory by-products. In ozonolysis, the strong oxidizing property of the ozone gas helps in the solubilization of the biomass. A study on ozonation pretreatment of mixed microalgal biomass reported that, depending upon the O_3 dosage, varying degrees of cell wall disruption with increment in dissolved organic carbon can be achieved (Cardeña et al., 2017). Although ozone pretreatment can change the biomass structure significantly, due to the large amount of ozone gas required, the economic feasibility of this process needs to be evaluated.

Biological pretreatment for the hydrolysis of the microalgal cell wall and biopolymers is regarded as a propitious alternative to energy-intensive pretreatment methods. Moreover, this method is eco-friendly and does not involve the formation of inhibitory by-products. Hydrolytic enzymes can efficiently convert the complex microalgal polysaccharides (cellulose, hemicelluloses, and starch) into low molecular weight carbohydrates, which are easily accessible to HPB. However, enzymes have high substrate specificity and the microalgal cell wall composition is enormously diverse. Thus, the applicability of single enzyme systems for efficient hydrolysis of biomass is limited. To address this limitation, use of enzyme cocktails has been recommended (Prajapati et al., 2015). Commonly used commercial enzymes for microalgal biomass hydrolysis and saccharification of carbohydrates include cellulases, lipases, xylanases, α-amylases, amyloglucosidases, and proteases (Hom-Diaz et al., 2016). Wieczorek et al. (2014) evaluated the efficiency of a hydrolytic enzyme mixture to pretreat the *Chlorella vulgaris* biomass for fermentative H_2 production. The results showed that the tested enzymatic mixture significantly enhanced the H_2 yield (135 mL H_2/g VS) from microalgal feedstock by seven times compared to the control (biomass without any pretreatment).

Although enzymatic pretreatment holds some technical advantages over physicochemical pretreatment, the high cost of the pure enzymes makes it economically unviable for microalgal biofuel production on a large scale. In this context, instead of commercial enzymes, use of bacterial or fungal crude enzymes has been suggested as a cost-effective approach for biomass pretreatment (Prajapati et al., 2015; Hom-Diaz et al., 2016; Barragán-Trinidad et al., 2017). Biological pretreatment is usually conducted under mild operating conditions and is a green approach to enhance the fermentability of the microalgal biomass. However, due to the lower rate of hydrolysis, the processing time is high, which limits its stand-alone application for biomass pretreatment. Therefore, in several studies, biological pretreatment has been conducted after physical or chemical pretreatment to achieve higher carbohydrate release in a shorter time (Cheng et al., 2012, 2014; Hernández et al., 2015). Biohydrogen production using microalgal feedstock via anaerobic fermentation is an area of extensive research, producing many publications globally. Table 15.2 summarizes the main findings on dark fermentative biohydrogen production from microalgal substrate.

Table 15.2 Biohydrogen production using microalgal biomass as feedstock via dark fermentation.

Microalgal biomass	Carbohydrate content (% w/w)	Pretreatment method	Inoculum	H_2 yield	References
Microcystis sp.	–	Alkaline (NaOH)	Anaerobic digester sludge	105 mL/g VS	Yan et al. (2010)
Lipid-extracted *Scenedesmus obliquus*	24.7	Alkaline (NaOH) + steam heating	Anaerobic digester sludge	45.54 mL/g VS	Yang et al. (2010)
Chlorella sp.	26.0	None	Anaerobic digester sludge	7.13 mL/g VS	Sun et al. (2011)
Arthospira maxima	–	Enzymatic	Activated sludge	78.7 mL/g TS	Cheng et al. (2011)
Chlorella vulgaris ESP6	57.0	Acidic (HCl) + steam heating	*Clostridium butyricum* CGS5	81.0 mL/g TS	Liu et al. (2012)
Chlorella vulgaris	38.8	Acidic (HCl) +ultrasonication	Anaerobic digester sludge	42.1 mL/g TS	Yun et al. (2013)
Lipid- and pigment-extracted *Nannochloropsis*	–	Milling + supercritical fluid extraction	*Enterobacter aerogenes* ATCC 13048	60.6 mL/g TS	Nobre et al. (2013)
Microcystis wesenbergii	12.0			47.1 mL/g VS	Cheng et al. (2014)

Table 15.2 Biohydrogen production using microalgal biomass as feedstock via dark fermentation—cont'd

Microalgal biomass	Carbohydrate content (% w/w)	Pretreatment method	Inoculum	H_2 yield	References
+ *Microcystis aeruginosa*		Acidic (H_2SO_4) + microwave heating + enzymatic	Anaerobic digester sludge		
Scenedesmus obliquus	30.7	Autoclave	*Clostridium butyricum* DSM10702	90.3 mL/g TS	Batista et al. (2014)
Chlorella sorokiniana	14.5	Acidic (HCl) + steam heating	Thermophilic mixed culture	333.5 mL/g hexose	Roy et al. (2014)
Chlorella vulgaris FSPE	51.3	Acidic (H_2SO_4) + steam heating	*Clostridium butyricum* CGS5	2.87 mmol/g TS	Chen et al. (2016)
Scenedesmus obliquus	30.7	Autoclave	*Enterobacter aerogenes*	40.9 mL/g TS	Batista et al. (2018)

15.3 Challenges and future perspectives of biohydrogen production from microalgae

Microalgae have great potential and seem to be an ideal candidate for the production of biohydrogen in a sustainable way. Nonetheless, doubts have been raised regarding the feasibility of microalgal-based hydrogen production systems due to the several research challenges associated with them. For instance, one of the major impediments of biophotolytic hydrogen production is the extreme O_2 sensitivity of the microalgal hydrogenases. This is a multifaceted problem because the presence of O_2 inhibits not only the activity of the [Fe-Fe] hydrogenases but also the transcription and the maturation of the protein (Oey et al., 2016). It has been reported that very low concentrations of O_2 can make the hydrogenases catalytically inactive by destroying the [4Fe-4S] domain of the H-cluster (Stripp et al., 2009). Several approaches have been taken to alter the O_2 sensitivity for sustained H_2 production including random mutagenesis, targeted mutagenesis, and recombinant system (Ghirardi et al., 2000; Xu et al., 2005). A different approach of O_2 sequestration was also developed by transforming the leghemoglobin proteins of the soybean plant into the chloroplast of *Chlamydomonas reinhardtii*. This strategy helped in rapid consumption of O_2, and the developed transgenic microalgal cultures produced H_2 greater than four times that of the control cultures (Wu et al., 2010). However, the issue of enzymes' sensitivity to O_2 is yet to be explored. Protein engineering-based research might be a promising avenue to achieve an oxygen-insensitive hydrogenase for sustainable H_2 production.

The other issue associated with biophotolysis is the limited availability of electrons for hydrogenases. This is due to the occurrence of other biochemical pathways that compete for the primary electron donors such as ferredoxin and NAD(P)H. These competitive metabolic pathways are mainly catalyzed by enzymes such as ferredoxin-$NADP^+$ reductase (FNR), nitrate reductase, glutamate synthase, and sulfite reductase (Das et al., 2014). Various molecular approaches including the downregulation of FNR, fusion of ferredoxin with hydrogenase, and fusion of ferredoxin with PS I have been followed to improve the electrons flow toward hydrogenase enzyme and enhance H_2 evolution (Sun et al., 2013; Rumpel et al., 2014; Yacoby et al., 2011; Wittenberg et al., 2013). Other than genetic engineering, strategies like culturing microalgal cells in medium containing ammonium instead of nitrate- and CO_2-depleted media have been proposed to enhance the availability of reduced ferredoxin for H_2 production (Maness et al., 2009).

Low photon conversion efficiency is the other major bottleneck of the microalgal H_2 production system. The maximum theoretical sunlight-to-hydrogen energy conversion efficiency by green microalgae is approximately 12%. However, practically, the solar conversion efficiency of the microalgal system is often below 1% (Srirangan et al., 2011). Microalgae assemble huge antennae systems (light harvesting complex, LHC) to capture maximum photons under natural environmental conditions. Under bright light conditions, the rate of photon absorption by LHC exceeds the rate of photon consumption for photosynthesis. As a survival strategy, 90% of these photons get dissipated via a mechanism known as nonphotochemical quenching (NPQ) in the form of heat. In dense algal cultures, NPQ occurs at the upper surface. The light does not penetrate efficiently to the lower surface, which causes a "self-shading effect."

Thus, NPQ and the self-shading effect result in poor photon conversion efficiency. To circumvent this problem, modifying the antenna complex and photobioreactor (PBR) design have been suggested as plausible strategies. A mutant strain of *Chlamydomonas reinhardtii* having smaller-sized antenna showed better photon conversion efficiency and H_2 production compared to the wild strain (Polle et al., 2002; Kosourov et al., 2011). PBR with better light penetration and distribution is crucial to obtain high photon conversion efficiency. The main parameter affecting the photosynthetic photon flux density (PPFD) is the surface area/volume (A/V) ratio of the reactor (Das et al., 2014). Tredici and Zittelli (1998) achieved photosynthetic efficiency of 6.6% with *Arthrospira platensis*, using helical tubular PBR having a high A/V ratio. Recently, for the uniform distribution of the light energy within the microalgal culture, planar waveguides doped with light-scattering nanoparticles were embedded into the flat-plate PBR. The modified PBR enhanced the illuminated surface area per unit volume and biomass production by 10.3 times and 220%, respectively (Sun et al., 2016). Although biophotolytic production of biohydrogen was first reported in the 1940s (Gaffron and Rubin, 1942), the research in microalgal hydrogen production system remains

in its infancy. Major breakthroughs are required to improve process efficiency and economic viability before this technology can be adopted on a large scale.

Dark fermentation (DF) utilizing microalgal feedstock is another promising approach for biological production of hydrogen. This is an anaerobic fermentation process, and hydrogenase is therefore never exposed to molecular O_2. Moreover, unlike biophotolysis, the efficiency of this system is not affected by solar radiation. Despite these clear benefits, DF also suffers from serious challenges that need to be addressed in order to gain commercial value. The major drawback is the low energy recovery from the substrate. In DF, a large part of the organic matter does not get converted into hydrogen and remains inside the liquid fraction in the form of soluble metabolites (acetate, butyrate, alcohols, etc.). This lowers the overall energy recovery and the effluent rich in organic acids may pose a threat to environment. To overcome this barrier at least partly, several integrative systems have been proposed where the spent media of DF process can be valorized in biomethanation, photo-fermentation, bio-electrochemical systems, and as feedstock for microalgal cultivation.

The integration of biohydrogen production process with biomethanation under the name of "biohythane" has been considered as an ideal integrative technology to harness maximum energy recovery from biomass (Kumari and Das, 2015). As per the authors' knowledge, there are many publications on biohythane production from various biomass-based feedstock in a two-stage process. However, few research articles have considered the subsequent utilization of the effluent of DF process for biomethane production from microalgal biomass (Cheng et al., 2011; Yang et al., 2011). Cheng et al. (2011) carried out the co-production of H_2 and CH_4 from *Arthospira maxima* biomass and evaluated the energy conversion efficiency (ECE) of the two-phase fermentation system. The cogeneration system resulted in significantly higher ECE (27.7%) compared to the hydrogen fermentation system alone (2.6%). The combination of photofermentation (PF) with DF is another interesting approach to maximize the substrate energy recovery. In the presence of light, photofermentative bacteria are able to utilize the spent media of DF rich in volatile fatty acids and convert it to molecular hydrogen (Dasgupta et al., 2010). By integrating both DF and PF processes, a theoretical maximum yield of 12 mol H_2/mol glucose can be achieved (Das and Veziroglu, 2008). So far, only a few studies have combined the DF and PF process to enhance biohydrogen production using microalgal feedstock (Kim et al., 2006; Cheng et al., 2012; Xia et al., 2013). Cheng et al. (2012) indicated the potentiality of the DF-PF integrated system for maximization of biohydrogen production from *Arthrospira platensis* wet biomass. The combined process significantly enhanced the hydrogen yield from 96.6 to 337.0 mL H_2/g DW. Moreover, a three-stage process (combining dark-fermentation, photofermentation, and biomethanation) has also been proposed for efficient valorization of DF effluent (Xia et al., 2013).

VFA-rich outlets of DF can be channeled to bioelectrochemical systems (BES) such as MFC and microbial electrochemical cells (MECs) to harness the valuable energy. These technologies

represent an elegant application of thermodynamic principles to manipulate the microbial metabolism for biofuel production. MFC provides a direct method of converting the organic feedstock into electricity by the help of exoelectrogens. The advent of MEC systems, based on MFC technology, delineates immense potential for biohydrogen production with high yield. In MEC systems, the addition of a small voltage (generally >0.2 V) between the electrodes could evolve hydrogen at the cathode by the reduction of protons. In this process, it is possible to achieve the stoichiometric potential (12 mol H_2/mol glucose) by overcoming the thermodynamic restrictions (4 mol H_2/mol glucose) of DF. A combination of DF process with BES has been already reported with substrates such as vegetable wastes, crude glycerol, and water hyacinth (Mohanakrishna et al., 2010; Chookaew et al., 2014; Varanasi et al., 2018). However, such types of integrative system are yet to be explored in terms of microalgal feedstock for the improvisation of the DF process and to reach practical levels. Another interesting approach to valorize the DF effluent could be its utilization for microalgal cultivation to produce biodiesel. Ghosh et al. (2017) developed an integrative system to utilize efficiently the VFA-rich spent media for the lipid production using an oleaginous microalgal strain. *Chlorella* sp. MJ 11/11 was mixotrophically cultivated for biodiesel production using the effluent of thermophilic dark fermentation. The spent media along with acetate were found to be suitable carbon sources corresponding to the higher level of lipid content (58% w/w) and unsaturated fatty acids (77.8%) achieved.

The other limitation of the fermentative biohydrogen production from microalgal feedstock is the requirement of a pretreatment step. This is a crucial step to enhance the accessibility of microalgal carbohydrates to HPB. To date, pretreatment has been considered as one of the most expensive processing steps for the conversion of biomass into low-value-added products like bioethanol and biohydrogen (Harun and Danquah, 2011; Roy et al., 2014). A number of physicochemical methods have been investigated for the pretreatment of the microalgal biomass. However, most of these methods are energy-intensive; they also result in formation of by-products (furfural, HMF and formic acid) that may inhibit the HPB (Monlau et al., 2014). Furthermore, the research based on biological pretreatment of microalgal biomass has received scarce attention. Therefore, for the development of an efficient microalgal biomass pretreatment process for H_2 production, it is imperative to address a few issues, such as the following:

(1) More investigations are required to determine the minimum inhibitory concentration (MIC) of each inhibitory by-product on HPB. It is necessary to explore whether the extent of inhibition depends upon the inoculum type (i.e., pure or mixed cultures), inoculum size, or on synergetic effects of different by-products generated.
(2) More focus is needed on biological pretreatment of biomass. In particular, development of crude enzymatic cocktails is required to hydrolyze the microalgal feedstock.
(3) The environmental, energetic, and economic assessments of various pretreatment methods should be carried out to define their feasibility for the microalgal-based DF process.

The other bottleneck of DF that needs to be overcome involves separation/purification of bioH_2. Unlike biophotolysis, which generates only H_2, the DF process primarily produces a mixture of H_2 (concentration less than 70%), CO_2, and moisture. Therefore, purification/separation of H_2 is essential before its practical usage (Show et al., 2018). Moreover, studies on a large scale are critical before releasing the microalgal hydrogen production process for commercialization. At a large scale, various operational and technical difficulties may arise that cannot be realized at laboratory scale. Hence, for conversion of any process into technology, a realistic pilot-scale study is crucial.

15.4 Conclusion

Hydrogen production through microalgal systems is an effective route for clean and renewable energy generation. Microalgae possess the inherent capacity to convert solar energy into molecular H_2 through water splitting. Moreover, carbohydrate-rich microalgal biomass has immense potential to serve as feedstock for H_2 production in anaerobic fermentation processes. However, the microalgal H_2 production has several limitations. The major challenges in the biophotolysis process are the incompatibility between the oxygenic photosynthesis and H_2 evolution and low photon conversion efficiency, while poor substrate conversion efficiency and requirement of biomass pretreatment step are the main hurdles in dark fermentation. Significant efforts have been made to overcome these bottlenecks through the development of various bioprocess and genetic engineering approaches. However, at present, H_2 production from microalgae is economically less appealing. Further technological advancements are needed to make the microalgal H_2 production process cost-effective and sustainable.

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Further reading

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CHAPTER 16

Microalgae single cell oil

Yusuf Nazir[a], Hafiy Halim[a], Pranesha Prabhakaran[a], Aidil Abdul Hamid[b], Yuanda Song[a]

[a]Colin Ratledge Center for Microbial Lipids, School of Agriculture Engineering and Food Science, Shandong University of Technology, Zibo, China [b]School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia, Bangi, Malaysia

Chapter outline

16.1 Introduction 419
- 16.1.1 Term definition 420
- 16.1.2 A brief history of MSCO development 420

16.2 Microalgae regarded as MSCOPS, their major fatty acid compositions and potential applications 424
- 16.2.1 Potential application of MSCO as biofuel feedstock 424
- 16.2.2 Potential application of MSCO in PUFA production 426

16.3 Biosynthetic pathway for MSCO biosynthesis and its regulation 427
- 16.3.1 Biosynthetic pathway for MSCO biosynthesis 427
- 16.3.2 Regulation of fatty acid biosynthetic pathway for improved MSCO production by manipulating the environmental stresses and metabolic engineering 431

16.4 Cultivation system of microalgae for MSCO production 435

16.5 Current market, challenges, and future prospects of MSCO 437

16.6 Conclusions 439

References 439

16.1 Introduction

Microalgae are either prokaryotic or eukaryotic and are phototropic, mixotrophic, or heterotrophic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. Prokaryotic microalgae include cyanobacteria (*Cyanophyceae*), while green algae (*Chlorophyta)* and diatoms (*Bacillariophyta*) are examples of eukaryotic microalgae. Under natural growth conditions, phototrophic microalgae require primarily three components to produce biomass: water, carbon dioxide (CO_2), and sunlight.

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Heterotrophic microalgae utilize organic carbon and do not depend on sunlight and CO_2 for growth, while mixotrophic microalgae may live in both conditions.

Microalgae are ubiquitous and it is estimated that more than 50,000 species exist, although only around 30,000 have been studied. Depending on the species, they can grow in many different environments including terrestrial and marine, some of which are considered very inhospitable (Serra, 2015). Exposure to different stress factors in the environment leading them to evolve and produce numerous metabolites including various high-value compounds such as lipids. Microalgal lipids, or lipids derived from oleaginous microalgae, are often termed microalgae single cell oils (MSCOs). Over the past decades, MSCOs have attracted considerable interest worldwide, due to their extensive application potential in the renewable energy, biopharmaceutical, and nutraceutical industries. Hence, efforts have been made to understand the overall aspects in MSCO biosynthetic regulation and development. This review aims to discuss comprehensively the historical perspective, the biosynthetic regulatory mechanisms involved in MSCO productions, cultivation systems, and challenges for commercial-level applications.

16.1.1 Term definition

The term MSCO was originally derived from the word "single cell oils" (SCOs) which was introduced by Ratledge in 1976 to describe lipids produced by single-celled microbes that would be suitable for human consumption as alternatives to plants, animal oils, and fats. The term SCO was invented as a means to avoid mentioning the microbial sources (i.e., bacteria, yeasts, and fungi) that the general public might find difficult to appreciate. However, the term has since been expanded to include all fatty acid-containing lipids within a single cell including algae and microalgae, giving rise to the term "microalgae single cell oil" (MSCO). The lipids produced by algae and microalgae are considered unique as they contain not only a major fraction of triacylglyceride (TAG) but also a complex array of other lipid types including many glycosylated and sulfur-containing lipids involved with the photosynthetic apparatus of these organisms (Ratledge, 2013).

16.1.2 A brief history of MSCO development

In the present day, MSCO is no longer an unfamiliar subject within the scientific world. This is evident from the many studies and publications over the past 20 years discussing the many potentials of MSCO. However, the term MSCO was still considered relatively new as recently as the 1990s. The interest in microbial lipids actually expanded 200 years ago, although it is only since the 1990s that microbial lipid production has received focused in terms of edible oils (Ratledge, 2004; Borowitzka, 2013).

Attempts to isolate microalgae began in the 1850s, with Ferdinand Cohn succeeding in keeping the unicellular flagellate *Haematococcus* in situ for some time; this gave rise to the term "*cultivation*." Unfortunately, Cohn did not use a culture medium and hence did not establish a way to maintain the culture. The plant physiologist Famintzin made the first attempt to culture algae in 1871 using a solution of a few inorganic salts. However, the earliest report of pure axenic cultures of microalgae originated from the Dutch microbiologist Beijerinck in 1890. Beijerinck was allegedly the first scientist to isolate free-living *Chlorella* and *Scenedesmus* in bacteria-free cultures, and to isolate symbiotic green algae from *Hydra* ("*Zoochlorella*") and lichens. He later reported successfully isolating pure cultures of other algae, including cyanobacteria and diatoms (Preisig and Andersen, 2005). After that, more and more pure cultures of microalgae began to rise from all over the globe using a variety of isolation and culturing techniques. In 1910, Allen and Nelson became the first to attempt the mass culturing of microalgae for aquaculture. The ability to isolate a pure culture was the start of better understanding and increasing the prospect in studying the physiology of microalgae (Borowitzka, 2013).

Microbial oil has long been a topic of interest; the oil content of certain molds and yeast was established in 1873 and 1878, respectively (Kyle and Ratledge, 1992). It was not until 70 years later, in 1942, that a report about the lipid content in microalgae was first published, when Harder and Witsch described the microalgae *Pennales* spp., which was able to accumulate 40%–50% lipid (Harder and von Witsch, 1942). At this point, more interest started to be focused on the study of lipids within microalgae. Initial microbial oil research and development were originally stimulated due to the intense political and economic disturbances of the First and Second World Wars. Research and development of microbial oil during these years were being focused on defensive initiatives—for example, to be used as fodder to feed horses being used by the army. However, over time, and during the years of peace, these defensive initiatives were slowly converted to a commercial focus with the development of microbial oil (Kyle, 2010).

In the early 2000s, the awareness of the importance of polyunsaturated fatty acids (PUFA) in the human diet, especially DHA and EPA, resulted in remarkable interest regarding its industrialization and commercialization. A heterotrophically grown MSCO-producing strain (*Crypthecodinium cohnii*) was developed by Martek (now Royal DSM) as a model organism for DHA production in the United States, with a production capacity exceeding 3000 m^3 by the end of 2004 (Wynn et al., 2010). Two years later, DSM's algal oil (DHASCO) was approved for use in infant formula by the European Union (EU) under Commission Directive 2006/141/EC. The model organism for DHA production is now expended to *Schizochytrium* sp. and is being commercialized under the name DSM "life's" as a novel food and dietary supplement. Several years later, Aurora Algae commercialized an EPA product under the name A2 EPA Pure, utilizing a patented MSCO-producing strain (*Nannochloropsis*) which contains more than 65% EPA in large seawater open ponds,

targeted for use in the supplements and pharmaceuticals sectors (Winwood, 2013). The early 21st century also witnessed significant scientific contributions in MSCO development as a new fatty acid biosynthetic pathway (PKS pathway) was discovered in *Schizochytrium* (Metz et al., 2001) as well as a new model for diatom (*Phaeodactylum tricornutum*) being developed (Kroth et al., 2008).

Even though there were many commercially developed MSCO-producing strains (MSCOPS) in the early 21st century, the industry still suffers from a major problem in the form of exorbitant production costs. Many efforts were made to tackle this constraint, and one of the most promising strategies was to enhance the yield of the production by developing genetically tailored microalgae with an enhanced growth rate and high lipid biosynthetic capacity. Therefore, the 21st century has witnessed tremendous advancements in the field of synthetic biology and lipid engineering in MSCO. Lipid engineering has been applied in microalgae with the aim to produce triacylglycerol (TAG) with better cold-flow properties, making them a better feedstock suitable for pharmaceutical and biodiesel production (Jagadevan et al., 2018). However, this is only being applied to a limited number of MSCO model strains such as *Chlamydomonas reinhardtii*, *Nannochloropsis* sp., *P. tricornutum*, *Cyanidioschyzon merolae*, *Ostreococcus tauri*, and *Thalassiosira pseudonana* (Hlavova et al., 2015). The studies that have been carried out so far are genome scale modeling (GSM), employment of advanced synthetic tools to engineer synthetic chromosome and metabolic pathways, etc. (Fig. 16.1) (Jagadevan et al., 2018; Tibocha-Bonilla et al., 2018). One early attempt is characterization of pathways to obtain extensive knowledge about carbon sharing that enables construction of artificial pathways for lipid biosynthesis in MSCO model organisms. For instance, investigations have been carried out to observe carbon partitioning between starch and lipids and the key players of TAG formation under stress in *C. reinhardtii* were identified, which led to a better understanding in lipid biosynthetic regulation in microalgae (Goncalves et al., 2016). In addition, employment of advanced synthetic biology tools such as CRISPR/Cas and TALEN aids in constructing complex programmable synthetic circuits that enrich fatty acid profiles for better biofuel and PUFA production in *C. reinhardtii*, *Nannochloropsis oceanica*, and *P. tricornutum*. For example, a recent study reported the production of TAG with a wide degree of unsaturation by rational modulation of type II diacyglycerol acyltransferases in *N. oceanica* via reverse genetics (Xin et al., 2017). Furthermore, construction of comprehensive genome-scale models for eukaryotic microalgae has enabled custom designing and production of novel molecules by accurately predicting chemical transformations taking place within cellular components and focuses on delineating genetic networks that are industrially desirable (Tibocha-Bonilla et al., 2018). de Oliveira Dal'Molin et al. (2011) constructed a genome-scale metabolic model (named AlgaGEM) based on the genomic sequence of *C. reinhardtii* with the functions of 866 unique ORFs, 1862 metabolites, 2249 gene-enzyme reaction association entries, and 1725 unique reactions. Rewiring the metabolic network in *C. cohnii* (Diao et al., 2019) as well as enhancing biosynthesis of both

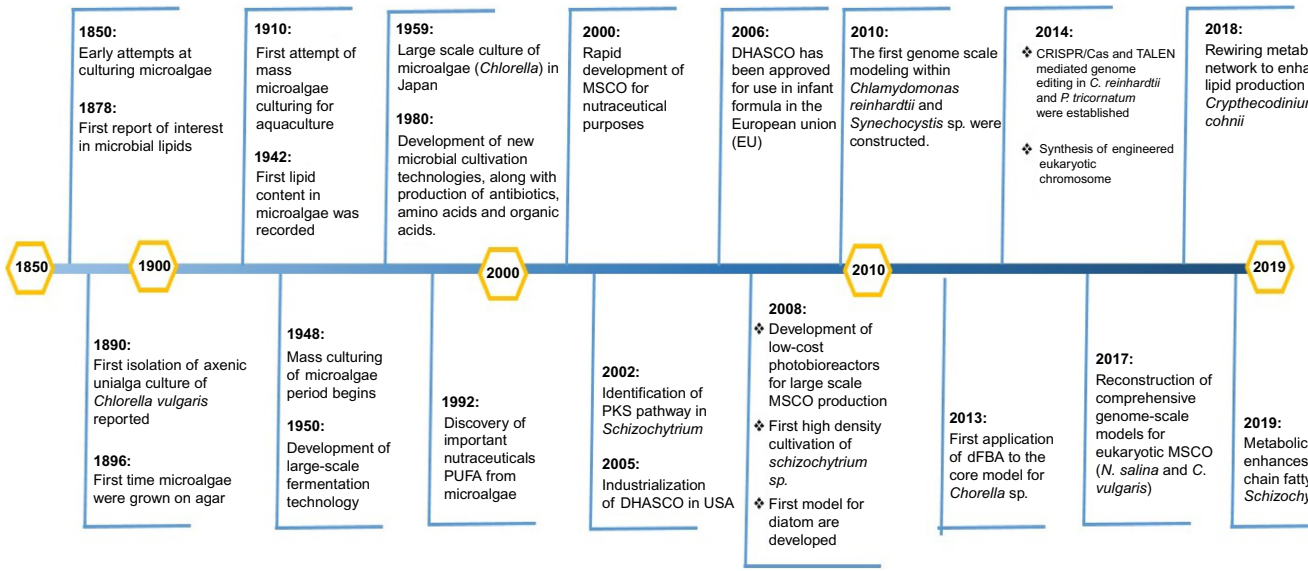

Fig. 16.1

Timeline showing the brief history of MSCO development (Ratledge, 2004; Borowitzka, 2013; Kyle and Ratledge, 1992; Harder and von Witsch, 1942; Kyle, 2010; Wynn et al., 2010; Winwood, 2013; Metz et al., 2001; Kroth et al., 2008; Jagadevan et al., 2018; Hlavova et al., 2015; Tibocha-Bonilla et al., 2018; Goncalves et al., 2016; Xin et al., 2017; de Oliveira Dal'Molin et al., 2011; Diao et al., 2019; Wang et al., 2019).

docosahexaenoic acid and odd-chain fatty acids in *Schizochytrium* sp. by metabolic engineering also marked a significant achievement in MSCO development (Wang et al., 2019) (Fig. 16.1).

16.2 Microalgae regarded as MSCOPS, their major fatty acid compositions and potential applications

Microalgae single cell oils (MSCOs) are defined as lipids produced by oleaginous microalgae that are capable of accumulating more than 20% lipid of the dry cell weight. Many microalgae species are capable of accumulating high lipid content with averages between 20% and 50%, while under certain cultural environments, some MSCOPS may accumulate lipid up to 70%–90% of the dry cell weight (Scott et al., 2010; Chen and Jiang, 2017). In addition, MSCOPS are also capable of generating various types of fatty acids, including short and long chain, composed mainly of saturated and unsaturated fatty acids, depending on the species, cultivation mode, and availability of key nutrients (Table 16.1). The basic key nutrients required for MSCOPS cultivation regardless of their cultivation mode includes the availability of organic or inorganic carbon, water, O_2 or CO_2, nitrogen, phosphorus, macronutrients (Na, Mg, Ca, and K), and micronutrients (Mo, Mn, B, Co, Fe, Zn, etc.). In addition, optimal temperature, pH, salinity, stirring and mixing, width and depth of the bioreactor, harvest frequency, and dilution rate also have great influence and impact on the overall yield of biomass and bio-products in microalgae (Khan et al., 2018). Optimization of these key nutrients and culture conditions has been proven to enhance lipids and specific fatty acids in microalgae (Khan et al., 2018; Nazir et al., 2018). The notable ability of microalgae in producing high lipid content and diverse fatty acid compositions has resulted in significant development for their commercial application, especially in biofuel and PUFA production.

16.2.1 Potential application of MSCO as biofuel feedstock

Rapid industrialization, coupled with an upsurge in global population, has significantly increased worldwide energy consumption and demand, which is currently being fulfilled by various nonrenewable energy sources, particularly fossil fuels. However, the energy consumption rate of these resources is reported to be 105 times greater than the replenishment rate, thus, triggering calls for immediate attention to look for alternative, renewable, and sustainable energy resources (Shuba and Kifle, 2018). Biomass-derived biofuels are currently being considered as the most sustainable alternative to fossil fuels. However, increased competition of crop-based first- and second-generation biofuels with food crops for arable land coupled with high corrosivity and hygroscopicity of bioethanol suggests that it may not be an ideal candidate to replace fossil fuels (Jagadevan et al., 2018). Therefore, third-generation biofuels derived from MSCO have been developed. This is because MSCOPS are able to produce 10–300 times more oil for biodiesel production than traditional crops on an area basis, shorter harvesting cycle, does not require arable land, using far less water, overcoming

Table 16.1: List of MSCOPS, their cultivation modes, and major fatty acid compositions.

List of MSCOPS	Cultivation mode	Lipid content (% dry weight of biomass)	Major fatty acid compositions (% total lipid)	References
Chlamydomonas reinhardtii	Photoautotroph	12–64	16:0 (4–19) 18:1 (1–11) 18:2 (1.5–10) 18:3 (2–7)	James et al. (2011)
Nannochloropsis oculata	Photoautotroph	22–37	16:0 (14.0–24.2) 16:1 (24.8–25.8) 20:5 (27.8–30.8)	Araujo et al. (2011)
Neochloris oleoabundans UTEX 1185	Photoautotroph	19–56	16:1 (34–42) 16:0 (15–22) 18:0 (14–18)	Gouveia et al. (2009)
Scenedesmus obliquus	Photoautotroph	21–58	18:1 (18–25) 18:2 (14–18) 18:3 (20–33)	Abou-Shanab et al. (2011)
Haematococcus pluvialis	Photoautotroph, mixotroph	15–34	16:0 (12–24) 16:1 (1–5) 18:1 (5–34) 18:2 (2–25) 18:3 (8–39)	Damiani et al. (2010)
Chlorella vulgaris	Heterotroph, and autotroph	20–42	16:0 (20.6–26.0) 16:1 (6.60–10.80) 18:0 (11.40–20.8) 18:1 (27.2–33.4)	Feng et al. (2011) and Widjaja et al. (2009)
Dunaliella sp.	Photoautotroph, mixotroph	12–30.12	16:0 (10–12) 16:1 (12–16) 18:1 (8–10) 18:3 (12–18) 22:6 (14–16)	Araujo et al. (2011)
Phaeodactylum tricornutum	Photoautotroph, mixotroph		16:0 (14–16) 16:1 (40–60) 20:5 (20–30)	Rodolfi et al. (2009)
Schizochytrium	Heterotroph	30–70	14:0 (2–8) 16:0 (20–45) 22:5 (5–12) 22:6 (35–55)	Manikan et al. (2015) and Chang et al. (2013)
Chlorella protothecoides	Heterotroph	50.5	17:0 (11.34) 18:1 (20–25) 19:1 (19.48) 19:0 (53.75)	Xiong et al. (2008)
Crypthecodinium cohnii	Heterotroph	25–50	16:0 (20–45) 22:6 (35–55)	Wynn et al. (2010)
Ulkenia sp.	Heterotroph	20%–52%	16:0 (25–30) 18:1 (10–12) 20:5 (5–15) 22:6 (15–30)	Chang et al. (2014)

food vs fuel dilemma and other shortcomings associated with the utilization of first-generation and second-generation biofuel (Mathimani and Pugazhendhi, 2018). In addition, many MSCOPS, especially photoautotrophs such as *Chlorella vulgaris* BDUG 91771, *Neochloris oleoabundans*, *C. reinhardtii*, *Chlorella protothecoides*, etc., are capable of producing high content of C:16, C16:1, C17, C18:1, and C19—fatty acids which are favorable for biofuel production (Table 16.1). Therefore, several initiatives and programs such as the National Renewable Energy Laboratory (NREL) have been launched for research and development, specifically for MSCO-based biofuel production (Sheehan et al., 1998). However, there are still several constraints and challenges that require significant attention to enable commercial application.

16.2.2 Potential application of MSCO in PUFA production

Long-chain polyunsaturated fatty acids (LC-PUFA) are the common product of interest of high-value product from the MSCO. PUFA such as omega-3 and omega-6 fatty acids, in particular, are essential for humans, but the human body is incapable of producing them in adequate quantities. Hence, intake from external sources is essential. PUFA like DHA, EPA, arachidonic acid (ARA), and gamma-linolenic acid (GLA) have been proven to reduce cholesterol levels, prevent cardiovascular diseases and Alzheimer's, delay aging, and more (Khan et al., 2018). Several MSCOPS such as *P. tricornutum* and *Nannochloropsis* sp. are able to accumulate high EPA content up to 50% of their total fatty acids, and are currently being used for commercialization of EPA and aqua feed (Table 16.1). In addition, *Dunaliella* spp. and *Haematococcus pluvialis*, which grow in both photoautotrophic and mixotrophic modes of cultivations, are capable of producing not only high lipid and PUFA content but also commercially viable pigments like astaxanthin and β-carotene (Damiani et al., 2010; Araujo et al., 2011). Recently, genetically modified *P. tricornutum* attracted attention as a potential source of EPA and DHA, as it could accumulate a maximum yield of 36.5% and 23.6% of DHA and EPA per total fatty acids, demonstrating its feasibility for production at commercial scale (Chauton et al., 2015). In addition, heterotrophic microalgae such as *Ulkenia* sp., *Schizochytrium, C. cohnii*, and *C. protothecoides* are able to accumulate lipid over 50% of DCW, which is mainly composed of PUFAs such as DHA, DPA, and EPA (Table 16.1). They are able to assimilate various types of organic carbon sources such as glucose, fructose, acetate, and mannose. Despite this promising potential, there are several hurdles and challenges for commercial-scale production, such as high production cost and complexity of downstream processing. Several initiatives to overcome these hurdles were piloted—for example, utilizing cheap, extensively available alternative carbon sources such as molasses hydrolysate, Jerusalem artichoke hydrolysate, cassava starch, sweet sorghum juices, glycerol, and many more (Xiong et al., 2008; Wei et al., 2009; Yan et al., 2011; Liang et al., 2010). For instance, several strains of *Schizochytrium* are able to accumulate high lipid content ranging

from 45% to 70% of DCW when cultivated using sweet sorghum juices and glycerol as an alternative carbon source (Liang et al., 2010; Chang et al., 2013).

16.3 Biosynthetic pathway for MSCO biosynthesis and its regulation

16.3.1 Biosynthetic pathway for MSCO biosynthesis

Lipid biosynthesis is a complex pathway that occurs naturally as all living organisms are obligated to synthesize a minimum amount of lipid for membranes and other structural and functional roles. This process involves many enzymatic reactions that take place in a different subcellular compartment which demands an adequate amount of carbon (acetyl-CoA) and NADPH. The major differences that distinguish autotrophic, mixotrophic, and heterotrophic microalgae are their dependency on organic and inorganic carbon as a precursor for the central carbon metabolic pathway that generates acetyl-CoA and NADPH, their starting components, and intermediate for the fatty acids synthesis (Fig. 16.2).

Typically, heterotrophic microalgae utilize organic substrates such as glucose, fructose, acetate, ethanol, and glycerol as sole carbon and energy sources as they lack photosynthetic machinery and do not depend on sunlight for energy. Utilization of these carbon sources requires various enzymatic systems for transport, activation via phosphorylation, intermediary anabolic and catabolic metabolism, and energy generation by substrate level and/or respiration (Morales-Sánchez et al., 2015). For instance, acetate is metabolized via two pathways, namely the glyoxylate cycle to produce malate in glyoxysomes and the tricarboxylic acid (TCA) cycle to produce citrate in mitochondria as carbon backbone and ATP; glycerol is converted into pyruvate through the Embden-Meyerhof-Parnas (EMP) pathways, which will later enter the TCA cycle (Khan et al., 2016). On the other side, production of acetyl-CoA pool in photosynthetic microalgae occurs not only from the endogenous source of plastidial acetyl-CoA, but also by carbon dioxide fixation using light energy (Garay et al., 2014; Taggar et al., 2014). The process takes place in the chloroplast, where photosynthetic machinery metabolizes atmospheric carbon to produce starch that will be catabolized via glycolysis with the Calvin cycle as a preliminary pathway for carbon fixation (Sharma et al., 2018).

Fascinatingly, for mixotrophic microalgae, both inorganic carbon dioxide and organic carbon sources can be used with the presence of light (Fig. 16.2). Carbon (carbon dioxide) fixation occurs via photosynthesis, whereas organic substrates are assimilated using aerobic respiration that influences by the accessibility of organic carbon (Perez-Garcia and Bashan, 2015). However, with the presence of organic carbon sources, the demands for light for carbon fixation in the mixotrophic condition are weaker when compared to autotrophic conditions (Chen et al., 2017). A green microalga *Chlorella zofingiensis*, which had been cultivated under both photoautotrophic and heterotrophic conditions, exhibited different growth, lipid, and fatty acid profile with significantly higher total lipids, comprising oleic acids and TAG under

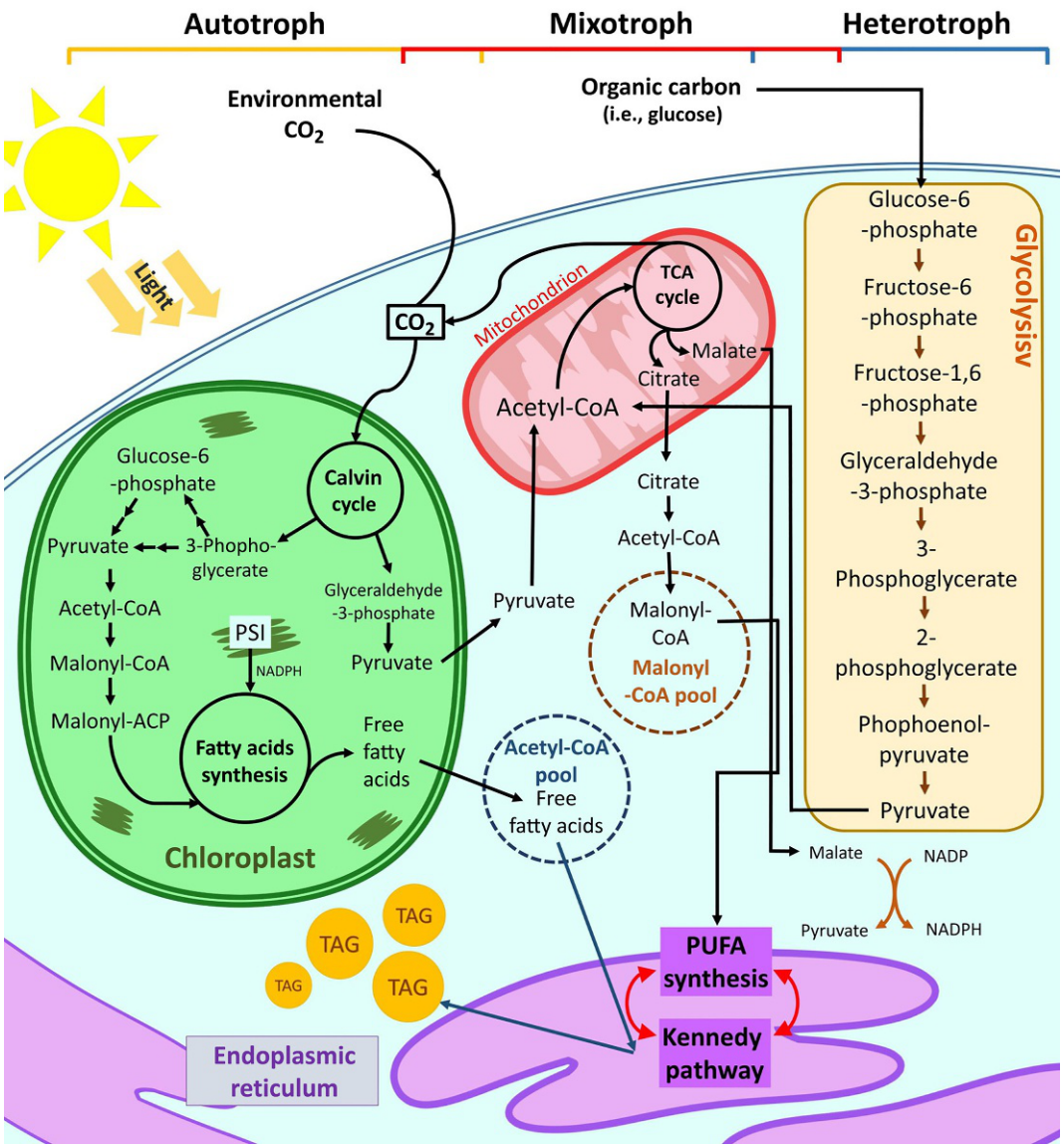

Fig. 16.2

Regulation mechanism of lipid biosynthesis in MSCO. The indicator (]) indicates the differences of the carbon fixation process in autotropic, mixotrophic, and heterotrophic MSCOPS.

heterotrophic conditions in comparison to photoautotrophic cells (Liu et al., 2011). This verified that different growth conditions could significantly influence lipid production in microalgae and manipulate the composition of the lipid content.

As mentioned earlier, acetyl-CoA and NADPH are the two main precursors for fatty acid synthesis. The formation of acetyl-CoA in MCSO is catalyzed by ATP: citrate lyase (ACL)

from its substrate, citric acid that is synthesized as part of the tricarboxylic acid (TCA) cycle. Interestingly this process does not occur in most nonoleaginous species (Ratledge, 2004; Zulu et al., 2018). Environmental stresses such as nitrogen or phosphorous depletion may interrupt the TCA cycle by inhibition of NAD^+-isocitrate dehydrogenase, resulting in citrate accumulation in the mitochondria and excretion into the cytosol. In the cytosol, the ACL will then catalyze citrate into oxaloacetate and acetyl-CoA (Bellou et al., 2014). The resulting acetyl-CoA will then be available and utilized for fatty acid synthesis. On the other hand, malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), and photosystem I (PSI) are known as major suppliers of NADPH for fatty acid synthesis in MSCOPS, depending on the cultivation mode. However, the NADPH supply from PSI only exists in photoautotrophic microalgae which are produced as a result of light-dependent reactions in the chloroplasts. ME is responsible in catalyzing the conversion of malate into pyruvate and NADPH via the transhydrogenase cycle pathway, which has been proven to have a positive effect on the increment of lipid content in oleaginous microorganisms (Liang and Jiang, 2015), whereas G6PDH is a rate-limiting enzyme in the pentose phosphate pathway (PPP) which catalyzes the conversion of glucose-6-phosphate into 6-phosphogluconolactone with the yield of one NADPH molecule (Xie and Wang, 2015). A study conducted by Bellou et al. (2014) showed that *Chlorella* sp. and *Nannochloropsis salina* when grown in photoautotrophic conditions have very low ME activity, indicating that the major donor of NADPH may be the photosystem I and PPP.

Fatty acid biosynthesis begins with the rate-limiting step pathway which is the ATP-dependent carboxylation of acetyl-CoA into malonyl-CoA, catalyzed by the enzyme acetyl-CoA carboxylase (ACC), which presents either in plastid or in the cytosol (Chen and Jiang, 2017; Bellou et al., 2014; Zulu et al., 2018). Carboxylation of one acetyl unit to produce one malonyl unit demands one molecule of ATP (Baba and Shiraiwa, 2013). This biosynthesis process continues with transfer of malonyl-CoA to an acyl-carrier protein (ACP) to form malonyl-acyl-carrier protein (malonyl-ACP) by malonyl-CoA ACP transacylase, which is one of the fatty acid synthase (FAS) multienzymatic complex subunits which typically ends with synthesis of saturated C16:0 and C18:0-ACPs (Sayanova et al., 2017; Chen and Jiang, 2017). Biosynthesis of long-chain fatty acids (LC-PUFA) will then continue with a sequence of desaturation and elongation reactions that result in production of PUFAs such as DHA and EPA (Ratledge, 2004).

In addition to the conventional FAS pathway for formation of LC-PUFSs, Metz et al. (2001) discovered the presence of the bacterial-like polyketide synthase (PKS) route in the microalgae *Schizochytrium* strain that involved seven proteins: 3-ketoacyl synthase (KS), 3-ketoacyl-ACP-reductase (KR), dehydrase (DH), enoyl reductase (ER), dehydratase/2-*trans* 3-*cis* isomerase (DH/2,3I), dehydratase/2-*trans*, and 2-*cis* isomerase (DH/2,2I) with an addition of C2 units and double bonds in a sequence (Monroig et al., 2013). This pathway does not demand anaerobic desaturation during the formation of double bonds in fatty acids biosynthesis (Khozin-Goldberg et al., 2011). This anaerobic system of the PKS

route involves acetyl-CoA and malonyl-CoA as the primary building block that does not require in situ reduction of the intermediate, as oxygen is not involved in double bond synthesis (Ratledge, 2004). This pathway has been suggested to occur in *Schizochytrium* sp. and in other *Thraustochytrid* organisms. The formation of DHA using the PKS pathway starts with the transfer of both acetyl-CoA and malonyl-CoA into their respective esters with ACP, and is then condensed by KS to produce the intermediate 3-ketobutytyl. This 3-ketobutytyl will later be converted into butyryl-ACP with the activity of KR, followed by DH, and will end with an ER. These reactions are identical to conventional FAS pathway except the successive addition of malonyl-CoA to the fatty acyl chain that enables double bonds formed by DH to be retained. These double bonds would initially be in the *trans* configuration, specifically at the 2,3-position. With the help of isomerase, the *trans* configuration could be transformed into the *cis* configuration and simultaneously transfer it to the 3,4-position. Addition of malonyl-CoA will further result in the lengthening of the retained double bond chain only if it is in the right position (Ratledge, 2004).

Upon completion of the elongation process, the synthesized FAs will be transported to the cytoplasm to be used as a substrate for acyl transferase, which is involved in TAG synthesis (Bilbao et al., 2017). TAG, which acts as a primary form of energy storage molecule, contains a glycerol backbone with three attached FAs that make up 60%–70% of the dry cell weight of MSCOPS. According to sequence homology and shared biochemical characteristics, it has been commonly recognized that the basic pathway of fatty acid and TAG biosynthesis in microalgae is directly analogous as described in higher plants (Cagliari et al., 2011). Two different pathways are involved in TAG synthesis: (1) the acyl-CoA-independent pathway; and (2) the acyl-CoA-dependent pathway (Zulu et al., 2018). The Kennedy pathway, which belongs to the acyl-CoA-dependent pathway, is involved in the synthesis of the storage lipid TAG via a chain reaction where synthesized FAs are incorporated into the glycerol backbone to form TAG (Sharma et al., 2018; Zulu et al., 2018). The reactions in the Kennedy pathway occurs in the endoplasmic reticulum, which comprises the production of lysophosphatidic acid (LPA) by transfer of FAs from the acyl-CoA pool to the *sn*-1 position of the glycerol-3-phosphate (G3P) as first committed step that catalyzed by glycerol-3-phosphate acyltransferase (GPAT) which could be further acylated to form phosphatidic acid (PA) with the action of acyl-CoA dependent acyl-CoA:LPA acyltransferase (LPAAT) enzyme (Sayanova et al., 2017; Zulu et al., 2018). The PA undergoes a further dephosphorylation process to produce DAG, which later undergoes a final acylation step to form TAG by the integration of a third acyl-CoA. While in the acyl-CoA-independent TAG synthesis pathway, phosphatidylcholine (PC) acts as an acyl donor in the formation of TAG from DAG. Followed by the completion of the TAG synthesis process, the TAGs will be assembled into lipid droplets in a specialized endoplasmic reticulum membrane domain and released into the cytoplasm (Zulu et al., 2018).

16.3.2 Regulation of fatty acid biosynthetic pathway for improved MSCO production by manipulating the environmental stresses and metabolic engineering

Lipid accumulation in microalgae is highly manipulated by intrinsic and extrinsic parameters including nutrient availability such as nitrogen and carbon, as well as cultivation factors such as temperature and salinity. Among these, nitrogen limitation has been proposed and proven as a key regulator that influences lipid biosynthesis, and has been commonly employed to enhance MSCO production in microalgae (Table 16.3). According to Yang et al. (2013) and Chen et al. (2017), nitrogen limitations frequently lead to perturbations in the synthesis of amino acids, nucleic acids, and various cellular constituents, leading to a drastic increase of lipid metabolism via upregulation of several pathways including GABA, glycolysis, and the TCA cycle. These findings were also supported by the transcriptomic analysis conducted by Sirikhachornkit et al. (2018) on microalgae *Scenedesmus acutus* TISTR8540, where nitrogen deprivation induced upregulation of glycolysis and starch degradation, whereas gluconeogenesis, photosynthesis, triacylglycerol (TAG) degradation, and starch synthesis pathways were downregulated. In addition, transcriptomic analysis revealed that nitrogen limitation also resulted in substantial upregulation of several key genes and pathways associated with lipogenesis in photoautotrophic microalgae, including the acyl carrier protein (ACP) gene, DGAT isoforms such as DGAT-1, DGAT-2A, DGAT-2B, and DGAT-2E (catalyzing the last step of TAG synthesis), biotin carboxylase (which regulates ACC activity through carboxylation of the biotin moiety of the enzyme), thioesterase genes (Fat A and thioesterease oleoyl-ACP hydrolase), acyl-ACP (acyl carrier protein) desaturase (AAD), delta 15 saturase, lipases, and saposin as well as downregulation in the activity of photosynthesis, nitrogen metabolism, starch synthase, ACC MAT, and 1,4,α-glucan branching enzymes (Table 16.2). Apart from nitrogen limitation, other stress factors such as phosphorus and silicon deficiency, light, temperature, and salinity have also been reported to boost MSCO production in microalgae (Table 16.3). A study conducted by Wahidin et al. (2013) showed that lipid accumulation in photoautotrophic microalgae *Nannochloropsis* sp. increased up to 31.3% after 8 days of cultivation under 100 $\mu mol\, m^{-2}\, s^{-1}$ light intensity with a photoperiod of 18 h light and 6 h dark cycle in comparison to a continuous light supply. Under low light intensity, more polar lipids will be produced due to an increase in chloroplast membrane synthesis; in reverse, increased light intensity induces accumulation of more neutral lipids without compromising the biomass yield (Breuer et al., 2013). Another study from Ma et al. (2015) showed that cold stress resulted in substantial upregulation of cellular lipid and PKS-related genes in *Aurantiochytrium* sp., which leads to significant enhancement in PUFA (DHA and DPA) production (Table 16.3).

Furthermore, several chemical modulator and minerals have also been reported to regulate lipid accumulation in MSCOPS. Addition of 10^{-5} M of phytohormones, indole-3-acetic acid (IAA) and diethyl aminoethyl hexanoate (DAH), resulted in 1.9–2.5-fold increment in lipid and

Table 16.2: Overview of different stress factors and chemical inducer that regulate MSCO biosynthesis in microalgae.

Microalgae	Stress factors/ chemical inducer	Genes/pathway upregulated	Genes/pathway downregulated	References
Botryococcus braunii 779 *Chlorella* sp., *C. reinhardtii* *Scenedesmus acutus* TISTR8540	**Stress factors**: nitrogen deprivation	**Pathway**: lipid metabolism, glycolysis, gluconeogenesis, pentose phosphate pathway, carbon fixation metabolism, starch degradation, diacylglycerol acyltransferases (DGTT3) **Genes**: ammonia permease, glutamine synthases, and glutamate synthases, DGAT1, DGAT2, PDAT1, TAG lipases biotin carboxylase, KAS II, KAS III, KAR	**Pathway**: photosynthesis (light harvesting), ribosomes, nitrogen metabolism, TAG lipase genes and starch synthesis **Gene**: starch synthase, ACCase, MAT, 1,4,α-glucan branching enzyme	Fang et al. (2015) and Li et al. (2016a,b) Boyle et al. (2012) and Sirikhachornkit et al. (2018)
Phaeodactylum tricornutum	Phosphorus starvation	**Pathway**: phosphate acquisition and scavenging	**Pathway**: photosynthesis, nitrogen assimilation and nucleic acid and ribosome biosynthesis	Alipanah et al. (2018)
Picochlorum strain SENEW3	Salinity stress	**Pathway**: photorespiration, proline synthesis, nitrate and urea assimilation, starch synthesis **Genes**: glycolate dehydrogenase	**Genes**: glycolate oxidase	Foflonker et al. (2016)
C. reinhardtii	Cold stress	**Genes**: ribosomal proteins, PSI, PSII, LHC, NADH, ATP synthases, dehydrogenase, NADPH ubiquinone oxidoreductase,	**Genes**: sucrose synthase, AGPase, transketolase, aconitase, pyruvate kinase	Kwak et al. (2017)

Table 16.2: Overview of different stress factors and chemical inducer that regulate MSCO biosynthesis in microalgae—cont'd

Microalgae	Stress factors/ chemical inducer	Genes/pathway upregulated	Genes/pathway downregulated	References
		triose phosphate isomerase, 7 bis phosphatase, fructose 1,6 bisaldolase, RuBisCO, SNF related kinase I, cytosolic ribosomal protein L22		
Aurantiochytrium sp.		Genes: FAS I, PUFA A, B, C, malic enzyme, acetyl-CoA carboxylase		Ma et al., 2015
Thalassiosira pseudonana	Silicon deficiency	**Pathway**: photorespiration, Calvin-Benson cycle, glycolysis, pigment biosynthesis **Genes**: ACCase, DGAT1, FAS II, LPLAT/AGPAT	**Pathway**: cell division, photosynthesis, translation, ribosome	Smith et al. (2016)
Chlamydomonas reinhardtii	**Phytohormones**: AA, GA3, KIN, TRIA, ABA	**Pathway**: enhance glycolysis, TCA cycle, lipid biosynthesis and upregulated the expression of antioxidant-related genes	**Pathway**: decreased metabolism in PPP and lipid peroxidation pathway	Sun et al. (2018, 2019)
Chlorella vulgaris *Crypthecodinium cohnii*	PAA, IBA, NAA BNOA, ETA and SA			Li et al. (2015)
Monoraphidium sp. *QLY-1*	**Minerals**: cadmium	**Genes**: genes associated with protein content antioxidases, Ca^{2+} and lipid biosynthesis	Alleviate ROS and lipid peroxidation	Zhao et al. (2019)

biomass production of *Scenedesmus obliquus*, *C. vulgaris*, *Chlorella pyrenoidosa*, and *Scenedesmus quadricauda* (Sun et al., 2018). On the other hand, the synergistic response of BNOA, ETA, and SA enhances lipid content of *C. cohnii* by more than 20% (Li et al., 2016a,b). This is due to the fact that phytohormone treatment upregulates several pathway such as glycolysis, the TCA cycle, and the expression of antioxidant-related genes which alleviate the oxidative stress and lipid peroxidation during the fermentation process, leading to enhanced

Table 16.3: Application of metabolic engineering to regulate and enhance MSCO production in MSCOPS.

Microalgae	Genetic modulation	Improvement (compared to wild type)	References
Chlorella minutissima	Overexpression of G3PDH, GPAT, LPAAT and DGAT	Twofold increment in TAG production relative to the wild-type	Hsieh et al. (2012)
Phaeodactylum tricornutum	Overexpression of DGAT1	35% increase of the neutral lipid content	Niu et al. (2013)
Nannochloropsis oceanica		TAG accumulation is 47% higher than wild type	Wei et al. (2017)
Chlamydomonas reinhardtii	Overexpression of DGAT 2	Ninefold increase of TAG content	Hung et al. (2013)
Nannochloropsis oceanica		69% increase of the neutral lipid content	Li et al. (2016a,b)
Aurantiochytrium limacinum	Overexpression of Δ5 desaturase from *Thraustochytrium*	4.6- and 13.2-Fold increases of EPA and ARA production, respectively	Kobayashi et al. (2011)
Nannochloropsis salina	Expression of WRI1 from *Arabidopsis thaliana*	Transformants exhibited 64% higher lipid content than the wild type	Kang et al. (2017)
Aurantiochytrium sp. SD116	Overexpression of glucose-6 phosphate dehydrogenase	Changed the fatty acid profile and enhanced 10.6% PUFA production	Cui et al. (2016)
Aurantiochytrium sp. SD116	Overexpression of malic enzyme	105% increment in SFA compared to the yield in the wild type	Cui et al. (2019)

lipid biosynthesis (Table 16.2). Several minerals (e.g., sulfur, cadmium, and copper) have been investigated for lipid accumulation in microalgae. Specifically, *C. reinhardtii* strains CC-124 and CC-125 exhibited more TAG content under sulfur starvation when compared with nitrogen deprivation (Cakmak et al., 2012). On another separate study, the addition of 80 and 40 μM cadmium enhances lipid content in *Monoraphidium* sp. QLY-1 by 1.59- and 1.39-fold, respectively, by upregulating the genes associated with lipid synthesis, antioxidases, and Ca^{2+} absorption as well as alleviating ROS and lipid peroxidation (Table 16.2).

In addition to the conventional stress factors and chemical modulator approaches, metabolic engineering strategies have been developed to enhance and improve the quality of MSCO production by increasing the pool of carbon precursors and/or the supply of reducing equivalents, enhancing photosynthetic efficiency, blocking competing pathways, and manipulating the expression of transcriptional regulators in microalgae (Sun et al., 2019). One of the most promising metabolic engineering strategies that has been commonly employed is overexpressing the key genes for the TAG biosynthesis pathway (the Kennedy pathway). For instance, overexpression of DGAT1 (the rate-limiting enzyme in the Kennedy pathway) in

P. tricornutum and *N. oceanica* resulted in 35% and 47% increments, respectively, in neutral lipid content in comparison to the wild type (Table 16.3). In addition, DGAT 2, which is localized in the endoplasmic reticulum, has been identified as a more potent enzyme for TAG biosynthesis than its type-I counterpart, especially for unusual fatty acids. This result was supported by studies by Hung et al. (2013) and Li et al. (2016a,b), who reported that overexpression of DGAT 2 resulted in ninefold and 69% increment of TAG content in *C. reinhardtii* and *N. oceanica*, respectively. In order to achieve a superior impact of TAG accumulation, a quintuple-gene construct that includes G3PDH, GPAT, LPAAT, PAP, and DGAT was constructed and overexpressed in *Chlorella minutissima* (Hsieh et al., 2012). However, this modification only resulted in a twofold increase of the storage lipid content, far less than was expected, indicating that other limiting factors are attributed to TAG biosynthesis in these microalgae. In addition, enhancing the expression of key genes for NADPH production in *Aurantiochytrium* sp. has also revealed some interesting findings. As mentioned earlier, *Thraustochytrids* are composed of two key pathways for lipid biosynthesis: the conventional FAS pathway, which is responsible for SFA production, and the PKS pathway, which has been proposed as the key pathway for PUFA biosynthesis. Studies conducted by Cui et al. (2016, 2019) have discovered and proved that PPP coupled with the PKS pathway supply NADPH for PUFA biosynthesis, while the malic enzyme (transhydrogenase pathway) coupled with the FAS pathway supply NADPH for SFA production. The results from the experiments revealed that overexpression of G6P enhanced the PKS pathway product (PUFA) by more than 10% while SFA production was boosted to over 105% when the transhydrogenase system was overexpressed, suggesting that manipulation of NADPH production is a promising strategy for increasing SFA and PUFA production in *Aurantiochytrium* sp. (Table 16.3). In addition, employment of CRISPR/Cas and TALEN aid in constructing complex programmable synthetic circuits that enrich fatty acid profiles for better biofuel and PUFA production in *C. reinhardtii*, *N. oceanica*, and *P. tricornutum* (Jagadevan et al., 2018).

16.4 Cultivation system of microalgae for MSCO production

Different microalgae require specific modes of cultivation, which can be classed into phototropic, heterotrophic, and mixotrophic cultivation systems. Photoautotrophic microalgae can usually be grown by two systems: open ponds or enclosed photobioreactors (i.e., flat-plate, tubular, and vertical-column photobioreactors). Each system is designed to suit specific needs and may have certain advantages compared to the other. Enclosed photobioreactors (EPBRs) may be more suitable to grow certain microalgae that may not work well with contamination from other microorganisms. These systems are generally available in the form of bags, tubes, or plates, which are commonly made up of plastic, glass, or other transparent materials, ensuring the microalgae receive an adequate supply of light, carbon dioxide, and nutrients (Medipally et al., 2015). Although many EPBR designs are available, only a few are

practically used for commercial productions, which include tubular, annular, and flat-panel reactors (Carvalho et al., 2006). In tubular photobioreactors, the microalgae cultures are pumped through long and transparent tubes by using mechanical pumps or airlifts to create the pumping force. The airlift also allows the exchange of CO_2 and O_2 between the liquid medium and the aeration gas. On the other hand, annular photobioreactors are more commonly used as bubble columns or airlift reactors (Lee et al., 2006). Generally, annular photobioreactors are arranged vertically, and aeration is provided from below, while light illumination is supplied through transparent walls. This system also has the advantages of best-controlled growth conditions, efficient mixing, and the highest volumetric gas transfer rates (Eriksen et al., 2007). Another type of EPBR, flat-panel photobioreactors, supports higher growth densities and promotes higher photosynthetic efficiency. In this system, a thin layer of more dense culture is mixed or sailed across a flat clear panel; the incoming light is absorbed within the first few millimeters at the top of the culture (Medipally et al., 2015).

Utilization of EFBR is well-suited not only for the production of biofuel but also for high-value long-chain fatty acids such as EPA and DHA. However, the biggest drawback of this system is that it is expensive. With high operation and capital investment, paired with the complexity of the bioreactor's design, EPBR might not be economic enough for large-scale production. Open pond systems, on the other hand, might be a better alternative, especially for the cultivation of microalgae for biodiesel application. The operating cost of this system is relatively cheap compared to that of the enclosed bioreactor. Open pond systems utilize natural waters such as lakes, ponds, and lagoons that are open to the atmosphere. This system might also offer the potential for integration with wastewater treatment processes or aquaculture systems (Bosma and Verdegem, 2011). Nevertheless, this system is not without flaws. Limitations of this system may include contamination from unwanted species, evaporation, diffusion of CO_2 to the atmosphere, and overall limited control over the environmental condition, especially temperature and availability of sunlight (Table 16.4).

Table 16.4: Comparison of open pond and EPBR cultivation system for MSCO production.

Parameter	Open pond system	Enclosed photobioreactor
Control over process parameters	Low	High
Contamination risk	High	Low
Water loss through evaporation	High	Low
Loss of CO_2	High	Low
O_2 build-up	Low	High
Area requirement	High	Low
Productivity	Low	High
Consistency and reproducibility	Low	High
Weather dependence	High	Low
Energy requirement	Low	High
Cost	Low	High

Modified from Medipally, S.R., Yusoff, F.M., Banerjee, S., Shariff, M., 2015. Microalgae as sustainable renewable energy feedstock for biofuel production. BioMed Res. Int. 2015.

Some MSCOPS can also be cultivated heterotrophically by providing organic carbon for their growth and development, instead of photosynthesis. Heterotrophic cultivation can be well-controlled so that a higher yield of valuable products, including lipids, can be obtained. It has been reported that heterotrophic growth of *C. protothecoides* accumulated a higher lipid content than the autotrophic growth cells (Miao and Wu, 2004). In addition, the heterotrophic cultivation system is relatively simple to operate and maintain in comparison to the photoautotrophic system. However, several drawbacks often associated with this mode of cultivation include a limited number of microalgae species that can be grown heterotrophically, increased cultivation costs due to the requirement of high concentrations of organic substrate, susceptibility to bacterial infection in a nutrient-rich medium, and inability to produce light-induced metabolites (Chen and Jiang, 2017).

In order to cope with these drawbacks, MSCOPS are grown in the photoautotrophic and heterotrophic systems, also known as the mixotrophic mode of cultivation. In the mixotrophic growth system, microalgae do not depend entirely on photosynthesis since light is not the only limiting factor, as either light or organic substrate can be mutually utilized for growth (Perez-Garcia and Bashan, 2015). Mixotrophic cultivation reduces the loss of biomass during dark respiration, and also decreases the utilization of organic carbon during cellular development and lipid biosynthesis. Based on these advantages, mixotrophic cultivation is often used in microalgae biofuel production. However, not all microalgae species can be cultivated mixotrophically. To date, some common microalgae that have been developed for mixotrophic cultivation are *Chlorella* sp., *Phaeodactylum* sp. and *Neochloris* sp. (Yoshikawa et al., 2011; Deng et al., 2014). In order to develop more MSCO strains which can be grown in the mixotrophic mode of cultivation, scientists have introduced several heterotrophic key genes into photoautotrophic microalgae, including *Chlamydomonas, Chlorella,* diatoms, etc. These genes included hexose uptake protein (HUP1, from *Chlorella*), Glut1 (from human erythrocyte), as well as Hxt1, Hxt2, and Hxt4 (from *Saccharomyces cerevisiae*) (Deng et al., 2014; Lee, 2003). Introduction of these key genes into photoautotrophic MSCOPS has successfully generated genetically modified microalga strains that can be grown in both heterotrophic and autotrophic cultivation systems for lipid production (Chen and Jiang, 2017).

16.5 Current market, challenges, and future prospects of MSCO

MSCO holds immense potential as an alternative source of bioenergy, as it can be produced by converting solar energy to lipid by fixing CO_2, and its efficiency is far greater than that of terrestrial, oil-producing plants. However, commercialization of microalgae biofuel technology is still far from reality, due to several factors, primarily the exorbitant overall production cost. The current production cost of biodiesel from MSCOPS biomass is still more than $5 per gallon, and some studies have suggested that the technology will only be feasible and economically viable if the cost decreases to $1/L. In addition, the current production cost of MSCO-based biofuel would also be economical if crude petroleum sells for more than 100 USD

per barrel, which is impossible, at least in the near future (Khan et al., 2018; Davis et al., 2014), as current crude petroleum fuel prices are around 55–60 USD per barrel. Therefore, many efforts in reducing the production cost of biofuel from MSCOPS have been developed, including improvement of MSCO strain by biotechnology intervention, development of efficient downstream processes, and more. Involvement of current and advanced biotechnological technologies such as cas9, synthetic biology, and genome editing has developed the necessary tools to design novel microalgae strains with superior oil-producing capacity. Development of superior, genetically modified MSCOPS, coupled with large-scale open pond (the cheapest mode of MSCO cultivation system) culture, has been reported to reduce the overall production cost of MSCO-based biofuel (Sharma et al., 2018). However, this can still not compete with the current production cost of petroleum-based fuels. In addition, the other major concern is the impact on human health and environmental risks from the transgenic microalgae if exposed to natural ecosystems (Rastogi et al., 2018). Being one of the primary producers in aquatic ecosystems, any involuntary introduction of GM microalgae could result in an ecological calamity (Singh et al., 2016). Strict monitoring and risk assessment analysis are therefore necessary to design the biosafety regulations for GM microalgae. In addition, some other drawbacks in cultivating MSCO in open pond systems for biofuel production include the requirement of sophisticated mechanisms to distribute CO_2 evenly throughout large cultivation areas, and also contamination by adventitious protozoa or weeds that would result in large-scale disruption of the MSCO cultures (Ratledge, 2013). Despite these drawbacks and constraints, there are companies in the United States, Europe, and other regions of the world that are still optimistic about producing algal fuels at commercial scale, such as Algenol, Sapphire Energy, and Seambiotic, with approximate outputs of 1 billion gallons/year (Duvall and Fraker, 2009). This is done, of course, with governmental support (Ratledge, 2013).

The constraints and challenges of developing oils just for biodiesel have also caused many companies, especially startups, to divert their focus into producing high-value, low volume MSCO products. Currently, there are more than 100 commercial producers of PUFA from MSCOPS globally, of which most are located in the United States, China (including Taiwan), and India (Sathasivam et al., 2019). The major producer of EPA and DHA, fish oil, with an annual production of over 600,000 tons, is expected to become inadequate to meet the demand of expanding markets within a few years. Many MSCOPS, including *Schizochytrium*, *P. tricornutum*, and *Nannochloris*, have shown a remarkable capacity to produce these fatty acids. Thus, companies such as Royal DSM, Lonza Group Ltd, and Cellana have invested heavily in developing profitable techniques of producing PUFA-rich lipids by MSCOPS. In 2012, the global market for microalgae-based DHA was estimated to be nearly $350 million and has been significantly augmented to nearly $4212 million in 2017, and this was predicted to increase further in subsequent years (Beligon et al., 2016). This is clearly due to the spiking demand for the superior quality of microalgal DHA that has been used especially in the infant formula milk industry, where it accounts for nearly 50% of global production of

microalgal DHA. Recently, some efforts have been made to enhance the production of EPA and DHA by altering the metabolic pathways via genetic manipulation (Jagadevan et al., 2018). The diatom *P. tricornutum* has been genetically sequenced and modified to produce a maximum yield of 36.5% and 23.6% of DHA and EPA per total fatty acids, respectively, making it a feasible producer of these fatty acids at commercial scale (Chauton et al., 2015). However, there are still a few limitations and challenges to be addressed, such as low product yields and high production costs. Numerous efforts such as the utilization of cheap carbon sources, development of efficient cultivation and extraction technologies, and culture development have been made to address these constraints.

The future commercial production of key PUFAs, such as DHA and EPA from MSCOPS, is likely to continue for many years to come. However, the prospects of producing biofuels from MSCOPS remain uncertain, due to the challenges and constraints discussed earlier. However, continuous ongoing anticipations and development of new technologies will ensure that in the near future, economically feasible large-scale production of biofuel from MSCOs will become reality.

16.6 Conclusions

The history of MSCO started centuries ago, but only in recent decades has its potential as an excellent renewable and sustainable source of biofuels and bioactive nutraceutical products been realized. Several MSCO strains are capable of producing high lipid content, up to 70%–90% of the DCW, making it a suitable candidate for commercialization purpose. Many efforts have been made to develop MSCO strains for commercial production, which include developing genetically modified strains, optimizing culture parameters, improving the efficiency of light and CO_2 absorption, utilizing cheap organic matter for cultivation and more. Although there are still many challenges in developing MSCO for commercialization especially for biofuel production, continuous ongoing efforts will ensure that in the near future, economically feasible large-scale production of biofuel from MSCOPS will become reality.

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CHAPTER 17

Microalgal bio-fertilizers

Arun Kumar[a], **Jay Shankar Singh**[b]

[a]*Department of Environmental Science, Guru Nanak Khalsa College, Yamuna Nagar, Haryana, India*
[b]*Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India*

Chapter outline

17.1 Introduction 445
17.2 Microalgae as bio-fertilizer 447
17.2.1 Nitrogen fixation 447
17.2.2 Improving availability of phosphorus 449
17.2.3 Improving physical properties of soil 450
17.2.4 Reclamation of saline soil 450
17.2.5 Plant growth promoters 451
17.3 Microalgae-based bio-fertilizer 452
17.3.1 Cultivation 452
17.3.2 Harvesting 454
17.3.3 Biomass valorization 455
17.4 Microalgae cultivation in wastewater 455
17.5 Integration of microalgae biomass production with wastewater treatment 457
17.6 Conclusions 457
References 459

17.1 Introduction

The term "microalgae" is primarily used for aquatic microscopic algae and cyanobacteria (Tomaselli, 2004; Priyadarshani et al., 2011), which are very common in both marine and freshwater environments. Unlike microscopic algae, cyanobacteria are gram-negative photosynthetic prokaryotes which specialize in oxygenic photosynthesis (Singh et al., 2016). Some cyanobacteria have specialized structures known as heterocysts; these are responsible for nitrogen fixation. Although they have a plant-like photosynthetic mechanism, due to their simpler cellular structure and adaptability to survive in aqueous environments, they are very

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efficient in capturing CO_2, nutrients and solar energy, and converting these into biomass. They are also an excellent source of oxygen and act as primary producers in aquatic food chains.

Microalgae is comprised of significant amounts of carbohydrates, lipids, and fatty acids. Some microalgal species have extracellular polysaccharides covering their cells, known as envelopes or sheaths. In addition to nitrogen fixation, cyanobacteria are known to produce growth-promoting substances, vitamins, organic acids, and antagonistic substances, which play a pivotal role in nutrient cycling (Singh et al., 2016). They also improve the soil's physical properties such as soil pore size and water-holding capacity through production of mucilaginous substances (Cohen, 2006; Singh et al., 2016).

Microalgae are primarily cultivated in open systems (open ponds, raceway ponds, and tanks) or closed systems (tubular, flat and column, photo-bioreactors) (Chisti, 2007; Dragone et al., 2011; Wang et al., 2012; Kumar and Singh, 2016). In comparison to open systems, closed systems provide better control in terms of growth conditions and environmental factors; this helps in successful cultivation of specific species and product of interest. However, open cultivation systems could be better alternatives in the case of using wastewater, and they have lower energy costs for the cultivation of many microalgal species in wastewater.

Microalgae could be cultivated in various wastewaters such as domestic, municipal, aquaculture, dairy, or industrial as they have sufficient amounts of nitrogen (urea, ammonium, or nitrate), phosphorus, carbon (organic or inorganic), and other trace elements. The use of microalgae for wastewater treatment could offer a low-cost and sustainable system which not only provides efficient wastewater bioremediation, but also produces microalgal biomass for bio-fertilizers (Singh, 2015), biofuel (Kumar et al., 2018b), and industrial production (Kumar et al., 2017) (Fig. 17.1).

Further wastewater treatment facilities such as sewage treatment plants (STPs) and effluent treatment plants (ETPs) and microalgal cultivation in wastewater could be integrated. Microalgal remediation integrated wastewater treatment could be helpful in various aspects like reduction of production cost, efficient and cost-effective wastewater treatment, and generation of extra funds for STPs' and ETPs' operation and modernization. The benefits of microalgal remediation integrated wastewater treatment are that it:

- improves the environmental and economic value of the wastewater through water and nutrient recovery;
- provides large volumes of water which can be used for agricultural and industrial purposes;
- allows cultivation of microalgal biomass for bio-fertilizers, biofuels, and other value-added products for industries; and
- reduces the carbon footprint by capturing CO_2 generated by STPs and ETPs during the wastewater treatment.

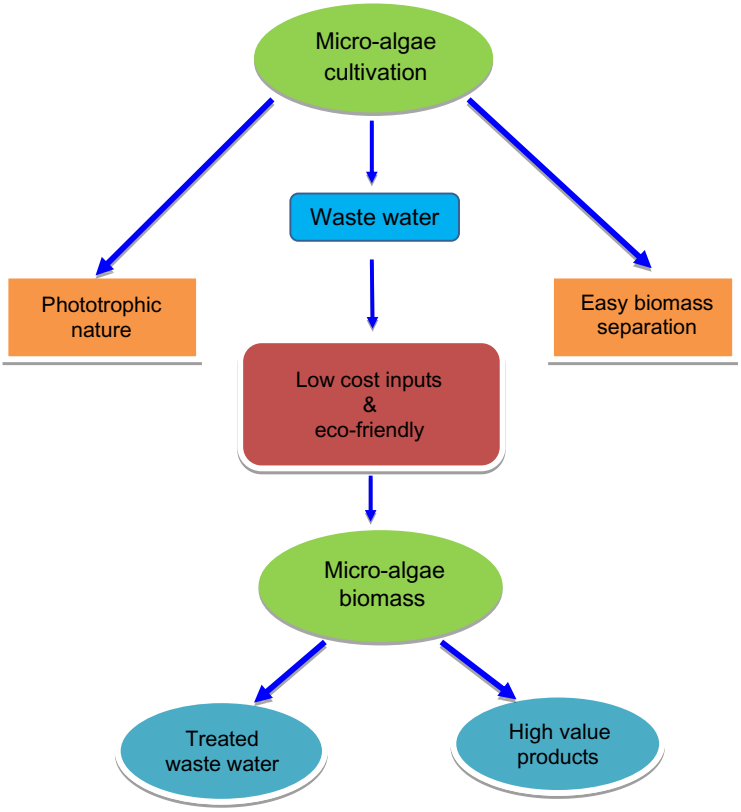

Fig. 17.1

Benefits of microalgae in wastewater treatment. *Modified from Kumar, A., Singh, J.S., 2017. Cyanoremediation: a green-clean tool for decontamination of synthetic pesticides from agro- and aquatic ecosystems. In: Singh, J.S., Seneviratne, G. (Eds.), Agro-Environmental Sustainability. Springer, Cham, Switzerland, pp. 59–83.*

17.2 Microalgae as bio-fertilizer

Many aspects of microalgae make them a potential entity for bio-fertilizer, such as nitrogen fixation, production of various substances for plant growth promotion, and improvement of soil physical properties.

17.2.1 Nitrogen fixation

Cyanobacteria are well-known to fix atmospheric nitrogen into a biologically useful form (i.e., ammonia) known as diazotrophy (Table 17.1). Some cyanobacterial strains have specialized thick-walled cells, i.e., heterocysts, which contain nitrogenase enzyme for the nitrogen fixation (Table 17.1). These heterocystous cyanobacteria are aerobic photodiazotrophs and naturally inhibit agricultural areas, especially paddies (Singh et al., 2016). This fixed nitrogen is released

Table 17.1: Important nitrogen fixing cyanobacterial genera.

Forms of cyanobacteria	Cyanobacterial genera
Unicellular	*Aphanothece*, *Chroococcidiopsis*, *Dermocapsa*, *Synechococcus*, *Gloecapsa* (*Gloethece*)[a], *Myxosarcina*, *Pleurocapsa* group[a], *Xenococcus*
Filamentous heterocystous	*Anabaena*[a], *Anabaenopsis*, *Aulosira*, *Calothrix*[a], *Camptylonema*, *Chlorogloea*, *Chlorogloeopsis*, *Cylindrospermum*, *Fischerella*[a], *Gloeotrichia*, *Heplosiphon*, *Mastigocladus*, *Nodularia*, *Nostoc*[a], *Nostochopsis*, *Rivularia*, *Scytonema*[a], *Scytonematopsis*, *Stigonema*, *Tolypothrix*, *Westiella*, *Westiellopsis*
Filamentous non-heterocystous	*Lyngbya*, LPP group, *Microcoleus chthonoplastes*, *Myxosarcina*, *Oscillatoria*, *Plectonema boryanum*, *Pseudoanabaena*, *Schizothrix*, *Trichodesmium*

[a]Some strains of these genera live symbiotically with other plants.
Courtesy: Sinha, R.P., Häder, D.P., 1996. Photobiology and ecophysiology of rice field cyanobacteria. Photochem. Photobiol. 64, 887–896; Singh, J.S., Kumar, A., Rai, A.N., Singh, D.P., 2016. Cyanobacteria: a precious bio-resource in agriculture, ecosystem, and environmental sustainability. Front. Microbiol. 7, 529.

into the soil either through secretion or by the degradation of cyanobacterial cells after death in the form of ammonia, polypeptides, free amino acids, vitamins, and auxin-like substances (Subramanian and Sundaram, 1986; Jhala et al., 2017).

It is estimated that cyanobacteria can contribute about 20–30 kg N/ha of nitrogen to the rice crop (Issa et al., 2014). Diazotrophic cyanobacteria such as *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertilissima*, and *Tolypothrix tenuis* could be used as inoculants for rice crops. *Anabaena-Azolla* fern symbiotic association was found to contribute nitrogen up to 60 kg N/ha/season and also provided a significant amount of organic matter to the soil (Moore, 1969). Apart from cyanobacteria, microalgae are also comprised of high amounts of macro- and micronutrients as well as amino acids (Mahmoud, 2001).

Microalgal mainly cyanobacterial bio-fertilizers are very frequently used in Asian countries like China, Vietnam, India, etc., in place of nitrogenous fertilizers for cultivation of paddies (Venkataraman, 1972; Lumpkin and Plucknett, 1982). Paddies offer favorable conditions for the growth of cyanobacteria such as their requirement for sunlight, water, temperature, humidity, and nutrients (Kumar et al., 2018b).

Venkataraman (1979a,b) suggested that cyanobacteria can switch over to nitrogen fixation in favorable situations, which also includes unavailability of combined nitrogen and aerobic

condition. It has also been observed that nitrogen fixation cannot be repressed up to the presence of 40 ppm ammoniacal-N in a soil-paddy-algae system (Venkataraman, 1979a,b), and in the same proportion, cyanobacterial diazotrophy was not inhibited at 30 ppm level of urea-nitrogen (Mekonnen et al., 2002). However, in the presence of high levels of combined nitrogen, the growth of cyanobacteria and its nitrogen fixation ability were inhibited.

It is well-established that microbial biomass carbon could be an indicator for measuring change in soil condition. Furthermore, all treatments regarding inoculation of microalgae or cyanobacteria showed a significant increase in microbial biomass carbon over uninoculated control (Albiach et al., 2000). It has also been observed using the $^{15}N_2$ labeled study that microalgal biomass significantly contributed to humus formation, which helps the soil to maintain its viability and fertility in dry conditions (Nekrasova and Aleksandrove, 1982). It has also been predicted that during suitable conditions, a good microalgal bloom can add about 6–8 t of fresh biomass in paddies. Kaushik (1985) observed that native algae can contribute an increase of about 0.03% (672 kg/ha) in soil organic carbon under in vitro conditions over a period of 6 months, while Subhashini and Kaushik (1984) reported that inoculation of halotolerant cyanobacterial strains to sodic soils could increase organic carbon about 5.3–7.6 t carbon/ha in a cropping season.

17.2.2 Improving availability of phosphorus

Phosphorus unavailability in the soil is a major problem; this needs to be considered as phosphorus gets fixed into the soil, but is unavailable to crops. Cyanobacteria are well-known to enhance bioavailability of phosphorus through solubilizing organic phosphorus by phosphatase enzyme production. They are able to solubilize the insoluble form of phosphorus such as $(Ca)_3(PO_4)_2$, $FePO_4$, $AlPO_4$, and hydroxyapatite ($Ca_5(PO_4)_3OH$) in soils and sediments (Bose et al., 1971; Dorich et al., 1985; Wolf et al., 1985; Cameron and Julian, 1988; Singh et al., 2016). Regarding this, two hypotheses about the mechanism of solubilization of phosphorus by cyanobacteria are as follows:

(a) Cameron and Julian (1988) and Roychoudhury and Kaushik (1989) suggested that cyanobacteria synthesize a chelator for Ca^{2+} which helps to dissolution to the light without changing the pH of growth medium as mentioned below:

$$Ca_{10}(OH)_2(PO_4)_6 \rightarrow 10Ca^{2+} + 2OH^- + 6PO_4{}^{3-}$$

(b) Bose et al. (1971) suggested that cyanobacteria secrete organic acids, which can solubilize phosphorus through the following reaction:

$$Ca_3(PO_4)_2 + 2H_2CO_3 \rightarrow 2CaHPO_4 + Ca(HCO_3)_2$$

In addition to the two above mechanisms, there is another hypothesis that suggests that firstly, solubilization of inorganic phosphate (PO_4^{3-}) takes place from where the cyanobacteria scavenged this solubilized PO_4^{3-} for their own nutrition needs. This cell-locked phosphorus is released into the soil after the death of the cyanobacterial cells, and is easily taken up by the plants and other organisms following mineralization (Saha and Mandal, 1979; Mandal et al., 1992, 1999; Singh et al., 2016; Rai et al., 2019).

It is also reported that phosphate uptake is much higher when it is delivered through microalgal inoculation compared to phosphorus available inorganically in soil (Fuller and Roger, 1952). Therefore, the most suitable mechanism used by cyanobacteria to increase availability of phosphorus is that cyanobacteria first scavenge the available phosphorus and then incorporate it into their cell biomass. From here, this fixed phosphorus inside the cells is provided to the plants by either its slow release through secretion, autolysis, or microbial decomposition of dead cells.

17.2.3 Improving physical properties of soil

Microalgae and cyanobacteria are well-known to release various compounds such as polysaccharides, peptides, and lipids during their growth in soil, which helps in binding the soil particles together to form micro-aggregates. Moreover, polysaccharides form fiber-like structures, which can trap clay particles, and this leads to maintaining the structure of micro-aggregates. These micro-aggregates further bind together to form larger soil aggregates known as macro-aggregates.

Due to their capacity to enrich soil aggregation, cyanobacteria can be used to improve the soil quality of degraded lands (Singh, 2014) in arid or semiarid regions. It has been observed that due to inoculation of cyanobacteria, the polysaccharide content of soils increases, which improves both the soil aggregate stability and water-holding capacity (Singh, 1961; Roychoudhury et al., 1979; Kaushik, 1998). Rogers and Burns (1994) suggested that macro-aggregates are further strengthened through the network of mucilaginous filaments of cyanobacteria, which can easily resist wind- and water-borne soil erosion. The cyanobacterial mediated macro-aggregates' stability is very helpful to substantial cropping in arid and semiarid conditions, i.e., a combination of sunny weather and sandy soils. Rogers and Burns (1994) also observed that cyanobacterial mediated macro-aggregates' stability is also responsible for better seedling emergence of upland crops sown after paddy cultivation. In addition, it has been reported that cyanobacterial growth improves aeration of soils, which reduces compaction in soils and favors below-ground biodiversity.

17.2.4 Reclamation of saline soil

Cyanobacteria are able to survive in extreme environments such as saline soils, and can therefore be used to improve these soils. Saline soils contain excessive amounts of salts in the upper layer, meaning that they are less productive, firm, and impermeable to water. Pandey

et al., (1992) suggested that due to the high soluble salt concentration in saline, soil creates high osmotic tension for plant roots for water and nutrient absorption. Singh (1961) observed that cyanobacteria are helpful in solubilizing nutrients from insoluble carbonate through the production of oxalic acids; these acids also help in lowering the pH, electric conductivity, and hydraulic conductivity of saline soil, which ultimately improves soil aggregation (Kaushik and Subhashini, 1985).

Cyanobacteria have some physiological benefits, which enable them to survive under saline stress: (a) restriction of sodium ion influx; and (b) concentrating inorganic (K^+ ion) or organic osmoregulators (Reed et al., 1984; Apte et al., 1987). Moisander et al. (2002) reported that some cyanobacterial strains such as *Anabaena oscillarioides*, *Anabaena aphanizomenoides*, and *Microcystis aeruginosa* can tolerate salt concentrations ranging from 7 to 15 g/L. It has also been observed that these cyanobacterial strains produce *exo*-polysaccharides, leading to better soil particle binding and water-holding capacity of soil.

17.2.5 Plant growth promoters

Cyanobacteria produce plant growth hormones (gibberellins, cytokinin, auxin, or abscisic acids) (Table 17.2), vitamins (vitamin B or amino acids), antibiotics, and toxins. Misra and Kaushik (1989a,b) studied the plant growth-promoting capability of cyanobacteria on paddy crops, and showed that cyanobacteria can promote the germination of paddy seeds and also the growth of roots and shoots. It has also been reported that cyanobacteria could improve root dry

Table 17.2: Phytohormone production among microalgae (especially in cyanobacteria).

Phytohormones	Microalgal genera	References
Auxins	*Anabaena*, *Anabaenopsis*, *Calothrix*, *Chlorogleopsis*, *Cylindrospermum*, *Glactothece*, *Nostoc*, *Plactonema*, *Synechocystis*	Sergeeva et al. (2002), Mohan and Mukherji (1978), Selykh and Semenova (2000)
Gibberellins	*Anabaenopsis*, *Cylindromum*	Mohan and Mukherji (1978)
Cytokinins	*Anabaena*, *Chalrogleopsis*, *Calothrix*	Selykh and Semenova (2000)

weight and chlorophyll content in wheat crop through the secretion of extracellular substances (Gantar et al., 1995a,b).

17.3 Microalgae-based bio-fertilizer

17.3.1 Cultivation

Microalgae can be cultivated in either open systems or closed systems (also called Photo-bioreactors, or PBRs) (Chisti, 2007; Posten, 2009; James and Boriah, 2010; Dragone et al., 2011; Wang et al., 2012; Takenaka and Yamaguchi, 2014; Fernandes et al., 2015; Kumar and Singh, 2016; Singh et al., 2016).

17.3.1.1 Open ponds and high-rate algal ponds (HRAP)

For the cultivation of cyanobacteria, open-pond systems are widely used. These are mainly natural ponds and lakes or high-rate algal ponds, and are well studied (Chaumont, 1993; Abdulqader et al., 2000; Tredici, 2004; Borowitzka, 2005; Spolaore et al., 2006; Dragone et al., 2011) and currently operated on large scales. High-rate algal ponds (HRAPs) are shallow raceway-type open ponds which have a depth of 0.2–0.4 m (sometimes up to 1 m) (Fig. 17.2A). They consist of single or multiple loops and a paddlewheel is used to obtain a water velocity of 0.15–0.3 m/s.

Compared to open ponds such as ponds, lagoons, and lakes, HRAPs are able to decrease the surface area needed by a factor of 5 (Picot et al., 1992), while improving microalgal production up to three-fold in terms of yield (Craggs et al., 2011). The biggest advantages of open-pond systems are their ease in operation and lower cost to construct; they also have greater biomass production than closed systems (Tredici, 2004; Carlsson, 2007; Dragone et al., 2011). However, they are very vulnerable to contamination and also to climatic conditions, especially variations in nutrient and cell numbers due to evaporation and rainfall (Tredici, 2004; Dragone et al., 2011).

17.3.1.2 Closed photo-bioreactors (PBRs)

In comparison to open systems, photo-bioreactors have better regulation and control of cell numbers, pH, temperature, and O_2 and CO_2 concentration (Pulz, 2001). They are more sustainable and flexible cultivation systems, and can be operated in outdoor or indoor conditions according to the type of product, production level, and local weather conditions. In the pharmaceutical and cosmetic industries, where high-value products are required, PBRs are very helpful as they are free from contamination (Dragone et al., 2011).

Currently the largest PBRs are the tubular type; two examples are a 25 m^3 PBR at Mera Pharmaceuticals (Hawaii) and a 700 m^3 PBR in Klotze (Germany). These PBRs are less expensive, possess a large illumination surface area, and also have a good biomass production

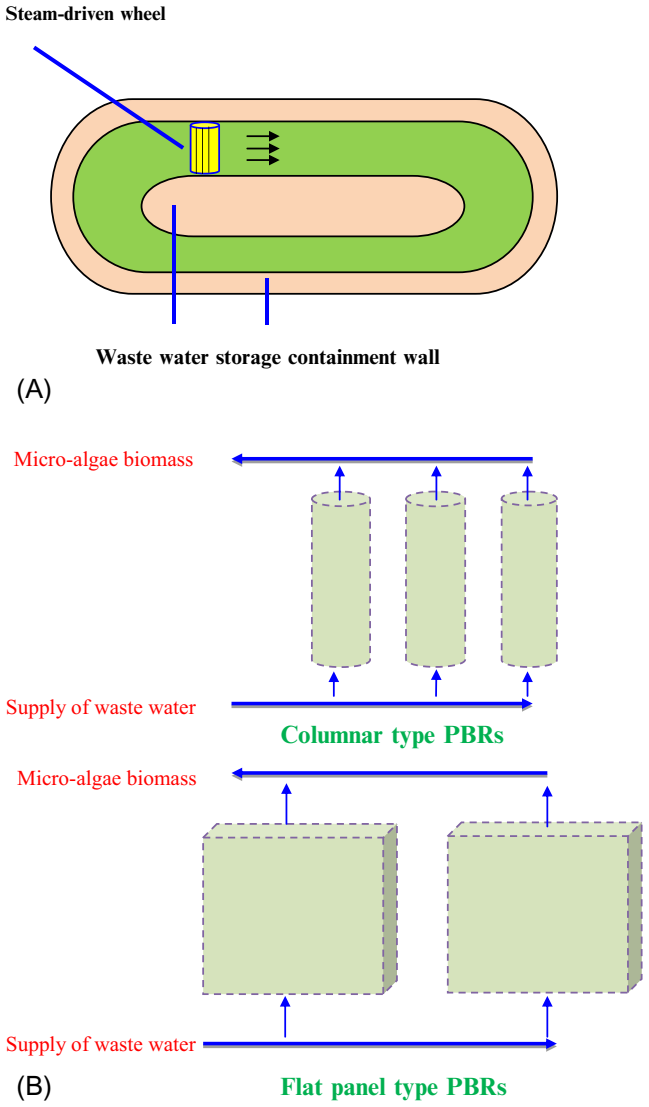

Fig. 17.2
(A) High rate algal ponds (HRAP) for microalgae cultivation. (B) Closed photo-bioreactor (PBRs) for microalgae cultivation.

capacity (Dragone et al., 2011; Brennan and Owende, 2010). In comparison to tubular PBRs, flat PBRs possess a larger surface area that is exposed to illumination and have a high density of microalgal cells (Brennan and Owende, 2010) (Fig. 17.2B). However, column PBRs are comparatively low-cost, compact, and relatively easy to operate (Fig. 17.2B). Tredici (2004) suggested that column PBRs are very efficient in mixing, provide the highest volumetric gas transfer rates, and also support better-regulated conditions for microalgal growth.

However, PBRs also have some disadvantages; they are very complex and expensive, and further light distribution could be affected, since as cell density of microalgae increases light penetration decreases. Furthermore, over time, microalgal growth on the walls of photo-bioreactors could affect the light penetration inside the reactor.

17.3.2 Harvesting

Harvesting microalgal biomass primarily considers the characteristics of microalgae like size, density, and the product required after harvesting. Brennan and Owende (2010) divided the harvesting into two phases: bulk harvesting and thickening.

17.3.2.1 Bulk harvesting

In this phase, microalgal biomass is separated from bulk suspension, which is collected from open ponds or closed photo-bioreactors. Brennan and Owende (2010) estimated that about 2%–7% total solid matter could be separated out from bulk suspension, and various methods like gravity sedimentation, flotation, or flocculation are used to achieve this.

Gravity sedimentation is the most common technique for bulk harvesting of microalgal biomass, but it is more useful in harvesting of large microalgae such as *Spirulina* (Brennan and Owende, 2010). It has also been suggested that although it is a very simple technique, it involves a very long time for pretreatment and also requires high temperature conditions. Furthermore, the efficiency of gravity sedimentation could be improved by the use of lamella separators and sedimentation tanks (Uduman et al., 2010).

In the flotation technique, microalgal biomass is harvested through gravity separation; air or gas bubbles are passed through a solid-liquid bulk suspension, from which solid biomass are separated with gaseous molecules. Uduman et al. (2010) observed that this technique is more useful for smaller microalgae, particularly cyanobacteria, as the smaller the cell size, the greater the chances of the cells to be floated by the bubbles. Depending on the size of the bubbles, this technique can be categorized further into dissolved air flotation, dispersed flotation, and electrolytic flotation.

Flocculation involves the use of chemical substances known as flocculants to increase the formation of flocs (aggregates formed by solute particles in a solution). This technique is used as a preliminary step prior to other methods like flotation or gravity sedimentation. Ugwu et al. (2008) suggested that ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$), and ferric sulfate ($Fe_2(SO_4)_3$) are commonly used flocculants. Flocculation is suitable for harvesting large quantities of microalgal biomass, and it could be applied for a wide range of microalgal species.

17.3.2.2 Thickening

In this step, the microalgal solid biomass obtained in bulk harvesting is further concentrated through various techniques like filtration, centrifugation, and ultrasonic aggregation (Brennan and Owende, 2010). The filtration method is commonly employed for large microalgae such as *Coelastrum* and *Spirulina* (cell size greater than 70 μm). This method is relatively expensive due to the use of membrane filtration; therefore it cannot be applied for large-scale harvesting (Brennan and Owende, 2010).

Ultrasonic harvesting is another method that is also used to concentrate the cells of microalgae by acoustic force; it is then followed by enhanced sedimentation. It has been observed that as ultrasonic harvesting is able to operate continuously without increasing shear stress on the microalgal cells, there could be the potential for destroying potentially valuable metabolites. Centrifugation is cost-effective and is the most reliable method used for thickening purposes. It is applicable for the harvesting and thickening of most microalgae and cyanobacteria. The efficiency of this method can attain very high levels, i.e., more than 95%.

17.3.3 Biomass valorization

After bulk harvesting and thickening of microalgal biomass, the concentrated solid biomass (dry weight) is generally 15%–25% (Uduman et al., 2010). This concentrated biomass could be developed as an animal feed or as a bio-fertilizer for agricultural purposes. To enhance the product value further, concentrated microalgal biomass could be dried. There are two type of drying, spray-drying and solar drying, which were developed as they avoid the denaturation of biomass. Although both spray-drying and solar drying are very efficient (Leach et al., 1998), spray-drying requires more energy through the use of hot gas (nitrogen or air), while solar drying needs a large surface area (Prakash et al., 1997). The dried microalgal biomass can be used as animal feed (Becker, 2007) or as a bio-fertilizer (Mulbry et al., 2005).

17.4 Microalgae cultivation in wastewater

There are many reports related to presence of various cyanobacterial genera such as *Synechococcus*, *Leptolyngbya*, *Nostoc*, *Merismopedia*, and *Limnothrix* in waste stabilization ponds, which are commonly used for wastewater treatment (Nandini, 1999; Vasconcelos and Pereira, 2001; Oudra et al., 2002; Furtado et al., 2009). Their cultivation in municipal wastewater has been extensively studied for nitrogen and phosphorus removal (Li et al., 2011; Chi et al., 2011; Bhatnagar et al., 2010; Ruiz-Marin et al., 2010) (Table 17.3). It is also established that microalgae have the ability to sequestrate nitrogen and phosphorus from

Table 17.3: Microalgae in nitrogen and phosphorus removal.

Microalgae	References
Synechococcus sp. PCC7942	Hu et al. (2000)
Phormidium laminosum	Garbisu et al. (1992)
P. bohneri	Chevalier et al. (2000)
P. tenue	Chevalier et al. (2000), Dumas et al. (1998)
Planktothrix isothrix	Silva-Benavides and Torzill (2012)
Synechocystis sp.	Suzuki et al. (1995), Rai et al. (2016)
Synechococcus elongatus	Aguilar-May and Sánchez-Saavedra (2009)
Synechocystis salina	Gonçalves et al. (2016)
Aphanothece microscopica	Queiroz et al. (2011)
Spirulina platensis	Lodi et al. (2003)

wastewater; *Phormidium bohneri*, *P. laminosum*, *P. tenue*, *Oscillatoria* O-210, and other species are efficiently applied for nutrient sequestration (Laliberte et al., 1997; Sawayama et al., 1998; Hu et al., 2000; Phang et al., 2000; Ogbonna et al., 2000; Chevalier et al., 2000; de Bashan and Bashan, 2004; Singh and Dhar, 2010).

It has also been observed that microalgae improve some conditions like oxygen, pH, and sunlight (Parhad and Rao, 1974; Asada and Takahashi, 1987), which may be helpful to reduce the number of total bacteria and coliforms in municipal sewage (Grigsby and Calkins, 1980; Curtis et al., 1992; Solo-Gabriele et al., 2000; Uma et al., 2002). Curtis et al. (1992) said that few study proposing that the number of coliforms can be reduced by improving oxygen, pH, and sunlight conditions in waste stabilization ponds, while there is no other report on cyanobacteria.

Laliberte et al. (1997) observed that if inorganic phosphates are added to wastewater, these enhance the biomass production of *P. bohneri* and there is no significant effect on the removal of the inorganic ammonium, nitrate, and phosphate. Therefore, this could be a useful strategy to add orthophosphate for enhanced production of microalgal biomass without affecting the capacity of nutrient removal.

There are many reports related to use of symbiotic bacteria with microalgae consortium to analyze the capability to remove nutrient from wastewater. Bhatnagar et al. (2010) isolated 15 microalgal species from wastewater and prepared a native microalgal consortium by mixing equal proportions. This consortium showed a significant biomass production for both the treated and untreated wastewater; it was also reported that native microalgae consortia are able to remove 98.8%–99.1% phosphate and 99.7%–99.8% nitrate in 72 h. Bhatnagar et al. (2010) compared the nutrient removal capability of microalgal consortia with unialgal cultures of *Botryococcus braunii*, *Chlorella saccharophila var. saccharophila*, *Dunaliella tertiolecta*, and *Pleurochrysis carterae*, and found that native microalgal consortia showed better biomass production than algal cultures.

17.5 Integration of microalgae biomass production with wastewater treatment

Wastewater treatment, i.e., sewage treatment plants (STPs) and effluent treatment plants (ETPs), primarily the processes or procedures involves: preliminary, primary, secondary, and tertiary. In the preliminary process, bar screens are used to remove large constituents which can clog or damage pumps, or interfere with subsequent treatment processes. In primary treatment, primary clarifiers are used to remove settable and floating materials; settable ones sink to the bottom while floating ones (grease and oils) can reach the surface and be skimmed off. After the primary process, sludge is separated for different treatment and homogeneous wastewater is produced, which further undergoes secondary treatment. In this secondary treatment, biological degradation of organic content takes place. Finally the tertiary treatment (unlikely) is used to remove the residual suspended matter and fine particulates like metals through sand filtration.

Further disinfection is followed to remove odor and pathogenic microbes from wastewater; for this, chlorine, ozone, and ultraviolet (UV) light are used before the wastewater is released into the environment. Usually, in wastewater treatment, STPs and ETPs do not undergo tertiary and disinfection processes; due to its requirements for many expensive techniques, wastewater treatment is a burden on municipalities and industries.

To solve this, microalgae can be used for the tertiary treatment and also for partial disinfection. Additionally, a value-adding approach would be to cultivate cyanobacteria in the wastewater treatment; this would enhance the profitability and sustainability of the wastewater treatment facilities. Microalgal remediation could be an effective approach for the removal of the excess nutrients that remain even after the secondary treatment, and it can also be helpful to remove odor and pathogenic microbes, improving overall aesthetic appearance. Further integration of microalgal remediation in STPs and ETPs could be a win-win strategy for researchers, governments, and industrialists in terms of working toward a clean environment. The high biomass productivity of microalgae grown in wastewater suggests that this method provides real potential for the production of a sustainable environment (Fig. 17.3).

17.6 Conclusions

Microalgae including cyanobacteria have proven to be a bio-resource for the production of various bioactive compounds which could be helpful for agriculture and environment sustainability. They have many advantages over other organisms because they are phototrophic and due to their wide distribution globally, due to their ability to survive in different and

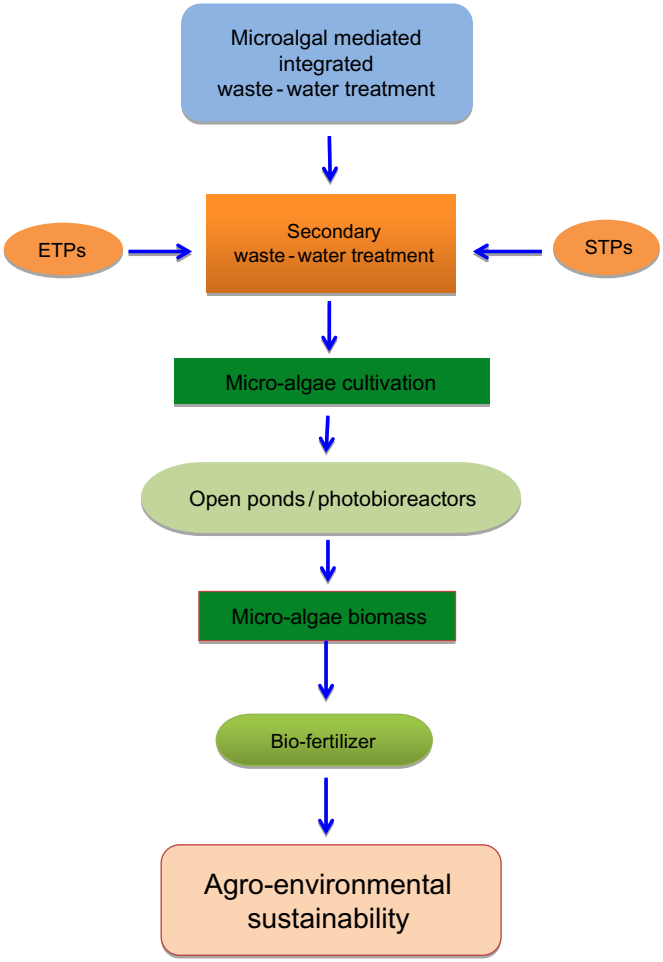

Fig. 17.3
Microalgae-mediated integrated wastewater treatment for agro-environmental sustainability.

extreme environments. Microalgae can be easily maintained due to their minimal nutrient requirements.

In recent years, microalgae have been successfully explored for the development of bio-fertilizers. Due to their nitrogen fixing and plant growth promotion ability, microalgal bio-fertilizers could offer a low-cost and sustainable alternate for chemical fertilizers. Further genetic engineering and biotechnology could be used for strain improvement to develop high-quality products.

Microalgal production technologies such as high-rate algal ponds (HRAPs) and closed photo-bioreactors undoubtedly improve the biomass yield in comparison to open ponds and waste

stabilization ponds. However, although the technical aspects of microalgal cultivation systems have been well demonstrated and examined, the economic sustainability aspects are the important challenges to the large scale or commercialization of such systems. These aspects could be overcome by technical improvements or innovations related to the design of photobioreactor and harvesting and processing of microalgal biomass.

Wastewater could be used as a resource for the cultivation medium of microalgae, ultimately helping to reduce costs. Furthermore, it could reduce the cost of wastewater treatment by replacing existing expensive chemical (coagulation) and biological methods (activated sludge); which are commonly performed during wastewater treatment. By using existing wastewater treatment facilities, integrated microalgal systems could be helpful in reducing capital costs and scalability challenges. Moreover, microalgal remediation integrated wastewater treatment provides value to wastewater through recovering nutrients and water, ultimately reducing the environmental footprint of wastewater treatment.

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CHAPTER 18

Pigments from microalgae

Fernando Pagels[a,b], Daniel Salvaterra[a,b], Helena Melo Amaro[a], Ana Catarina Guedes[a]

[a]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, Novo Edifício do Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal [b]Department of Biology, Faculty of Sciences, University of Porto (FCUP), Porto, Portugal

Chapter outline

18.1 Introduction 465
18.2 Industrial application of microalgal pigments 468
18.2.1 Pharmaceutical use and prospecting 468
18.2.2 Food and nutraceuticals 470
18.2.3 Feed 471
18.2.4 Other applications 472
18.3 Pigments bioprocesses 472
18.3.1 Phycocyanin 472
18.3.2 β-Carotene 475
18.3.3 Astaxanthin 477
18.3.4 Lutein 477
18.4 Economical interest and challenges 479
18.4.1 Market size 480
18.4.2 Constraints 482
18.4.3 Prospects and solutions 484
Acknowledgments 485
References 485

18.1 Introduction

Microalgae (including cyanobacteria) are one of the oldest photosynthetic organisms on the planet and were responsible for the creation of the Earth's oxygenous atmosphere (Masojdek et al., 2007).

Microalgal pigments are the molecules responsible for light harvesting and energy transfer to the reaction centers—processes essential for photosynthesis. They are structured in the form of complexes called antennae, which are located in the thylakoid membranes. In eukaryotic microalgae, this membrane is located inside the chloroplasts, while in cyanobacteria

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(prokaryotic microalgae), it is placed close and parallel to the cell surface (Mullineaux, 1999; Masojdek et al., 2007; Geada et al., 2017). Pigments are usually grouped into three major classes: chlorophylls (chl), carotenoids, and phycobiliproteins (Masojdek et al., 2007).

Although the pigment composition present in the photosynthetic machinery differs between different microalgal groups, all photosynthetic organisms have chl *a* as a part of their core reaction center. Chl *b*, *c*, or *d* can also be present as accessory pigments in the antenna, widening the light absorption range (Masojdek et al., 2007), in specific microalgal groups. Furthermore, all chlorophylls show two absorption bands: 450–475 and 630–675 nm (Fig. 18.1).

In cyanobacteria and red algae, the main class of light harvesting pigments-protein complexes is phycobiliproteins. These pigments are organized to form complexes called phycobilisomes, which are attached to the cytoplasmatic face of the thylakoid membranes (Gantt, 1980; Bogorad, 1975; Pagels et al., 2019). In these microorganisms, the photosystem II (PS II) is composed mainly of phycobilisomes while the photosystem I (PS I) is exclusively constituted by chl *a* (Masojdek et al., 2007). These hydrophilic pigments absorb light in the visible spectrum between 500 and 650 nm (Fig. 18.1), and work together with chl *a* to maximize light harvesting (Gantt, 1980). Phycobiliproteins are divided into four main classes: phycocyanin, phycoerythrin, phycoerythrocyanin, and allophycocyanin. The most common phycobiliprotein class in cyanobacteria is phycocyanin, whereas in red algae it is phycoerythrin. Nevertheless, some cyanobacteria can synthesize phycoerythrin or can have phycoerythrocyanin as a substitute. The last class, allophycocyanin, works as a mediator in the energy transfer between other phycobiliproteins and the photosynthetic reaction centers (Dumay and Morançais, 2016; Pagels et al., 2019).

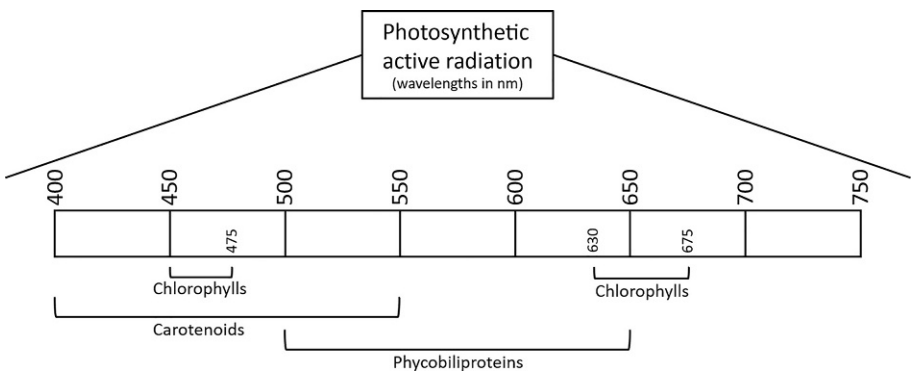

Fig. 18.1
Schematic representation of the abortion range of the three main classes of pigments (chlorophylls, carotenoids, and phycobiliproteins) in the visible light spectrum.

Carotenoids are a vast group of terpenoid pigments considered essential for the survival of photosynthetic organisms (Cogdell and Frank, 1987; Guedes et al., 2011a). Within this large group of more than 600 pigments, there are two major classes: carotenes and xanthophylls. Carotenes, e.g., α and β-carotene, are composed only of carbon and hydrogen while xanthophylls, which include pigments like violaxanthin and astaxanthin, also have oxygen-containing functional groups in their composition (Del Campo et al., 2007; Guedes et al., 2011a; Higuera-Ciapara et al., 2006; Masojdek et al., 2007).

Carotenoids can also be classified according to their role in the cell. Primary carotenoids (α-carotene, β-carotene, and lutein) are crucial for cell survival and are directly involved in photosynthesis. Secondary carotenoids (astaxanthin and canthaxanthin) are only produced and accumulated when cells are exposed to certain stimuli by a process denominated carotenogenesis (Guedes et al., 2011a; Zhang et al., 2014).

Carotenoids play two major roles in photosynthesis. On the one hand, they work as accessory light-harvesting pigments, with a light absorption range between 400 and 550 nm (Fig. 18.1), by transferring energy to chl *a* and expanding the wavelength range in which light can be used for photosynthesis (Cogdell and Frank, 1987; Masojdek et al., 2007). On the other hand, they are also important agents for the protection of the photosynthetic machinery (membranes, photosystems, and antenna). Simultaneously, carotenoids can quench reactive oxygen species (ROS) and chlorophyll triplets, resulting in triple state carotenoids, which release the excess energy safely as heat (Varela et al., 2015). These pigments are also involved in a process called nonphotosynthetic quenching, which lowers the levels of chlorophyll singlets by thermal dissipation, meaning that the probability of chlorophyll triplets formation decreases and consequently that ROS production, specifically singlet oxygen, is prevented (Muller and Mu, 2001).

In addition to their important biological role, microalgae pigments have been used for many applications, since they have properties that are highly attractive for food, feed, pharmaceutical, nutraceutical, and cosmetic industries—for example, the already described antioxidant capacity of β-carotene and phycocyanin (Abed et al., 2009; Eriksen, 2008; Guedes et al., 2011a; Pandey et al., 2013). Microalgae, due to the fact that they can produce and accumulate valuable pigments, are also attractive for industrial production since they gather characteristics that are advantageous when compared to other vegetable sources, e.g., their wide natural diversity and distribution, and their lack of need of arable land to be produced. Moreover, their photosynthetic nature could also reduce the cost of production since, in contrast to heterotrophic organisms, microalgae do not need carbon supplementation and some species can also fixate nitrogen, which would remove the need to use nitrogen supplements (Panda et al., 2006).

Industries are already using these microorganisms to produce pigments and other products. However, pigments represent the main source of revenues for industries when compared to

other components from microalgae, particularly in the food, cosmetics, and healthcare industries, as pigment market prices are higher than those for other microalgae components (Ruiz et al., 2016). The most used microalgae are *Dunaliella salina* to produce β-carotene, *Haematococcus pluvialis* to produce astaxanthin, and *Arthrospira platensis* to produce C-phycocyanin. Pilot-scale projects for the production of lutein using *Muriellopsis* sp. and *Scenedesmus almeriensis* are also being carried out (Del Campo et al., 2007).

In this chapter, the industrial applications of microalgae pigments will be described, as well as the biotechnological advances of their production, focusing on phycocyanin, β-carotene, lutein, and astaxanthin; finally, the economic interest and challenges for the production of these compounds will be considered.

18.2 Industrial application of microalgal pigments

Microalgal pigments have been known for their potential for industrial applications; this is due to their antioxidant, antiinflammatory, neuroprotective, and hepatoprotective properties (Spolaore et al., 2006). They are already extensively used in several industries, including food, nutraceuticals, pharmaceuticals, aquaculture, and cosmetics. β-Carotene from *Dunaliella*, astaxanthin from *Haematococcus*, and phycocyanin and other phycobiliproteins from *A. platensis* are already being exploited in some of the industries mentioned above (Dufossé et al., 2005). Microalgal pigments have been also used in clinical/research laboratories, as they are effective as a label for antibodies and receptors (Santiago-Santos et al., 2004). In addition, due to the eco-friendly nature of microalgae production, there is increasing interest in using microalgal pigments in aquaculture for feed formulation/supplement (Begum et al., 2016).

18.2.1 Pharmaceutical use and prospecting

Pigments from microalgae have long been known to possess several bioactivities, the best known being antioxidant. Indeed, this may sometimes be the mechanism underlying other bioactivities, such as antiinflammatory or antitumor (Guedes et al., 2011b).

An example is phycobiliproteins, such as phycocyanin, phycoerythrin, and allophycocyanin. Among them, phycocyanin, a pigment isolated from cyanobacteria, holds the most potential to be used in pharmaceutical formulations due to its proved antioxidant, antiinflammatory, neuroprotective, and hepatoprotective properties (Sekar and Chandramohan, 2008). Phycocyanin, derived from *Aphanizomenon flos-aquae*, proved to be a strong antioxidant when applied in vitro against oxidative damage (Benedetti et al., 2004). In addition, phycocyanin radical scavenging properties were shown to inhibit microsomal lipid peroxidation (Sekar and Chandramohan, 2008), and C-phycocyanin derived from *A. platensis* exhibited hypocholesterolemic activity by modeling serum cholesterol concentrations (Nagaoka et al., 2018). In terms of hepatoprotective properties, phycocyanin was also revealed to play a role in a

human hepatitis animal model. This pigment reduced the serum alanine amino transferase, aspartate amino transferase, and malondialdehyde (González et al., 2003). As an antiinflammatory, phycocyanin also decreases edema, histamine release myeloperoxidase activity, and levels of prostaglandin and leukotrienes in inflammation tissues (Sekar and Chandramohan, 2008). Phycocyanin antitumor effects have also been proven by decreasing the tumor necrosis factor in mice blood serum treated with endotoxin, and in particular, one isolated from *A. platensis* was found to inhibit the growth of human leukemia K562 cells (Liu et al., 2000).

Another phycobiliprotein, R-phycoerythrin, was shown to be useful in improving selectivity of photodynamic therapy and treatment of mouse tumor cells S180 and human liver carcinoma cells SMC 7721 (Bei et al., 2002). In addition, allophycocyanin was reported to have an antiviral capacity, by inhibiting enteron virus 71-induced cytopathic effects, viral plaque formation, and viral-induced apoptosis (Shih et al., 2003).

Carotenoids (xanthophyll and carotenes) are the group of microalgal pigments most studied in terms of bioactivities; among the more than 600 carotenoids available in nature, β-carotene is possibly the most significant one. β-Carotene exerts several benefits for the human body where it can be converted to vitamin A, having a wide range of diverse biological functions related to human health, such as helping to increase immunity and prevent cataracts, night blindness, and skin diseases (Pisal and Lele, 2005). Antioxidant properties of β-carotene aid in immune system stimulation, free radicals quenching, life-threatening diseases such as arthritis, coronary heart diseases, premature aging, some types of cancer, and specifically Alzheimer's disease, which is caused by persistent oxidative stress in the brain (Mattson, 2004; Törnwall et al., 2004). In this way, Nakashima et al. (2009) also observed that cognitive impairment was prevented in transgenic mice fed with extracts from *Chlorella* sp. containing β-carotene and lutein. As specific examples, it was found that β-carotene from *Dunaliella* sp. contains 40% 9-cis and 50% all-trans stereoisomers, which are able to lower the incidence of several types of cancer and degenerative diseases (Ben-Amotz and Fishler, 1998), and one isolated from *Chlorella ellipsoidea* and *Chlorella vulgaris* was able to inhibit the development of colon cancer (Plaza et al., 2009).

Among xanthophylls, astaxanthin stands out due to its bioactive potential, and like the pigments described so far, it has a strong connection with antioxidant capacity. Indeed, astaxanthin antioxidant capacity is 10 times higher than β-carotene and more than 500 times higher than α-tocoferol, acting as a super vitamin E (Jyonouchi and Gross, 1995). Due to its powerful bioactive antioxidant properties and its ability to cross the blood brain barrier, this pigment can be used in prevention of neuronal damage associated with age-related macular degeneration, in treatment of Alzheimer's and Parkinson's diseases, ischemic reperfusion injury, and spinal cord and other types of central nervous system injuries (Begum et al., 2016; Snodderly, 1995). In addition, in vitro studies found that astaxanthin is effective in prevention of oxidation of

low-density protein, which disclosed a potential application for prevention of arteriosclerosis, coronary heart disease, and ischemic brain development (Miki, 2007). It should be noted that a peculiarity of this pigment is that antioxidant capacity has been reported under both hydrophilic and hydrophobic conditions (Kobayashi and Sakamoto, 1999). A photoprotectant capacity of astaxanthin was also observed against ultraviolet irradiation, which might be used in preparations for prevention of light-induced aging of skin (Savouré et al., 1995). Administration of astaxanthin in diet has proven to inhibit carcinogenesis in the mouse urinary bladder, rat oral cavity, and rat colon (Tanaka et al., 2012). Moreover, astaxanthin is able to induce xenobiotic metabolizing enzymes in rat liver and exhibited a preventive effect against aflatoxin carcinogenicity (Gradelet et al., 1996; Miki, 2007).

Astaxanthin also revealed an immune-stimulatory effect in vitro (Jyonouchi and Gross, 1995), modulating humoral and nonhumoral immune systems enhancing the release of interleukin-1 and the tumor necrosis factor in mouse to a greater extent (Okai and Higashi-Okai, 1996). Astaxanthin also stimulates the production of immunoglobulin A, M, G, and the T-helper cell antibody production (Jyonouchi and Gross, 1995).

In addition, astaxanthin has been promising in treatment of *Helicobacter* infections of the mammalian gastrointestinal system, leading to the development of a patent for the production of an oral preparation for treatment of *Helicobacter* sp. infections (Alejung and Wadstroem, 1998).

Other xanthophylls like lutein and zeaxanthin have also been shown to hold specific biological roles in decreasing cancer development, enhancing immune function, and reducing age-related macular degeneration (Lakshminarayana et al., 2010). Moreover, in terms of antioxidant capacity, lutein proved to be more effective than β-carotene in inhibiting auto-oxidation of cellular lipids, and in protecting against oxidant-induced cell damage (Lakshminarayana et al., 2010). Indeed, lutein has been recommended for prevention of cancer and diseases related to retinal degeneration. Xanthophyll can significantly inhibit growth of androgen-dependent and androgen-independent prostate cancer cell lines in vitro, as well as prevent colon carcinogenesis in vivo (Narisawa et al., 1996); it also plays a role in inhibiting proliferation of human mouth epithelial cancer line KB (Sun et al., 2006). Additionally, Reynoso-Camacho et al. (2011) demonstrated a chemoprotective effect of lutein against colon cancer induced by DMH. Indeed mice, feed with 0.002% of lutein in the diet, had a preventive effect diminished the number of tumors by 55%, and 32%, when given as a treatment after DMH administration (Reynoso-Camacho et al., 2011).

18.2.2 Food and nutraceuticals

Among the different types of microalgae-derived compounds, those with antioxidant properties are probably the most interesting ones for industrial applications. In the food industry, during industrial processing or storage, oxidation of essential nutrients occurs, destroying them and potentially generating toxic compounds.

Thus, solutions commonly applied include the use of synthetic antioxidants, like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and EDTA, among others (Ngo et al., 2012). However, despite their utilization being regulated by law and controlled, adverse effects on health have been reported for some synthetic additives (Carocho et al., 2014). Moreover, consumers associate synthetic with unhealthy, leading to their preference for more "natural" products; and here, microalgal bioactive compounds such as pigments may play an important role (Caporgno and Mathys, 2018; Carocho et al., 2014). Moreover, in the food industry, synthetic pigments have been banned in many countries due to their adverse effects on human health—mainly their toxic effects (Begum et al., 2016). Therefore, the preference for the use of dyes obtained from different natural sources has been increasing, making microalgae an effective environmentally friendly alternative (Dufossé et al., 2005). Pigments such as phycocyanin (blue pigment from *A. platensis*), β-carotene (yellow pigment from *Dunaliella*), and astaxanthin (yellow to red pigment from *Haematococcus*) are becoming more popular as nontoxic and noncarcinogenic natural solutions (Dufossé et al., 2005).

Phycocyanin obtained from *A. platensis* is already used as a colorant in food items such as candies, jellies, chewing gum, ice sherbets, popsicles, soft drinks, dairy products, and wasabi (Santiago-Santos et al., 2004; Spolaore et al., 2006). Moreover, phycocyanin extracts and whole *A. platensis* have been incorporated to produce cookies to enhance protein and fiber content, with potential health benefits (Abd El Baky et al., 2015). In addition, *C. vulgaris* has been incorporated in cookies as a coloring agent as well as for its antioxidant potential and nutritional value (Gouveia et al., 2007).

Other phycobiliproteins such as phycoerythrin isolated from *Phorphyridium aerugineum* and *A. platensis* are used as a dye in ice creams, gelatin deserts, fermented milk products, sweet cake decoration, milk shakes, and cosmetics. In addition, phycoerythrin, due to its yellow fluorescence properties and fluorescent color, is used to make transparent lollipops, dry sugar-drop candies for cake decoration, soft drinks, and alcoholic beverages (Dufossé et al., 2005).

Carotenes like β-carotene from *Dunaliella* are commonly used as food colorants to improve the appearance of margarine, fruit juices, cheese, baked goods, canned foods, dairy products, and confectionary, to attract consumers. It is approved by the United States Food and Drug Administration (US FDA) as a safe and natural food color. As reported previously, β-carotene is also used as pro-vitamin A (retinol) in the formulation of healthy foods (Spolaore et al., 2006).

European countries have also allowed astaxanthin as a color additive in human dietary supplements (Begum et al., 2016).

18.2.3 Feed

In salmon aquaculture, the color of the fish is crucial. Usually, synthetic astaxanthin is the predominant source of carotenoids. As an alternative, canthaxanthin, astaxanthin, and lutein from *Chlorella* sp. have been regularly used, and have consequently been included as

ingredients of feed for salmonid fish, trout, and poultry, to boost their reddish color or the yellowish color of egg yolk (Guerin et al., 2003; Lorenz and Cysewski, 2000; Plaza et al., 2009).

Additionally, astaxanthin can enhance fish and shrimp immunity, promoting efficient growth and survival (Dufossé et al., 2005).

It should be noted that astaxanthin is allowed as a color additive in fish feed by the US FDA (Begum et al., 2016). β-Carotene is also used as a colorant in feed to improve the appearance of fish and shellfish (Cantrell et al., 2003; Dufossé et al., 2005).

18.2.4 Other applications

In fluorescent-based detection systems, phycobiliproteins play a significant role, mainly in flow cytometry, due to their spectral properties (Kronick and Grossman, 1983). In addition, phycoerythrin, due to its absorbance spectrum properties, has been used as a second color in fluorescent-labeling antibodies, and phycoerythrin labeled with streptavidin can be used for the detection of DNA and protein probes (Sekar and Chandramohan, 2008). Other low-molecular weight cryptomonad-derived phycobiliproteins may also be applied in flow cytometry for extracellular and intracellular labeling applications (Telford et al., 2001).

In addition, phycocyanin is being used as a colorant agent in lipsticks and eyeliners (Santiago-Santos et al., 2004).

18.3 Pigments bioprocesses

A microalgae-based bioprocess for the production of pigments consists of several steps to obtain a specific product. It goes from biomass production to harvesting, cell disruption, and extraction, and finally (if needed) purification. In the case of pigments, the purification can represent the most expensive step of the bioprocess; however, a high purity grade can increase the value and safety of the products. Most of the bioprocess optimization is done at laboratory scale, and its scale-up is still a concern. Large-scale processes are rarely available in the literature and are protected by patents. In this section, the main pigment bioprocesses will be discussed in order to give a perspective in terms of patterns on the production of pigments and provide information for future optimizations. Table 18.1 summarizes the most common processes for the production of the four main pigments for industrial use (astaxanthin, lutein, β-carotene, and phycocyanin).

18.3.1 Phycocyanin

Phycocyanin, the cyanobacteria blue pigment, is largely extracted from *A. platensis* at an industrial level, although many other species can be alternative sources, such as the *Anabaena* and *Nostoc* species (Hemlata and Fatma, 2009). The optimization of the bioprocess has again

Table 18.1: Bioprocess optimization of the main industrial-applied microalgae pigments. *L*, light; *T*, temperature; *S*, salinity; *N*, nitrogen source.

Bioprocess	Pigment			
	Phycocyanin	**β-Carotene**	**Astaxanthin**	**Lutein**
Main microalgae source	*Arthrospira platensis*	*Dunaliella salina*	*Haematococcus pluvialis*	*Murielopsis* sp. and *Scenedesmus almeriensis*
Optimal production conditions	**L:** red light; low intensity; 16 h:8 h (light:dark) **T:** high temperatures (>30°C) **pH:** 8.0–10.0 **S:** varies from strains **N:** nitrate, ammonium and/or urea	**L:** high intensity; UV-A supplementation **T:** low temperature for β-carotene accumulation **pH:** 7.5 **S:** high salinity **N:** nitrate associated to starvation stress	**Green phase** **L:** 250 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ **T:** 20°C **pH:** 7.5–8.0 **N:** 10 mM nitrate **Red phase** **L:** 500 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ **T:** 27°C **S:** salt stress **N:** 3 mM nitrate	**L:** high intensity **T:** high temperature **pH:** 8.0–9.5 **S:** 100 mM of NaCl for salt stress **N:** nitrate associated to starvation stress
Extraction process	– Cell disruption by mechanical methods – Chemical extraction with aqueous solvents	– Solvent extraction – Supercritical fluid extraction – Direct extraction into edible oil	– Bead milling – Spray-drying – Supercritical fluid extraction	– Bead milling – Sonication – Hexane extraction–supercritical fluid extraction
Purification process	– Precipitation by ammonium sulfate – Chromatographic isolation	– Use of oleoresin – Chromatographic isolation	– Use of oleoresin (20% of astaxanthin) – Chromatographic isolation	– Use of oleoresin (25% of lutein) – Chromatographic isolation
References	Pagels et al. (2019)	Hosseini Tafreshi and Shariati (2009) and Raja et al. (2007)	Panis and Carreon (2016)	Cerón et al. (2008) and Fernández-Sevilla et al. (2010)

been focused mainly on the growth and obtaining of biomass, but some approaches on extraction and purification were also followed.

Phycocyanin production is mainly influenced by abiotic factors, such as light, nutrients, temperature, pH, and salinity. Once phycocyanin is part of the photosynthetic apparatus of cyanobacteria, light represents the most important factor, and consequently is one of the most studied.

Light can be optimized in terms of quality, intensity, and photoperiod. In terms of light quality, it has been shown that red light can enhance the production of phycocyanin in several species (Keithellakpam et al., 2015; Mishra et al., 2012; Pagels et al., 2019). Concerning light intensity, a preference has been observed for lower intensities for the production of this pigment (Castro et al., 2015; Gris et al., 2017; Hemlata and Fatma, 2009; Oliveira, 2014). Moreover, the photoperiod of 16 h of light and 8 h of dark has been described as ideal for phycocyanin production (Hemlata and Fatma, 2009; Johnson et al., 2014; Maurya et al., 2014).

Regarding nutrients, in special nitrogen, the choice of source determines the production and accumulation of phycocyanin, which, inside the cell, also has the role of nitrogen storage. Cyanobacteria production uses nitrates in most cases (Ürek and Tarhan, 2012), although a few studies have suggested ammonium and urea as alternatives (Ajayan et al., 2012; Khazi et al., 2018). On the other hand, some cyanobacteria are nitrogen fixers, and the addition of nitrogen-based salts can decrease the amount of pigment in the cell (Hemlata and Fatma, 2009; Khattar et al., 2015; Simeunović et al., 2013).

In terms of temperature, the production of phycocyanin has been described as being enhanced by higher temperatures, equal to or higher than 30ºC (Chaneva et al., 2007; Hemlata and Fatma, 2009; Hifney et al., 2013; Johnson et al., 2014; Maurya et al., 2014).

Concerning pH, it has been described that cyanobacteria tend to prefer more alkaline environments for both growth and phycocyanin accumulation, in a range from 8 to 10 (Kaushal et al., 2017; Keithellakpam et al., 2015; Maurya et al., 2014; Poza-Carrión et al., 2001).

Furthermore, salinity is a fundamental factor for the production of marine cyanobacteria. High NaCl concentrations (ca. 0.5 M) have increased the phycocyanin production in some species, such as *Phormidium* sp., *Oscillatoria* sp., and *Arthrospira* sp. (Fuenmayor et al., 2009; Jonte Gómez et al., 2013). However, in other species, the high amounts of NaCl led to a decrease in the phycocyanin accumulation, as in the case of *Anabaena* sp. and *Limnothrix* sp. (Hemlata and Fatma, 2009; Lemus et al., 2013).

Beyond the abovementioned factors, the addition of chemical compounds to the culture (e.g., glucose and phytohormones) can enhance the production of phycocyanin. Supplementing cyanobacterial cultures with glucose switches the metabolism to mixotrophy and may increase the production of phycocyanin. In the case of *Nostoc* sp., the addition of sugar

increased the production of phycobiliproteins 12-fold (Andrade and Costa, 2007; Borsari et al., 2007; Chen and Zhang, 1997). Glucose addition also increased the phycocyanin production in *Arthrospira* sp. and *Anabaena* sp. (Kovač et al., 2017). Moreover, the use of phytohormones is based on the similarity of all photosynthetic organisms. It has already been described that low concentrations of gibberellic acid 3 (GA_3) and indole-3-butyric acid (IBA) can induce a positive effect on phycocyanin accumulation (Mansouri and Talebizadeh, 2016, 2017; Pan et al., 2008).

Regarding the extraction and purification processes, phycocyanin is sometimes sold in the form of raw biomass product (*Spirulina* containing phycocyanin), although the biological benefit of the pigment can be increased within the purification process (Pagels et al., 2019; Yu et al., 2017). The extraction process requires a mixture of mechanical and chemical extraction due to a high resistance of the cyanobacteria cells. The methodology needs to be chosen according to the organism (Pan-utai and Iamtham, 2018). Different mechanical cell disruption methodologies have been used in order to extract phycocyanin, such as sonication, homogenization, high-pressure, and glass beads-assisted extraction, among others (Pagels et al., 2019). Thus, the chemical extraction is based on the high solubility of phycocyanin in aqueous solvents, using one (classical) or more (two-phase or successive) solvents, according to the desired purity. It is also possible to use an enzymatic extraction, although it is necessary to treat the extract in order to separate the enzymes (Moreno et al., 1997; Stewart and Farmer, 1984).

Moreover, the purification process of phycocyanin is based on two main steps: first, a precipitation using ammonium sulfate is done, in order to separate phycocyanin from other pigments; the second step can then be done in a chromatographic column using both polarity and size of phycocyanin as factors for separation and consequent purification (Binder et al., 1972; Piero Estrada et al., 2001).

18.3.2 β-Carotene

β-Carotene, present in most of microalgae as an accessory pigment, has been widely explored industrially from hypersaline microalgae *D. salina* production. The hypersaline characteristic gives this microalga culture the advantage of a low contamination profile, although a process of salt removal is needed in order to purify the compound.

Concerning the production of β-carotene, and growth of *D. salina*, abiotic factors such as light intensity, salinity, temperature, and nutrients have been optimized. Light intensity can cause stress to the culture, and in response, a high production of secondary pigments such as β-carotene is expected; however, by reaching the saturation of the photosynthetic apparatus, the cells can die (Ben-Amotz and Fishler, 1998). In addition, it is possible to modulate the production of β-carotene by supplementing the light source with ultraviolet (UV) radiation.

High intensity light supplemented with UV-A can increase the production of carotenoids more than twofold (Raja et al., 2007; White and Jahnke, 2002).

Another way of increasing the production of β-carotene is by controlling the nutrient availability in the culture medium. During nutrient starvation stress, the culture changes its metabolism and starts to accumulate some compounds, including β-carotene. It has been described that nitrate and sulfate starvations can induce a higher production of β-carotene (Becker, 2004); the same can be observed by reducing nitrate and phosphates (Geider et al., 1998; Milko, 1962; Raja et al., 2007). The optimal condition for *D. salina* growth is $5\,\mathrm{mM\,L^{-1}}$ of nitrate, and it is possible to induce nutrient starvation in a late state of the culture and increase the total productivity of the pigment (Hosseini Tafreshi and Shariati, 2009).

In terms of temperature, it has been described that low temperatures (ca. 10°C) can lead to a fourfold increase in carotene production compared to higher temperatures (>26°C). In addition, the effectiveness of the temperature can increase when a high intensity is simultaneously applied (Ben-Amotz and Fishler, 1998; Gómez and González, 2004; Raja et al., 2007). On the other hand, the optimal condition for growth of *D. salina* is around 30°C, making it possible to cultivate it in open ponds, using as an advantage the temperature variation during the day (Ben-Amotz, 1996; Hosseini Tafreshi and Shariati, 2009).

At industrial scale, pH control in open ponds is achieved by injecting CO_2 and HCl, and keeping the pH around 7.5 for a higher β-carotene accumulation, although the optimal range of pH for *D. salina* growth is between 9 and 11 (Hosseini Tafreshi and Shariati, 2009).

Finally, the physiological ability of growing in a hypersaline medium allows the production of *D. salina* with minor problems of contamination. Although the optimal condition for β-carotene production occurs with an intermediate salinity, high concentrations are used in order to avoid predation and contamination (Borowitzka and Borowitzka, 1990; Hosseini Tafreshi and Shariati, 2009; Raja et al., 2007).

Regarding the downstream process, harvesting is one of the most critical steps in mass production of β-carotene. Filtration seems to be challenging due to the size of the cells, which easily clog the filters. Some attempts with diatomaceous earth have provided useful perspectives on their use in filtration. As alternatives, chemical flocculation and precipitation of cell agglomerates can be employed; however, the chemical contamination of the biomass is still a concern and needs a better methodology for purification, increasing the final costs of production (Hosseini Tafreshi and Shariati, 2009).

Moreover, the extraction of β-carotene is achieved from dried biomass and consists of several patented methodologies, including conventional extraction using organic solvents (e.g., hexane, ethanol, chloroform), CO_2 supercritical fluid extraction, and direct extraction into edible oil (Gamlieli-Bonshtein et al., 2002; Nonomura, 1987; Ruane, 1974). The direct extraction of carotene oils has a better acceptability in the market compared to organic solvents, in terms of safety and purity (Hosseini Tafreshi and Shariati, 2009).

18.3.3 Astaxanthin

Astaxanthin is the most successful case of a pigment from microalgae that is widely produced. The pigment comes from *H. pluvialis*, a freshwater green microalga. *H. pluvialis* has a two-stage life cycle: one vegetative stage, where the culture is mostly green and it is able to divide and grow, and one latent stage, where the culture turns red, and the cells stop dividing and become nonmotile. The production of astaxanthin must take into account both stages, as these are necessary for biomass accumulation and then pigment overproduction.

Regarding the green phase, the optimal condition for the growth of *H. pluvialis* has been set at a temperature of 20°C and a light intensity of 250 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, a CO_2 concentration of ca. 2.2 $mg\ L^{-1}$, nitrate concentration up to 10 mM, and a pH set around 7.5–8.0 (García-Malea et al., 2005; Giannelli et al., 2015; Jonker and Faaij, 2013; Panis and Carreon, 2016).

Moreover, related to the red phase, the microalga reaches this stage when stressed, mainly from nutrient starvation and light, temperature, or salt stresses. The pigment accumulation can be triggered by increasing the temperature to higher than 27°C, a light intensity of 500 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, and a nitrate concentration of ca. 3 mM (Evens et al., 2008; Fábregas et al., 2001; Giannelli et al., 2015; Panis and Carreon, 2016).

After the culture reaches the red stage, the biomass can be harvested, and there are one or two steps of concentration and dewatering of the algal suspension. Harvesting can be done by mechanical and chemical methods. In the case of *H. pluvialis*, the size allows mechanical harvesting by gravity sedimentation and/or centrifugation (Barros et al., 2015).

Then, in order to extract the astaxanthin from the cells, a process of cell disruption is necessary, followed by dehydration, and finally the recovery of the compound. The cell disruption is still the most problematic process because of the thick wall of *H. pluvialis*, and mechanical process such as bead milling can be used. After the cell disruption, the biomass must be treated in a short period as the compound is easily degraded. Dehydration can overcome this issue and prolong the storage of the biomass. Spray-drying is usually carried out to dry high-value microalgae-based products (Brennan and Owende, 2010; Mata et al., 2010; Mendes-Pinto et al., 2001; Molina Grima et al., 2003). The final step of astaxanthin obtainment can be a supercritical fluid extraction; although this kind of extraction is still expensive, the applications for pharmaceutical and nutraceutical sectors justify the cost (Mercer and Armenta, 2011; Sudhakar et al., 2014). The final product is called oleoresin, and contains about 20% of astaxanthin. It is also possible to isolate the pigment in a pure substance (>95%), although the cost is not justifiable (Panis and Carreon, 2016).

18.3.4 Lutein

Lutein, a yellow pigment, exists in microalgae and in higher plants. Industrially, the main source of lutein nowadays is marigold flowers, although microalgae are gaining attention and may be a sustainable source of lutein in the coming years. Lutein content and biomass

productivity are the main concerns in the choice of suitable strains for industrial application. Among the several screened microalgae, *Murielopsis* sp. and *S. almeriensis* seem to be the most promising strains for the production of lutein (Del Campo et al., 2007).

The production and accumulation of this pigment, as for the other ones, is majorly influenced by light, pH, temperature, nitrogen source, and salinity. High temperatures can favor the accumulation of lutein, although some strains are less tolerant and extra temperature increases can be damaging to the culture (Fernández-Sevilla et al., 2010).

Regarding light intensity, it seems that high intensities can also induce the production of lutein (Fernández-Sevilla et al., 2010). A synergetic effect between light and temperature was also observed (Sánchez et al., 2008), which may affect the photosynthetic efficiency of the cells and modify the pigment composition.

Regarding pH, the effect and range varies according to the strain and type of the culture (batch or continuous). In general, a pH around 8.0–9.5 seems to be the best range for the production of this pigment (Fernández-Sevilla et al., 2010).

In terms of the culture medium, the nitrogen source supplied as nitrate can be used for growth, although an induced stress of nitrogen starvation can cause an increase in lutein accumulation. The use of an intermediate concentration of nitrates can be an alternative to the two-step cultivation, where the biomass grows and accumulates some lutein. It is also possible to induce lutein production by salinity stress, with use of a moderate salinity of around 100 mM of NaCl (Sánchez et al., 2008).

Finally, it is also possible to increase the productivity of lutein with some oxidative stresses, such as the addition of H_2O_2 and NaClO. The induction of oxidative stress occurs due to the fact that lutein plays an important antioxidant role in the cell (Fernández-Sevilla et al., 2010).

In the matter of downstream processing, the use of microalgae as a source of lutein may simplify the extraction. The differences between microalgae and marigold are basically the chemical composition and the thickness of the cell wall. The marigold extraction is usually achieved by a simple solvent extraction to an oleoresin containing 80% of lutein. Microalgae lutein can also be extracted in the form of oleoresin, but contains only 25% of lutein and can be used as a direct product for further purification with chromatography (Cerón et al., 2008).

The major problem with using *Murielopsis* sp. or *S. almeriensis* is the need for a cell disruption step prior to extraction; again, milling or ultrasound are the most suggested and competitive methodologies for industrial applications. A classical solvent extraction can then be done with hexane, although for some applications the solvent contamination can be a concern. The supercritical fluid extraction with CO_2 can be used as an alternative for the chemical contamination; however, the cost of this kind of extraction is not competitive to market (Cerón et al., 2008).

18.4 Economical interest and challenges

The market use of microalgae is mainly related to health and cosmetics applications, where the scale of biomass production is relatively small when compared to other application such as the food industry, aquaculture, or even energy production (Acién et al., 2012; Richmond, 2000). In recent years, the total microalgae biomass production was about 20 kt per year; this was four times higher than in 2004, when the total production was around 5 kt per year (Benemann, 2013). Regarding cost, in 2004 the cost per ton was ca. $25,000 (USD) and had reduced by 20% by 2013 (Acién et al., 2012; Benemann, 2013; Pulz and Gross, 2004). The total revenue for *Chlorella* biomass is actually about $38 billion per year (USD) (Odjadjare et al., 2017; Yaakob et al., 2014).

Microalgae are basically sold in two main categories in the market: as raw biomass or processed for the achievement of high added-value compounds. As raw biomass (dried), microalgae are sold as a high source of protein and vitamins, and used for health dietary supplements and nutraceutical formulations. When processed for the achievement of secondary metabolites, the second category is a high added-value market, e.g., carotenoids, phycobiliproteins, and polyunsaturated fatty acids that can be use as food additives or in nutraceutical and pharmaceutical formulations (Mehta et al., 2018). The most challenging issue with high added-value bioproducts coming from microalgae is producing and processing the biomass in a cheap and effective way in order to fulfil the market requirements. In addition, the competition from synthetic equivalents to the natural compounds is still a great concern, even though in recent years society has been changing behavior and has started to give priority to natural sources for such compounds (Guedes et al., 2011b).

The market for natural products coming from microalgae is a growing one, yet its growth is limited by the technology used and bioprocess constraints. The costs of such products are still too high when compared to other sources, such as plants, which have competitive advantages to similar products (Guedes et al., 2011a,b). Compared to plants, microalgae share some of the advantages as sources for natural compounds, such as patterns of protein glycosylation and low risks of contamination by viruses that could infect the final product (Guedes et al., 2011a,b). On the other hand, microalgae have advantages over plants in terms of growth and space occupation (a much higher concentration of cells can be attained when in optimized conditions), and they do not compete for arable land or potable water. In relation to competition for other microorganism production, microalgae production is advantageous regarding the kind and diversity of compounds produced, but disadvantageous in terms of scale (Olaizola, 2003). The overcoming of such constraints could give the microalgae industry a boom in the world market, and both fundamental and applied research in the microalgal field are necessary in this regard.

In terms of the pigments market, the increasing demand for natural colors is raising the need to increase the product appeal and the research for new applications in the food and feed industry.

In the last few years, this kind of application for microalgae has risen, and it is expected that the global food colors market alone will reach over $4 billion (USD), with Europe being the largest market, followed by North America and Asia (Markets, 2019).

Apart from the pigments, a microalgae-based bioprocess can be used for valuable co-products, such as proteins, fatty acids, enzymes, or even biodiesel. Therefore, the use of the remaining biomass for application as biofuels may provide a solution to reduce the production costs. Some co-production has been already described: lutein and biodiesel co-production and also fucoxanthin and lipids from hexane-ethanol systems are established (Gong and Bassi, 2016).

From a biotechnological point of view, microalgae are a group of organisms with great potential but are as yet not well-studied. While more than 100,000 species are believed to exist (Guiry, 2012), only a few thousand strains are maintained in culture collections. From these collections, only some hundreds have been chemically analyzed, and even fewer species are produced at industrial scale. However, it is believed that microalgae can be a real source of bioactive compounds that can be exploited for several applications (Guedes et al., 2011b).

18.4.1 Market size

The global market for microalgal pigments is well-established and has expanded due to the extensive use in human health applications. Carotenoids represent the majority of this market, having reached ca. $1.5 billion (USD) in 2016 (Acién et al., 2012; Gong and Bassi, 2016; Markets and Markets, 2016) and with the potential to reach $1.8 billion (USD) in 2019. The carotenoid market can be categorized by compounds—β-carotene, lutein, and astaxanthin—which are responsible for 60% of the market (Gong and Bassi, 2016). Regarding application, the carotenoid market can be segmented into feed, food, supplements, cosmetics, and pharmaceuticals, with feed having the largest share in the carotenoid market (Markets and Markets, 2016). Microalgae share the carotenoid market with higher plants, but the former can be efficiently applied on the top three carotenoids production (Gong and Bassi, 2016). In addition, due to the improvement of fundamental research, the knowledge about the health benefits of microalgal carotenoids has increased the demand for these compounds (Markets and Markets, 2016). According to market studies (Fact.MR, 2018), the global market for carotenoids is expected to have a demand of more than 5000 tons per year in the near future.

The strict norms related to the use of synthetic carotenoids in the European Union have driven the use of microalgae as a source of carotenoids. Europe plays a key role in the carotenoid market, encouraging the use of natural sources for feed and food. The Asia Pacific region comes in second, and is the most promising and growing producer, with increasing consumption (Markets and Markets, 2016). Thus, it is expected that multinational manufacturers will invest in this market in the next years, and that there will be an increase in research and development initiatives for exploring new applications of carotenoids. The main concerns of the market

are the associated health risks related to high dosage and the strict regulation in emerging countries (Markets and Markets, 2016).

To date, two successful cases of commercialized microalgal carotenoids are known: astaxanthin from *H. pluvialis* and β-carotene from *D. salina*. Lutein is the third major market component, but the main source of lutein is still a higher plant (marigold flowers) with a price of $500 (USD) per kilo—a price even lower than that of synthetic products (Gong and Bassi, 2016).

The astaxanthin market is expected to reach ca. $800 million (USD) by 2022 and the demand for astaxanthin is still growing due to new functions and applications (Markets and Markets, 2017a). The most significant use for astaxanthin is as feed and animal pigmentation in aquaculture; these applications represent more than 40% of the general market. In second place appears the use in food and then in cosmetics (Mehta et al., 2018). However, synthetic astaxanthin still represents the majority of the market, although attitudes among buyers about medical advantages and their preference for biological items make synthetic pigments substantially less attractive (Mehta et al., 2018). The Asia Pacific region has a key role in this market, representing about 40% in 2016, with potential growth in the near future due to the increasing demand for healthy and nutritious products in the region and the changing habits of consumers (Markets and Markets, 2017a). *H. pluvialis* is the microalga used for the production of astaxanthin nowadays. This microalga has around 3% of astaxanthin in its composition, and it is the natural and safest choice in aquaculture and some other markets. Astaxanthin from microalgae is approved in the United States for dietary supplements and in some European countries for human utilization. The biggest producers of astaxanthin from microalgae are the United States, Israel, Japan, and India (Davinelli et al., 2018; Mehta et al., 2018).

The β-carotene share of the market is expected to have the quickest growth among the pigments within the next years, mainly due to its high market acceptability and healthy properties. The β-carotene market is overall focused on its use as colorant and multivitamins supplements (as provitamin A compound). *D. salina* was the first microalgae to be produced and commercialized as a source of β-carotene, with cultivation dating from the 1960s (Acién et al., 2012). *D. salina* has around 10% of carotenoids in its biomass. Therefore, commercial production of microalgal β-carotene has increased worldwide, with Australia being the major producer of this pigment, with a production of 1200 tons of *Dunaliella* per year. Israel, United States, India, and China are also significant markets for β-carotene. The worldwide market represents around $270 million (USD) (Mehta et al., 2018).

In the case of lutein, the main source is still higher plants, mainly due to production costs. However, the high content of lutein in microalgae might be an excellent opportunity for the market. The market for lutein is receiving more attention in the pharmaceutical industry due to its potential in new formulations for human health. The lutein market is expected to reach ca. $350 million (USD) by 2022, and in terms of demand, it is projected to reach 2000 tons by 2022. The interest in this pigment has been driven by the demand for dietary supplements and

companies focusing on the application for food and beverages (Markets and Markets, 2017b; Mehta et al., 2018). Europe has been a key player in the lutein market in the last few years, and is expected to grow in the future, in terms of both production and demand, mainly due to new regulations from the European Union that discourage the use of synthetic ingredients (Markets and Markets, 2017b). India is expected to be the fastest-growing market, mainly due to the changes in lifestyle and consumption behavior of its inhabitants. These changes have increased the demand for natural sources of carotenoids and are driving the growth of the lutein market in India. This market is mostly concentrated, with a reduced number of companies driving the market. However, small-scale partners have adopted strategies to expand their businesses globally by investing in the establishment of manufacturing facilities and technical service centers in various regions (Markets and Markets, 2017b).

Phycobiliprotein pigments have been used in several applications for human consumption. In the particular case of phycocyanin, its approval in the United States as food safe led to high demand for its use in the colorant industry (Pagels et al., 2019). In the case of phycoerythrin, the main use for this pigment is for fluorescent probes and analytical reagent in photodynamic therapy for cancer patients (Mehta et al., 2018). The market value of phycobiliproteins is around $100 million (USD), and this is estimated to rise to more than 200 million (USD) by 2028. The key player is Europe, where the use of phycocyanin as a colorant has increased due to its natural, nontoxic, and biodegradable characteristics (Pagels et al., 2019). The use of phycobiliproteins is still limited due the purification process cost and complexity (Markets and Markets, 2017b).

Finally, chlorophyll is used as a colorant in the food industry due to the demand for natural sources of pigments. Although some microalgae contain significant amounts of chlorophyll, extraction from inexpensive sources such as grass is still more common. However, it is possible that coproduction of chlorophyll with other pigments could provide sustainability to the use of microalgae as a source of this pigment (Odjadjare et al., 2017).

18.4.2 Constraints

Despite the increase of the potential of microalgae as a natural source of pigments for industrial application, there is still a gap regarding the technology scale-up and consequently a higher production of microalgae biomass and a lower price of production. Therefore, there are still constraints regarding biological, engineering, and economic aspects of microalgae-based processes (Acién et al., 2012; Richmond, 2000). Some of the main issues are the high installation and operation costs, the difficulties in terms of controlling culture growth, and contamination by other microorganisms (Rizwan et al., 2018). As already observed, the lack of knowledge about microalgal basic biology is one of the main constraints in industrial production, especially regarding the biosynthesis and regulation of bioproducts. For industrial-

scale cultivation, an in-depth understanding of the growth and metabolic characteristics of microalgae is still needed (Chen et al., 2019).

The microalgae-based process for the production of pigments requires several associated costs, from production to purification. Regarding culture growth, water, nutrients, CO_2 source, and energy costs are the main issues. Furthermore, the energy demand represents a key factor for the sustainability of the process (Acién et al., 2017). In addition, there are concerns regarding the culture medium and the contamination risk. In the case of astaxanthin and lutein, which are produced from *Haematococcus*, cultures can be easily contaminated by other microalgae and bacteria, and it is necessary to clean and sterilize the culture medium by filtration, UV-radiation, and/or heat, increasing the final cost. However, in the case of β-carotene production by hypersaline microalgae *Dunaliella*, the culture medium does not require much previous treatment, decreasing its cost (Acién et al., 2017).

In addition, as pigments are intracellular components, the recovery of biomass and further downstream processing is needed. However, there is no universal protocol for harvesting biomass, and that step must be adapted to each situation and strain. Sedimentation, centrifugation, and membrane filtration are the most commonly used and easiest approaches; an alternative is induced flocculation, with the disadvantage of possible contamination from the flocculant. Finally, it is possible to concentrate the biomass by spray- or freeze-drying, but this has a high associated cost (Guedes et al., 2011b). After the harvesting and before the downstream process, the waste needs to be released or recycled, and many costs are associated with this.

For the extraction of pigments, two major steps are fundamental: cell disruption and then extraction, using food-grade solvents or supercritical CO_2 (Acién et al., 2017). It should be noted that the cell disruption of microalgae is more difficult and consequently more expensive when compared to plants, as their cell walls may be composed of several layers with structural rigidity (Bastiaens et al., 2017).

The constraints are also related not only to the process itself but also to the installation of the process and the creation of the production facilities. The total investment takes into account the main equipment cost, including assembly and installation, in addition to other costs such as the infrastructure of the facility. The production costs also include raw materials, labor, supervision, and maintenance, among others (Acién et al., 2017).

Finally, the use of pigments for food products faces the requirements of society for vibrant colors and appealing products, which are easier to obtain with synthetic compounds. Furthermore, with natural colorants it is difficult to obtain a standard and continuous product due to the variability of the color in the final product of the microalgae bioprocess. In addition, strict storage is necessary for preserving the integrity of natural pigment. Finally, another challenge for the carotenoid market is ingredient compatibility, as the oil-based naturally

sourced colors of carotenoids can lead to permanent stains, thereby creating added challenges for food and beverage processors (Fact.MR, 2018).

18.4.3 Prospects and solutions

Considering the list of constraints described in Section 18.4.2, several solutions are possible and may help to improve production, reduce cost, and increase the market acceptability of microalgae pigments. The solutions include the screening for new strains with better composition based on the desired product or that resist more extreme conditions in order to avoid contamination, the optimization of culture conditions in order to increase pigment productivity, and the development of highly efficient and low-cost downstream processes (Rizwan et al., 2018).

As already stated, only a few species of microalgae have been studied and produced at large scale. Therefore, the prospection and discovery of new microalgae species with higher content of pigments may increase the production of these compounds. Furthermore, fundamental research into the analysis of different metabolic pathways is indispensable; thus, genome, postgenomic, proteomic, and metabolomic technologies would be a significant step forward (Chen et al., 2019). Using genetic engineering, it would be possible to increase pigment production. For example, an astaxanthin-producing *Saccharomyces cerevisiae* strain was created by successively introducing the *H. pluvialis* genes (Ye et al., 2018), although this technology could be applied in the microalgae itself.

Moreover, microalgae, and especially cyanobacteria, have developed unique adaptations to survive harsh environments, and it is possible to use these organisms to produce pigments avoiding contaminations, such as halotolerant species (Ye et al., 2018).

Regarding the bioprocess, it is possible to reduce the production cost by assembling the production facility with standard materials and equipment, in order to avoid custom-made designs or expensive materials (Acién et al., 2012). The equipment cost can also be reduced by increasing the size and production capacity of the equipment. Thus, the power consumption could be reduced by redesigning the photobioreactor, taking into account the energy balance of the system (Acién et al., 2012). Another way to reduce the production cost is the automatization of as many operations as possible in the facilities, reducing also the labor required (Acién et al., 2012, 2017).

Regarding extraction and purification, there is no standard method for microalgae pigment products, mainly due to the variability of cell composition and biochemical profile. In addition, the costs of extraction can be up to 60% of the total costs (Molina Grima et al., 2003). The choice of methodology may determine the final price and the use of the pigments. When used for food industries, extracts with lower purity are allowed, depending also on toxicity. On

the other hand, for pharmaceutical applications, a high purity of compound is necessary and the cost can be a hundred times higher (Morais et al., 2018).

Furthermore, in an optimal scenario, it would be possible to use the biomass for a variety of products, leading to a biorefinery process. The total revenues generated by the different coproducts in a biorefinery could increase the economic feasibility of algal-based applications (Bastiaens et al., 2017). However, theoretical concepts that have been reported are mostly related to small-scale experiments, and there is still a gap in technical feasibilities. Therefore, the upscaling of the biorefinery is needed to evaluate the viability of the process and its practical implementations (Bastiaens et al., 2017).

Ultimately, both optimization in technical process and biological advances in algae production must be tested by economic assessments in large-scale production systems by integrating life cycle analysis into the evaluation process (Chen et al., 2019).

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CHAPTER 19

Nutritional quality and bioactive properties of proteins and peptides from microalgae

Caleb Acquah[a], Sean M. Tibbetts[b], Sharadwata Pan[c], Chibuike Udenigwe[a]

[a]School of Nutrition Sciences, University of Ottawa, Ottawa, ON, Canada [b]Aquatic and Crop Resource Development Research Centre, National Research Council of Canada, Halifax, NS, Canada
[c]School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany

Chapter outline

19.1 Introduction 493
19.2 Microalgae proteins 495
19.2.1 Microalgae protein extraction 497
19.3 Bioactive peptides from microalgae 498
19.4 Nutritional quality of microalgae proteins 500
19.4.1 Microalgae amino acid profile 505
19.4.2 Digestibility of microalgae proteins 506
19.4.3 Protein utilization parameters 507
19.5 Challenges and opportunities 518
References 520

19.1 Introduction

Given the explosion of research activities surrounding microalgae in recent years, which has been largely ignited by seemingly Sisyphean algal biofuels initiatives (Peng et al., 2018; Yeong et al., 2018; Demirbas and Fatih Demirbas, 2011; Edeseyi et al., 2015), one might be forgiven for thinking that the use of microalgae for nutritional gain is a modern endeavor. People have been actively utilizing microalgae for millennia. Some of the first documented reports of microalgae consumption by humans and animals come from South America, Asia, and Europe, and date back more than 2500 years (Tseng, 2004; Becker, 2013; Wells et al., 2017). While the strategic scientific evaluation of the nutritional value of microalgae is a more modern development, many groups are now heavily invested in studying and producing microalgae proteins as alternatives to conventional sources widely used in food/feed products to help alleviate the world's anticipated "protein gap."

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As noted by Caporgno and Mathys (2018), in the context of nutritional quality, much of this is a consequence of some inherent benefits of microalgae-based proteins over other terrestrial plant-based proteins. It may be noted that the increased attention toward microalgae-based functional foods and their nutritional examination and evaluation is not a recent development; rather, it has been noted and documented in earlier studies (Brown, 1991; James et al., 1989). The protein nutritional quality, in particular, has received attention with factors such as toxicity, assimilation capability, and variations in biochemical composition across different microalgal species influencing the final attributes (Brown, 1991). Recent reviews have augmented these findings, adding to the repertoire of preexisting health advantages concerning microalgal biomass-based nutraceuticals (Bleakley and Hayes, 2017; Caporgno and Mathys, 2018; Wells et al., 2017). Conspicuously, the plasticity of microalgae species leads to the inherent production of antioxidants and pigments such as phycobiliproteins, chlorophylls, and carotenes as a protective mechanism against environmental stress (Koyande et al., 2019). Although accurate information regarding the amino acid sequence, cultivation conditions, and biochemical composition define and provide a better understanding of the protein nutritional quality, the proportions of the biochemical fractions themselves do not influence these qualities to a large extent (Brown, 1991). Instead, the particular contents of the specific fractional component, e.g., amino acids, are more important factors.

Past studies have focused on a diverse range of evaluations in the context of outlining several parameters, which directly or indirectly influence the protein nutritional attributes of isolated bioactive peptides. Hayes (2018) commented on the processing of microalgae-based food proteins and bioactive peptides, while taking into account the characterization and production strategies. Furthermore, the utilization of microalgae as a potential source of single-cell proteins has been reviewed by Barka and Blecker (2016), including collating the protein contents of a range of microalgal species, which varies between $<10\%$ and $>70\%$. The researchers assessed the technical, functional, nutritional, and physicochemical characteristics of the microalgal proteins based on parameters such as digestibility, biological significance, protein efficiency ratio, and net protein consumption. In addition, the subtle tendencies in the utilization of microalgae in novel food products along with their associated health benefits and their marketability have been reviewed recently by Caporgno and Mathys (2018). Furthermore, a systematic and in-depth review of general algal proteins as functional food components was done by Wells et al. (2017), with special attention on microalgae-based proteins. Pursuant to commenting on several aspects including bioavailability and the hordes of bioactive compounds derived from microalgae, the researchers noted a considerable information gap on the antioxidative (free-radical scavenging) abilities of microalgal products (Wells et al., 2017).

A careful review of past studies indicates a major focus on sustainability while investigating diverse nutritional aspects of microalgae including protein quality, novel origins of biologically

active peptides, and a multitude of strategies for their innovative and systematic characterizations, and advocacy for their increased use in the food sector. As mentioned earlier, the nutraceutical characteristics of bioactive peptides from microalgal proteins make them one of the most promising sources of peptides. However, despite a wide range of relevant but isolated studies, there is a need for a systematic documentation of outlooks on the current progress, challenges, and opportunities on utilization of microalgal proteins as food and feed. Furthermore, the database concerning relevant references on microalgal proteins, digestibility, and utilization efficiency is significantly smaller compared to their macroalgae (seaweed) counterparts. Even more limited are the research studies and reviews that focus on biologically active peptide fragments derived from microalgal proteins.

This chapter provides readers with a holistic account of the dietary attributes of the native proteins and short protein fragments derived from various microalgal species. Microalgal proteins are discussed with respect to their origins, proportions, and variation in different species, as well as diverse strategies adopted for their extraction from the complex microalgal matrices. Additionally, an overview of the generation of biologically active peptides from microalgal proteins is presented in Section 19.3, whereas Section 19.4 focuses on the nutritional attributes of the proteins including digestibility and bioavailability, and amino acid composition. Section 19.5 discusses some challenges that impede practical applications, including maintenance of microalgal protein nutritional quality, processing issues during isolation, and purification of proteins and peptides, among others.

19.2 Microalgae proteins

Proteins are essential dietary nutrients and macromolecules necessary for the existence of all living organisms. According to Henchion et al. (2017), the major sources of feed proteins across the globe are vegetarian proteins (~57%), meat (18%), dairy (10%), finfish and shellfish (6%), and other animal products (9%). Global protein demand has been projected to increase from about 202 million tonnes in 2015 to about 360–1250 million tonnes by 2050 (Soto-Sierra et al., 2018). Additionally, the ever-increasing global population, sociodemographic changes, climate changes, urbanization, and growing levels of affluence in emerging economies continue to exacerbate the challenges in meeting current protein demands (Henchion et al., 2017; Kim et al., 2019).

Microalgae proteins can be utilized in several forms like conventional proteins to produce primary protein concentrates or in the formulation of novel foods. They are generally characterized by high protein content (30%–55% of the dry weight), competitive nutritional qualities, minimal risk of allergenicities, and sustainable means of production (Henchion et al., 2017; Villarruel-López et al., 2017). Formulation of novel foods and edible products using microalgae proteins require a fundamental understanding of their nutritional and techno-functional properties. Techno-functional properties of proteins are their inherent

physicochemical properties that are essential in forming a stable interaction between each ingredient constituting the food matrix during preparation, storage, and consumption. The techno-functional properties of food proteins are determined by their surface-related and hydrodynamic properties (Schmitt et al., 1998). These properties are responsible for determining the quality of food in terms of the texture, structure, palatability, and stability. Despite the competitive protein content of most microalgae species, there have been limited studies regarding the characterization of their nutritional and techno-functional properties. Some microalgae species such as *Arthrospira* sp. (spirulina), *Chlorella vulgaris*, *Scenedesmus* sp., and *Dunaliella salina* have been reported to have comparable protein contents, if not higher, to some conventional protein sources such as dried eggs (47%), dried skimmed milk (36%), soy flour (37%), fish (22%), chicken (24%), beef (22%), and peanuts (26%) (Moorhead et al., 2012; Barka and Blecker, 2016). In addition, microalgae species have a superior protein yield of about 4–15 tonnes/Ha/year relative to the lower yields derived from soybean (0.6–1.2 tonnes/Ha/year), wheat (1.1 tonnes/Ha/year), and pulse legumes (1–2 tonnes/Ha/year) (Bleakley and Hayes, 2017). A summary of the protein contents for various microalgae species is presented in Table 19.1.

It must be emphasized that the protein content and nutritional value of microalgae are affected by numerous biotic and abiotic factors, genomic variation in species, and method of protein extraction. Protein content in similar microalgae species can vary based on factors such as the

Table 19.1: Estimated protein content for selected microalgae species.

Microalgae species	Protein content (% dry weight)	Reference(s)
Acutodesmus dimorphus	28.1	Tibbetts et al. (2015a)
Botryococcus braunii	~39	Tibbetts et al. (2015a)
Chlorella pyrenoidosa	58	Safafar et al. (2016)
Chlorella vulgaris	53.3	Tibbetts et al. (2015a) and Becker (2007)
Dunaliella tertiolecta	20–28	Welladsen et al. (2014)
Entomoneis punctulata	15–25	Welladsen et al. (2014)
Melosira dubia	6–12	Welladsen et al. (2014)
Neochloris oleoabundans	30.1	Tibbetts et al. (2015a) and Rashidi et al. (2019)
Nannochloropsis gaditana	44.9	Vizcaíno et al. (2019)
Porphyridium aerugineum	31.6	Tibbetts et al. (2015a)
Porphyridium cruentum	28–39	Tibbetts et al. (2015a) and Becker (2007)
Phaeodactylum tricornutum	45–54	Tibbetts et al. (2015a)
	39.6	Becker (2007)
Spirulina platensis	43–63	Tibbetts et al. (2015a)
Scenedesmus almeriensis	42.8	Vizcaíno et al. (2019)
Tetraselmis chuii	46.5	Tibbetts et al. (2015a)
Tisochrysis lutea	43.6	Vizcaíno et al. (2019)
Tetraselmis suecica	36.0	Vizcaíno et al. (2019)

analytical approach, strain of species, pretreatment method, growth medium, and harvesting and production methods (Barka and Blecker, 2016). For instance, *Chlorella spaerckii* and *Chlorella ovalis* have been reported to have protein contents of 6.87% and 10.87%, respectively, whereas *Chlorella vulgaris* has a protein content of 58% (Slocombe et al., 2013; Becker, 2007). Similarly, *Dunaliella tertiolecta* was reported to have a protein content of 11.4% whiles *Dunaliella salina* has a protein content of 57% by dry weight (Becker, 2007; Barbarino and Lourenço, 2005).

19.2.1 Microalgae protein extraction

Some of the major nutrition-related setbacks in the consumption of raw microalgae are their poor digestibility, bioavailability, and bioaccessibility of their proteins due to the recalcitrant nature of many microalgae cell walls. However, this can be circumvented through various extraction and isolation methods, namely, chemical, mechanical, and enzymatic extraction methods.

Typically, the extraction of microalgae proteins can be done using any of the following: aqueous, acidic, and/or alkaline media under optimum conditions such as biomass concentration, pH, ionic strength, temperature, and extraction time. Thereafter the media may be centrifuged, and protein recovered by applying separation techniques such as precipitation, ultrafiltration, and chromatography. Proteins have a net neutral charge at their isoelectric point (p*I*). Adjustment of pH $>$ p*I* results in the deprotonation of amino acid residues to enhance the solubility of proteins from microalgal biomass, whereas pH $<$ p*I* results in the protonation of amino acids to precipitate proteins from the media. This technique is simple for the extraction of crude protein isolates; however, there is no fit-for-all readily available pH value for microalgae protein extraction. For instance, the highest obtained protein solubility for *Spirulina platensis* was reported at pH 11 (Parimi et al., 2015), *Chlorella vulgaris* at pH 12 (Ursu et al., 2014), whereas *Nannochloropsis* sp. had an increase in protein recovery at a higher value, up to pH 13 (Gerde et al., 2013). That notwithstanding, harsh acidic and alkaline conditions have the potential to denature and aggregate proteins, resulting in the decrease in solubility and protein quality (Haque et al., 2005). Furthermore, for subsequent downstream biorefinery operations, where the residual biomass after protein recovery is designated for other bioprocesses, formic acid (HCOOH) could be used as a suitable alternative to hydrochloric acid (HCl) in protein precipitation. This is to prevent the triggering of corrosion reactions in thermochemical reactor vessels as well as minimizing the risk of toxicity on microbes in biochemical processes (Parimi et al., 2015).

Another extraction method is the aqueous two-phase system (ATPS), which consists of two incompatible aqueous phases, such as two polymers, two salts, or a combination of a polymer and certain salts, for the extraction of proteins and other valuable biomolecules in microalgae

(Suarez Ruiz et al., 2018). ATPSs are useful because they are mild techniques to prevent degradation of extracted biomolecules; they are also nontoxic, efficient, economical, and can readily partition extracted biomolecules and be easily scaled-up (Suarez Ruiz et al., 2018; Suarez Garcia et al., 2018). That notwithstanding, ATPSs have been rarely explored in microalgae protein extraction to date, and their principles of operation are less understood.

19.2.1.1 Microalgae cell lysis

Due to the recalcitrant nature of most microalgae cell walls, thereby limiting protein recovery, various techniques (e.g., chemical, enzymatic, mechanical, thermal, ultrasound) are applied in cell lysis prior to protein extraction. Recently, Ahn et al. (2019) compared the efficiency of lysis buffers containing different surfactants for the treatment of *Heterocapsa triquetra* for protein and DNA extraction. Optimal extraction of biomolecules was obtained at 1% of sodium dodecyl sulfate (SDS) whereas the exclusion of SDS resulted in insignificant protein extraction. The proposed technique was observed to be more efficient than the use of glass beads, which is commonly used in cell lysis.

Enzymatic disruption of cell walls is another technique useful in the extraction of multiple bioproducts from microalgae species. Choice of enzymes is critical and is predicated on the biochemical composition and cell wall structure of microalgae species (Sierra et al., 2017). Examples of enzymes for cell lysis include xylanases, cellulases, lipase, proteases, lysozyme, amylases, autolysin, and pectinases (Sierra et al., 2017; Chen et al., 2016).

Parimi et al. (2015) explored the effect of high-pressure homogenization, ultrasonication, and autoclaving as pretreatment techniques for *Spirulina platensis* prior to protein extraction. High-pressure homogenization yielded a protein recovery of 83.5%, ultrasonication 69.9%, conventional protein extraction without pretreatment 32.1%, and autoclaving 29%. It was observed through cell counting that high-pressure homogenization resulted in near-complete cell lysis of *Spirulina platensis*, which correlated with the highest release of proteins. However, the use of high-pressure homogenization resulted in the degradation of some low molecular weight proteins within the 25–100 kDa size range (Parimi et al., 2015). Other applicable techniques beyond cell counting for the analysis of degree of microalgae cell lysis include ultraviolet and visible light absorbance, and turbidity measurements (Huang et al., 2016).

19.3 Bioactive peptides from microalgae

Bioactive peptides are short fragments of proteins, often consisting of about 2–20 amino acid residues with biological activities and health benefits. Bioactive peptides can be obtained through digestion of food proteins, inherently active peptides in food, enzymatic hydrolysis, microbial fermentation, or a combination of various hydrolytic approaches (Ejike et al., 2017). Prior to hydrolysis, the fragments are often biologically inactive within their precursor proteins.

Consequently, relative to their native proteins, the generated peptides have a high specificity to their biological targets, and are thought to be bioaccessible, bioavailable, and to have reduced allergenicity (Agyei et al., 2015).

Currently, the commonly used food proteins for bioactive peptide production are from animals (egg, milk, fish, and meat) and plants (soy, pulses, canola, and flaxseed) (Chakrabarti et al., 2018; Udenigwe and Aluko, 2012). Considering the ever-growing population and concomitant effect on the increasing protein gap, a more sustainable approach is warranted for bioactive peptide production. Despite their high protein content and economical and sustainable means of production, microalgae species have received limited attention as protein sources for bioactive peptide production. Generation of bioactive peptides from microalgae species is affected by several factors, viz. differences in microalgal proteins (species and strain), seasonal variation, choice of proteolytic enzyme(s), and processing conditions (temperature, duration of hydrolysis, enzyme-to-substrate ratio, and pH). Optimization of these factors for high yield can be achieved using one-factor-at-a-time or design of experiment methods. Barkia et al. (2019) hydrolyzed six strains of marine diatom microalgae with different proteases including trypsin, Flavourzyme, papain, and pepsin at their optimal conditions for catalytic activity and an enzyme-to-substrate ratio of 1:100 (w/w). The generated peptides had bioactivities in inhibiting angiotensin I-converting enzyme (ACE) and oxidative stress markers, with the different strains of marine diatoms showing a wide variability in bioactivities of the peptide fractions. The biological properties and health benefits of bioactive peptides derived from microalgae are illustrated in Fig. 19.1.

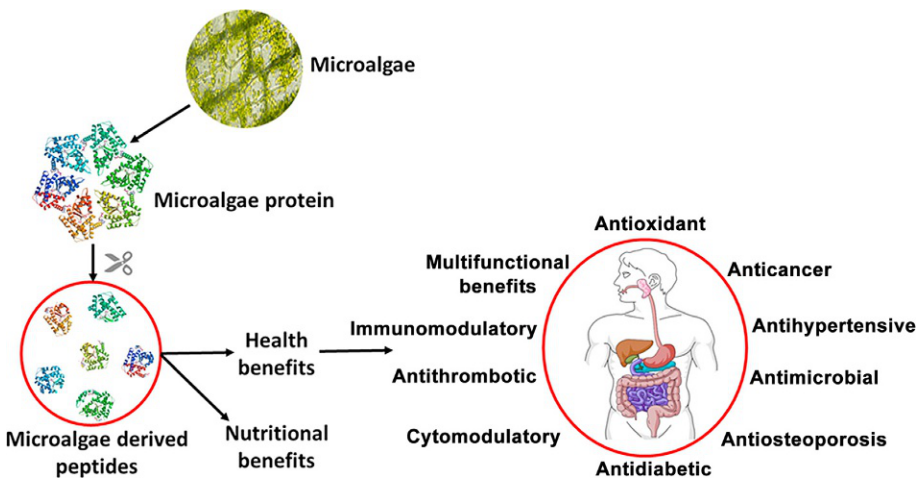

Fig. 19.1
Nutritional and health benefits of proteins and peptides derived from microalgae.

Identification of bioactive peptides from microalgae proteins can be facilitated using bioinformatic and proteomic tools (Tejano et al., 2019; Montone et al., 2018) for high-throughput screening and structure-function relationship studies. After hydrolysis, the generated bioactive peptides (hydrolysates) can be isolated and purified with a wide array of separation techniques such as membrane filtration, electrophoresis, size-exclusion chromatography, reversed-phase high performance liquid chromatography (RP-HPLC), ion-exchange chromatography (IEX), fast protein liquid chromatography (FP-LC), and affinity chromatography (Acquah et al., 2019; Aluko, 2018). These techniques can be configured into one-dimensional, multidimensional, or orthogonal multidimensional separation tools depending on the extent of peptide purity expected and end application of the products (Acquah et al., 2019).

Peptides exhibit structural diversity (amino acid sequence, molecular weight, net charge, chain length, and conformation) that determines their functionality (Yao et al., 2018). For instance, the structural features of bioactive peptides in inhibiting ACE includes the occurrence of aliphatic or aromatic hydrophobic amino acids at the C-terminal, aliphatic amino acids at the N-terminal, and short peptide chain length (Iwaniak et al., 2015; Lee and Hur, 2017). Antioxidant capacity of peptides are characterized by structural features such as the presence of antioxidant amino acid residues (e.g., cysteine, histidine) in the sequence, and peptide hydrophobicity (Sarmadi and Ismail, 2010; Rajapakse et al., 2005). Antimicrobial peptides are characterized by longer chain lengths and cationic and amphiphilic features that enhance their interaction with microbial cell membrane (Mohanty et al., 2016; Nielsen et al., 2017). Biological activities and health benefits reported for peptides generated from microalgae are summarized in Table 19.2.

19.4 Nutritional quality of microalgae proteins

One of the foremost criteria when evaluating novel food/feed resources for their nutritional value is the assessment of their "protein quality." Protein quality can be described as a series of measures aimed to evaluate the capacity of a dietary protein source (or dietary mixtures) to satisfy the metabolic needs (e.g., maintenance, growth, tissue repair, reproduction, lactation) of a target animal for amino acids and nitrogen. This is predominantly related to the amino acid composition, digestibility, and bioavailability of the protein source itself and the daily dietary nitrogenous requirements of the consuming animal (Boye et al., 2012). For microalgae, protein quality has been investigated by food scientists and applied nutritionists for approximately 60 years. The earliest studies, focusing primarily on *Arthrospira* (Spirulina), *Chlorella*, *Scenedesmus*, and *Spongiococcum*, began in the 1950s with the intent to assess their potential as superfoods for the growing global human population and as a compact, nutrient-dense food for planned space missions (Becker, 2013).

Table 19.2: Microalgae species and method of generating bioactive peptides for health applications.

Microalgae species	Method of hydrolysis	Bioactivity	Test subject	Reference(s)
Arthrospira maxima	Enzymatic hydrolysis	Antioxidant, antimicrobial, chelating, antihyaluronidase, antioxidant, and anticollagenase	In vitro	Montalvo et al. (2019)
Chlorella ellipsoidea	Enzymatic hydrolysis	Antioxidant	Monkey kidney cell line	Ko et al. (2012b)
Chlorella ellipsoidea	Enzymatic hydrolysis	ACE-inhibition and antihypertensive	Spontaneously hypertensive rats	Ko et al. (2012a)
Chlorella ellipsoidea	Enzymatic digestion	Antioxidant	African green monkey kidney cell line	Lee et al. (2009)
Chlamydomonas reinhardtii	In vitro simulation of gastrointestinal digestion	Antihypertensive	Spontaneously hypertensive rats	Carrizalez-López et al. (2018)
Chlorella pyrenoidosa	Hot water extraction	Antioxidant	Human skin fibroblast	Shih and Cherng (2012) and Chen et al. (2011)
Chlorella sorokiniana	Enzymatic action simulation	DPP-IV inhibition, ACE inhibition, antioxidant, stimulating	In silico analysis	Tejano et al. (2019)
Isochrysis galbana	Enzymatic hydrolysis and in vitro digestion	ACE-inhibition	In vitro	Wu et al. (2015)
Navicula incerta	Enzymatic hydrolysis	Antioxidant	Human hepatocellular carcinoma (HepG2) cells	Kang et al. (2012)
Nannochloropsis oculata	Enzymatic hydrolysis	ACE-inhibition	Human umbilical vein endothelial cells	Samarakoon et al. (2013)
Spirulina maxima	Enzymatic hydrolysis	Anti-HIV-1	Human T cell line	Jang and Park (2016)
Spirulina maxima	Enzymatic hydrolysis	Antiallergic therapy	Rat basophilic leukemia mast cells	Vo et al. (2014)
Tetraselmis suecica	Enzymatic digestion	Antioxidant	African green monkey kidney cell line	Lee et al. (2009)

Whether for food or feed, the scope of protein quality evaluation of novel ingredients like microalgae generally takes a logical stepwise approach (Fig. 19.2). This involves: (1) determination of total protein content and characterization of essential and nonessential amino acid profile; (2) estimation of protein and essential amino acid digestibility, which may be

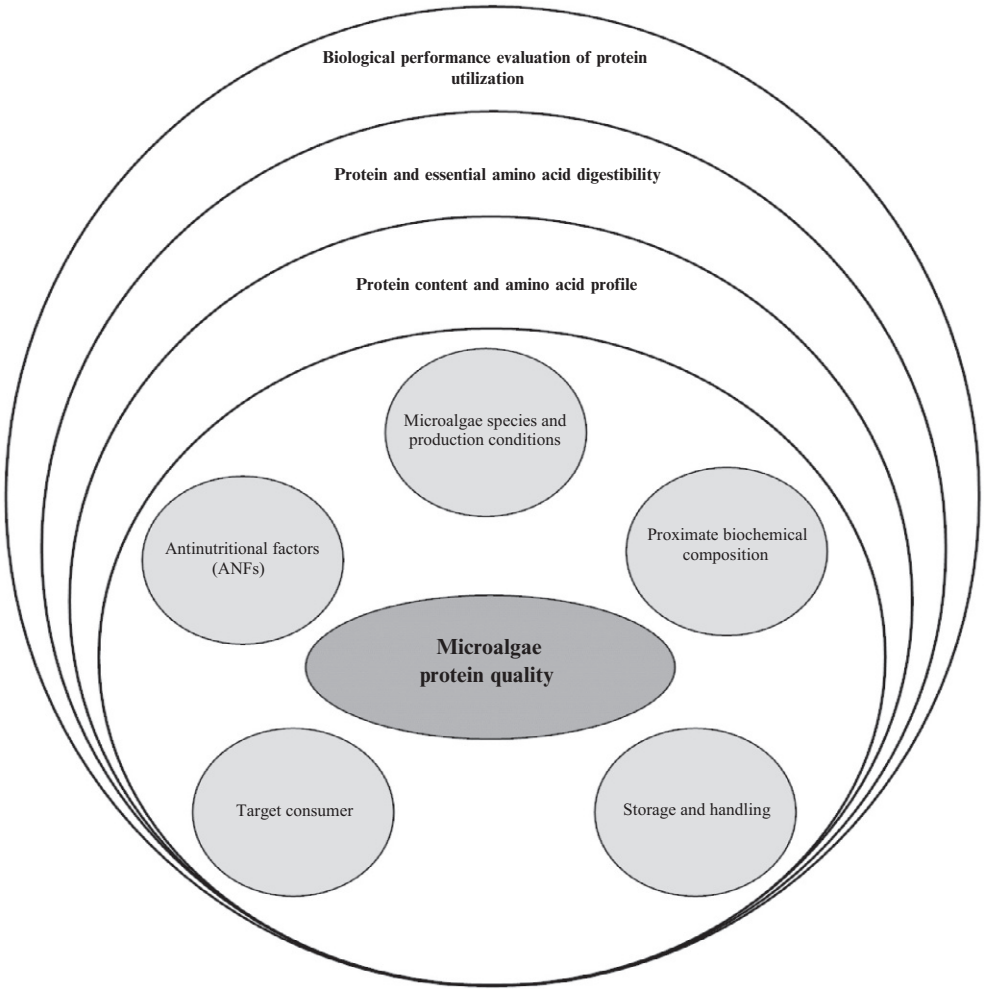

Fig. 19.2

Scope of microalgae protein quality evaluation and general factors that affect it. *Modified from Tibbetts, S.M., 2012. Protein and Energy Nutrition of Marine Gadoids, Atlantic Cod (Gadus morhua L.) and Haddock (Melanogrammus aeglefinus L.). https://www.semanticscholar.org/paper/Protein-and-energy-nutrition-of-marine-gadoids%2C-cod-Tibbetts/ea388eee1e5e603f817f6042bb363d0ca012d69c.*

conducted in vitro, in vivo, or using a combination of both methods; and (3) in vivo biological evaluation of dietary protein quality through feeding studies with test subjects of the target consumer. Within the context of protein quality, it is important to consider that several biotic and abiotic factors influence the protein quality of food/feed ingredients. For microalgae, these factors include the species/strain of microalgae, the cultivation and harvesting strategy, the postharvest processing conditions used in its production, its general proximate biochemical composition, the presence or absence, concentrations, and types of antinutritional factors, and

the environmental conditions in which the product has been transported and stored through the commodity market chain. Another important consideration for microalgae comes from the pioneering work of Chronakis (2001) who demonstrated that, unlike that of terrestrial plant proteins, the three-dimensional structures of *Spirulina* proteins are highly regulated by strong hydrophobic protein-to-protein bonds, disulfide bonds, and relatively weak hydrogen bonds. All of these forces influence the biological functions and solubility of proteins. Of course, protein solubility is often a requisite for protein quality, and some differences between conventional plant proteins and algal proteins might be expected due to relative hydrophobicity of their constituent amino acids. It has been reported that nonpolar (hydrophobic) amino acids represent 35% of the total amino acid pool of soy-based proteins, while those of proteins derived from the microalga *Cladophora* sp. are higher (44%), which may indicate that algal proteins are more resistant to digestion (Kumar et al., 2002; Borgen, 2012). Lastly, and often less considered, is that protein quality is highly dependent upon what specific animal species is intended to consume the microalgae. Different potential target consumers have very different feeding habits and are thus equipped with a diverse range of digestive capabilities, which can be broadly generalized as those of monogastric vs ruminant animals, carnivorous vs herbivorous animals, homoeothermic vs poikilothermic animals, and, as in the case for aquaculture, tropical warm- and freshwater fish vs temperate marine species.

The first and seemingly most simplistic step in the evaluation of microalgae protein quality is determination of its total protein content, and this has been an area of some debate in recent years (Chronakis, 2000; Becker, 2013; Tibbetts et al., 2015a,b,c,d). For applied nutrition applications, three analytical methods are the most commonly used for microalgae and these include: (1) spectrophotometric copper- or dye-binding assays (e.g., Lowry, Bradford, etc.); (2) elemental nitrogen analysis (e.g., Dumas, Kjeldahl, etc.) and the application of an "appropriate" nitrogen-to-protein (N-to-P) conversion factor (k_p); and (3) comprehensive amino acid analysis and calculation of protein content by the sum of the quantified amino acids (e.g., ΣAA). The latter method is generally agreed to provide the most accurate estimation of the true total protein content; however, it is often cost-prohibitive for some research and industrial applications. Elemental nitrogen analysis and the application of a reliable k_p factor is likely to be the most broadly suitable method to determine the protein content of microalgae, and a generalized factor of $N \times 4.78$ seems appropriate (Laurens et al., 2012; Tibbetts et al., 2015a,b), while several other species- and cultivation phase-specific k_p factors have been published for various microalgae (Table 19.3). This method is fully destructive so there is no requirement for protein extraction preparation steps, such as cell wall disruption, and it also eliminates potential interferences of microalgal carotenoids and chlorophyll that make spectrophotometric methods easily erroneous and difficult to replicate. However, the indiscriminate application of the conventional "Jones factor" ($N \times 6.25$; Jones, 1931) after elemental nitrogen analysis remains problematic and has led to unreliable overestimations of protein contents of microalgae in the published literature. This conventional k_p factor disregards the assumption that the protein source in question contains 16% N, which is rarely

Table 19.3: Published nitrogen-to-protein (k_p) conversion factors for various genera of microalgae proteins.

Microalgae genus	k_p factor	Reference(s)
Amphidinium	5.13	Lourenço et al. (2004)
Arthrospira	4.44	González López et al. (2010)
	6.21–6.27	Safi et al. (2013)
Chlorella	4.25–4.50	Lourenço et al. (2004)
	4.33	Laurens et al. (2012)
	5.96–6.35	Safi et al. (2013)
	3.66–4.33	Slocombe et al. (2013)
	4.82–5.13	Templeton and Laurens (2015)
	5.14–5.21	Tibbetts et al. (2015b)
Dunaliella	3.99–4.34	Lourenço et al. (2004)
	5.37	Slocombe et al. (2013)
Haematococcus	5.63–6.25	Safi et al. (2013)
Hillea	4.61–4.72	Lourenço et al. (2004)
Isochrysis	3.99–4.59	Lourenço et al. (2004)
Micractinium	4.86–4.91	Tibbetts et al. (2015b)
Monodopsis	2.72	Slocombe et al. (2013)
Muriellopsis	4.44	González López et al. (2010)
Nannochloris	5.01–5.14	Tibbetts et al. (2015b)
Nannochloropsis	4.87–4.95	Lourenço et al. (2004)
	5.86–6.28	Safi et al. (2013)
	3.46	Slocombe et al. (2013)
	4.77–5.35	Templeton and Laurens (2015)
Nannofrustulum	5.01	Templeton and Laurens (2015)
Neochloris	4.80	Templeton and Laurens (2015)
Phaeodactylum	4.72–4.87	Lourenço et al. (2004)
	4.68–5.08	Templeton and Laurens (2015)
Porphyridium	4.44	González López et al. (2010)
	6.34–6.35	Safi et al. (2013)
Prorocentrum	3.77–4.43	Lourenço et al. (2004)
Rhodella	3.00	Slocombe et al. (2013)
Scenedesmus	4.44	González López et al. (2010)
	5.55	Dickinson et al. (2013)
	4.95–5.08	Templeton and Laurens (2015)
	5.29–5.65	Tibbetts et al. (2015c)
Skeletonema	3.82–4.63	Lourenço et al. (2004)
Synechococcus	4.66–5.22	Lourenço et al. (2004)
Synechocystis	4.44	González López et al. (2010)
Tetracystis	5.29–5.30	Tibbetts et al. (2015b)
Tetraselmis	4.37–4.80	Lourenço et al. (2004)
	3.23	Slocombe et al. (2013)
Thalassiosira	5.40	Lourenço et al. (2004)

the case for microalgae that generally contain higher levels of nonprotein nitrogenous (NPN) compounds (e.g., chlorophyll, phycoerythrin, nucleic acids, amino sugars, ammonia, inorganic N) than other conventional terrestrial food/feed protein sources (Barbarino and Lourenço, 2005; Gilani, 2012). The literature reports a high variability in NPN content of microalgae (4%–54%) depending upon species, season, and growth phase (González López et al., 2010; Becker, 2013; Templeton and Laurens, 2015).

19.4.1 Microalgae amino acid profile

While the total protein content of microalgae varies by several magnitudes in the literature, with ranges of 7%–70% (Lourenço et al., 2004; Becker, 2007; Laurens et al., 2012), the amino acid profile of microalgae protein remains relatively conserved, regardless of algal species and cultivation strategy. From a nutrition standpoint, of the 20 amino acids that typically make up the proteins of the body, some are considered as being either nonessential (dispensable) or essential (indispensable). Nonessential amino acids (NEAAs) can be synthesized de novo in quantities sufficient to support daily metabolic protein demands. By contrast, essential amino acids (EAAs) cannot be synthesized endogenously in sufficient quantities to support daily protein demands and, therefore, must be provided in the diet. In effect, some amino acids are "dietary essential" because the consumer lacks the biochemical mechanisms required to synthesize the chemical configurations of the carbon chain skeletons of the particular amino acids (Jobling, 1994).

Most monogastric animals (e.g., poultry, swine, fish, rodents) generally require the same 10 EAAs in their diet: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Arginine is not considered essential for humans, with the exception of specific developmental stages, under conditions of ill-health, and for preterm infants (Reeds, 2000; McDonald et al., 2010). Of course, there are specific exceptions to the 9–10 EAAs, whereby some classical NEAAs may be considered as "conditionally essential" (CEAAs) given the particular physiological status of the human/animal and the other constituents making up their diet. In addition, the absolute daily amounts of each EAA required to meet metabolic demands is complicated because it is influenced by multiple factors such as temperature, age, growth rate, sex, genetics, reproductive status, state of health, caloric intake, and other dietary interactions (Boye et al., 2012; WHO, 2007). When expressed in relation to total protein content, microalgae protein is typically richest in the EAAs leucine, arginine, and lysine (at an average of ~7% of total protein), moderate in isoleucine, phenylalanine, threonine, and valine (at an average of ~4% of total protein), and lowest in methionine, histidine, and tryptophan (at an average of ~2% of total protein) (Brown et al., 1997; Tibbetts, 2018). With regard to the NEAAs, microalgae are most characteristically richest in the amidic amino acids, aspartic acid and glutamic acid, which typically predominate at 20%–30% of total protein (Becker, 2007, 2013).

Although microalgae amino acid profile data is readily available in the literature, a simple and rapid initial estimate of protein quality based on those profiles is usually lacking. This parameter is referred to as the EAA index (EAAI), which can be calculated for any novel food/feed protein resource in reference to an "ideally" balanced protein (e.g., egg albumin, EAAI = 1.0) according to (Oser, 1951). It has been suggested that microalgae proteins for human and animal consumption are of high quality if they have an EAAI of at least 0.9, moderate quality at 0.7–0.9, and inadequate quality when less than 0.7 (Brown and Jeffrey, 1992). When considering the EAAI strictly as an initial estimation of protein quality, reported values almost exclusively suggest that the balance of EAAs making up microalgal proteins compare well and often exceed those of other conventional terrestrial plant-based protein food/feed resources. Reported EAAI values for microalgae are *Alexandrium* (1.59), *Ansanella* (1.12), *Arthrospira* (0.81), *Botryococcus* (1.03), *Chlorella* (0.80–1.35), *Dunaliella* (0.98), *Gymnodinium* (0.79), *Heterocapsa* (1.03), *Micractinium* (0.90–0.92), *Micromonas* (0.86–0.91), *Nannochloris* (0.86–1.07), *Nannochloropsis* (0.90–1.18), *Pavlova* (0.82–0.83), *Phaeocystis* (0.90–0.93), *Phaeodactylum* (0.91), *Porphyridium* (1.25), *Scenedesmus* (0.51–1.00), *Stichococcus* (0.92), *Takayama* (0.84–0.90), *Tetracystis* (1.02–1.10), and *Tetraselmis* (0.89) (Brown and Jeffrey, 1992; Kent and Mangott, 2015; Tibbetts et al., 2015a,b,c,d, 2017a, 2019; Vidyashankar et al., 2014; Waghmare et al., 2016; Lim et al., 2018).

High protein content and a well-balanced EAA profile (as reflected by the EAAI) may provide investigators and product developers with good initial indicators of microalgae protein quality, but the measures alone do not guarantee high dietary protein utilization by the consumer. In fact, there is no one single parameter to describe the protein quality of food/feed ingredients (Bender, 1982; WHO, 2007). Rather, it must be a combination of biochemical analysis, EAA profiles, protein and amino acid bioaccessibility, and the efficiency of protein utilization measured through biological evaluations. Specifically, many microalgae come with an additional nutritional barrier that may be less prevalent in other conventional protein resources. Many microalgae are known to possess highly recalcitrant cell walls, and digestibility represents the first bottleneck for adequate protein assimilation by humans and animals once consumed.

19.4.2 Digestibility of microalgae proteins

Digestibility of microalgae protein has been measured using both in vitro and in vivo methods. In vitro methods involve the "simulation" of digestion using exogenous enzymes that may or may not be species-specific. Over the past half-century, most of the in vitro protein digestibility assays applied to food and feed have evolved from foundational methods employing commercial enzymes (e.g., gastric pepsin, alkaline proteases, papain, trypsin, chymotrypsin, aminopeptidases) and most assays employed today utilize commercially sourced mammalian (typically porcine) enzyme cocktails (Sheffner et al., 1956; Ford and Salter, 1966; Buchanan, 1969; Maga et al., 1973; Hsu et al., 1977; Satterlee et al., 1979; Adler-Nissen et al., 1983;

Pedersen and Eggum, 2009). While in vitro methods are generally quick to perform and relatively inexpensive, they typically only provide general approximations and are useful for preliminary sample screening, but ultimately require in vivo validation to be of quantitative value. By contrast, costly but extremely reliable in vivo methods involve feeding the target consumer (or a suitable animal model) with test diets containing varying inclusion levels of the microalgae products and then comparing protein composition in the test diets with that of the collected fecal samples [either fully passed from the rectum (fecal digestibility) or surgically collected from the terminal ileum (ileal digestibility)], in relation to the analyzed levels of a suitable indigestible inert marker added to the test diet (e.g., metal oxides, acid insoluble ash) (Boye et al., 2012). Protein digestibility values generated in this manner are referred to as "apparent digestibility" while those that take into account "endogenous" protein losses in the feces (by feeding a protein-free diet) are considered "true digestibility"; the latter is more difficult to reliably obtain for many consumers (WHO, 2007). The in vitro and in vivo protein digestibility for various genera of microalgae reported in the literature is highly variable (Tables 19.4 and 19.5) and is, to a large extent, related to algal species and strain, cultivation strategy, and postharvest processing (predominantly the extent of algal cell wall disruption). This is also influenced by the wide range of assay conditions used by various investigators to measure protein digestibility.

Ultimately, the most definitive measure of protein quality comes from in vivo biological studies that involve feeding trials with the target test subjects or a suitable animal model. For microalgae, these studies have traditionally involved laboratory rodents (rats and mice as models for human nutrition), terrestrial livestock (predominantly mink, poultry, rabbits, and swine), and, in a few rare cases, actual human test subjects. By far the most exhaustive body of knowledge on microalgae protein quality comes from aquaculture research, where innumerable evaluations have been conducted with a vast array of aquatic animals (e.g., farmed finfish and shrimp). It should be noted that many of the protein quality parameters to be discussed were initially developed as standardized assays using laboratory rats under a controlled set of experimental conditions, test diets, and feeding exposure times (Gilani, 2012). Despite this, during the past decades, many of these protein quality parameters have been applied to other domestic farmed livestock and fish, and results from these examples are also presented herewith.

19.4.3 Protein utilization parameters

The criteria used by researchers to evaluate protein quality of microalgae (beyond protein and amino acid content and digestibility, previously discussed) have varied somewhat between studies and target consumers. The most commonly used parameters are (in order of complexity): chemical score (CS), EAA index (EAAI), protein efficiency ratio (PER), protein digestibility-corrected amino acid score (PDCAAS), digestible indispensable amino acid score (DIAAS), productive protein value (PPV), net protein utilization (NPU), and biological value (BV). The nitrogen retention efficiency (NRE) has appeared more recently, particularly for

Table 19.4: Published protein digestibility values for various genera of microalgae measured using simulated in vitro enzymatic assays.

Microalgae genus	Enzyme source	Protein digestibility (%)	Reference(s)
Acutodesmus	Mammalian	78	Tibbetts et al. (2015a)
Arthrospira	Mammalian	84	Lipinsky and Litchfield (1974)
		70–85	Devi et al. (1981)
		71–98	Mišurcová et al. (2010)
		88–89	Tibbetts et al. (2015a)
		71–83	Wild et al. (2018)
Botryococcus	Mammalian	87–97	Tibbetts et al. (2015a)
Chlorella	Mammalian	55–66	Casey and Lubitz (1963)
		27–93	Hedenskog et al. (1969)
		64–96	Yoshida and Hoshii (1982)
		80–81	Komaki et al. (1998)
		70–97	Morris et al. (2008)
		61–79	Mišurcová et al. (2010)
		86–87	Tibbetts et al. (2015a)
		69–80	Tibbetts et al. (2016)
		72–94	Wild et al. (2018)
Micractinium	Mammalian	69–80	Tibbetts et al. (2016)
Microcystis	Mammalian	69	de la Fuente et al. (1977)
Nannochloris	Mammalian	49–64	Tibbetts et al. (2016)
Nannochloropsis	Gilthead seabream	78	Vizcaíno et al. (2019)
	Mammalian	85–91	Tibbetts et al. (2015a)
		48–80	Wild et al. (2018)
	Pacific white shrimp	69–78	Tibbetts et al. (2017b)
	Rainbow trout	79–88	Tibbetts et al. (2017b)
	Senegalese sole	80	Vizcaíno et al. (2018)
Neochloris	Mammalian	89–90	Tibbetts et al. (2015a)
Nostoc	Mammalian	40–48	Hori et al. (1990)
Pavlova	Mammalian	82	Tibbetts et al. (2020)
Phaeodactylum	Mammalian	89	Tibbetts et al. (2015a)
		76–84	Wild et al. (2018)
Porphyridium	Mammalian	93–94	Tibbetts et al. (2015a)
Scenedesmus	Gilthead seabream	59	Vizcaíno et al. (2019)
	Mammalian	28–93	Hedenskog et al. (1969)
		52–84	Tibbetts and Fredeen (2017)
	Senegalese sole	57	Vizcaíno et al. (2018)
Tetraselmis	Gilthead seabream	52	Vizcaíno et al. (2019)
	Senegalese sole	57	Vizcaíno et al. (2018)
Tetracystis	Mammalian	52–62	Tibbetts et al. (2016)
Tisochrysis	Gilthead seabream	78	Vizcaíno et al. (2019)
	Senegalese sole	72	Vizcaíno et al. (2018)

Table 19.5: Published protein digestibility values for various genera of microalgae measured using species-specific in vivo digestion bioassays.

Microalgae genus	Test subjects	Protein digestibility (%)	Reference(s)
Arthrospira	African catfish	81	Teuling et al. (2017)
	Arctic charr	82	Burr et al. (2011)
	Atlantic salmon	85	Burr et al. (2011)
	Caspian sturgeon	69–76	Safari et al. (2016)
	Nile tilapia	86	Sarker et al. (2016)
		82	Teuling et al. (2017)
		74–81	Ekpo and Bender (1989)
	Poultry	61	Velten et al. (2018)
	Rats	74–77	Clément et al. (1967)
		77	Chung et al. (1978)
		75–81	Narasimha et al. (1982)
		74–84	Becker (2013)
		83–90	Hoseini et al. (2013)
		85	Gutiérrez-Salmeán et al. (2015)
	Siberian sturgeon	80–87	Palmegiano et al. (2005)
	Silver carp	60–75	Ekpo and Bender (1989)
	Swine	77–81	Neumann et al. (2018b)
Aurantiochytrium	Nile tilapia	83	Fernandes et al. (2019)
Chlorella	African catfish	81	Teuling et al. (2017)
	Atlantic salmon	77–87	Tibbetts et al. (2017a)
	Humans	57–59	Dam et al. (1965)
		66	Lee et al. (1967)
	Mice	76–88	Neumann et al. (2018c)
	Nile tilapia	80	Sarker et al. (2016)
		81	Teuling et al. (2017)
		90	Barone et al. (2018)
Poultry		81	Lipstein and Hurwitz (1980)
Rainbow trout		64	Tulli and Tibaldi (2017)
Rats		69–75	Erchul and Isenberg (1968)
		80	Thananunkul et al. (1977)
		89	Herrero et al. (1993)
		87–89	Komaki et al. (1998)
		45–66	Janczyk et al. (2005)
		59–89	Becker (2007)
		44–63	Janczyk et al. (2007)
		80–89	Becker (2013)
Coelastrum	Rats	78	Pabst (1974)
		75–88	Ciferri (1983)
		89	Saleh et al. (1985)
		89	Herrero et al. (1993)
Desmodesmus	Atlantic salmon	54–67	Gong et al. (2018)

Continued

Table 19.5: Published protein digestibility values for various genera of microalgae measured using species-specific in vivo digestion bioassays—cont'd

Microalgae genus	Test subjects	Protein digestibility (%)	Reference(s)
	Poultry	76–91	Ekmay et al. (2015)
Entomoneis	Atlantic salmon	83–85	Norambuena et al. (2015)
Isochrysis	Mink	19	Skrede et al. (2011)
Nannochloropsis	African catfish	72	Teuling et al. (2017)
	Atlantic salmon	72–73	Gong et al. (2018)
	Mice	84–89	Neumann et al. (2018c)
	Mink	35	Skrede et al. (2011)
	Nile tilapia	75	Teuling et al. (2017)
		73–81	Sarker et al. (2018)
		61–78	Teuling et al. (2019)
	Poultry	78–79	Gatrell et al. (2018)
Phaeodactylum	Mice	69–88	Neumann et al. (2018c)
	Mink	80	Skrede et al. (2011)
Porphyridium	Rainbow trout	94	Tulli and Tibaldi (2017)
Scenedesmus	African catfish	68	Teuling et al. (2019)
	Humans	68	Dam et al. (1965)
	Nile tilapia	67	Teuling et al. (2017)
	Rats	65–73	Cook (1962)
		51–65	Erchul and Isenberg (1968)
		11–87	Hedenskog et al. (1969)
		81	Saleh et al. (1985)
		72–88	Becker (2007)
		51–89	Becker (2013)
	Swine	54	Hintz et al. (1966)
		72	Hintz and Heitman (1967)
Schizochytrium	Nile tilapia	82	Sarker et al. (2016)
Staurosira	Poultry	75–88	Ekmay et al. (2015)
Uronema	Rats	82	Pabst (1974)

livestock and aquaculture feeds. With the exception of CS and EAAI, all of the parameters generally take into account the nutritional properties of the protein source itself (e.g., its protein content, amino acid profile, and digestibility) and the proteinaceous use by the animal consuming the protein (Brody, 1994). The CS of a protein source is simply determined based on its analyzed EAA profile relative to an "ideal" EAA profile such as egg albumin. The percentage difference between the two protein sources for the most limiting EAA is taken to be its CS. Unlike the EAAI index, which was discussed previously, also determined by comparison with ideally balanced egg albumin, the definition of CS is restricted to the single EAA concentration that is the most "distant" from its egg albumin counterpart, while the EAAI makes use of the concentration of all EAAs within the test protein. Both CS and EAAI rely

solely on EAA profile data and do not require animal feeding trials, so these parameters, for a particular protein source, remain the same regardless of the target consumer, and thus are limited in their usefulness. A notable advancement to these basic scoring parameters is the PDCAAS (FAO/WHO Expert Consultation, 1991), which requires a digestibility study conducted with the target consumer whereby the amino acid concentrations are quantified in the test protein source, experimental diet, and collected fecal samples to determine the species-specific digestibility of the protein. Thus, while similar to CS and EAAI, PDCAAS is an improvement as it reports the EAA profile of the test protein source, corrected for its overall protein digestibility, but some concerns still remain with its use (Gilani, 2012). As such, a recent extension of this parameter is known as the DIAAS, which corrects each EAA for its own individual digestibility as opposed to the overall combined protein digestibility (FAO, 2013; Rutherfurd et al., 2015). However, until such a time where sufficiently more reliable protein and EAA digestibility data are generated for microalgae proteins, PDCAAS and DIAAS will not be widely adopted.

To measure the PER of a protein source, a feeding trial is required, but the necessary measurements are rather minimal, and questions have been raised in recent years about the PER's continued usefulness (Boye et al., 2012; Gilani, 2012). Briefly, the subject must be fed a diet containing the test protein and accurate determination of feed intake (total feed consumption over the entire trial) and total body weight gain recorded over the trial period. The PER is then estimated based on the total amount of live body weight gain given the amount of protein that the subject has consumed, regardless of the EAA profile or digestibility of that protein; it completely excludes consideration of nitrogen balance in the test subject. A moderate improvement on this deficit is the PPV, which also takes into account the amount of protein that the subject has consumed but is expressed as a function of the subject's daily protein deposition rate (thus requiring whole-body carcass composition analysis) as opposed to simply their live body weight gain (Liebert, 2015). The parameters NPU and BV represent the most comprehensive assessments of protein quality as they take into account several nutritional factors, including the test subject's nitrogen balance. Determination of the NPU of a protein source requires a feeding trial conducted in a manner that the investigators can accurately account for total feed intake and subsequent nitrogenous fecal and urinary losses from test subjects fed both a diet containing the test protein source and a nitrogen-free diet. The NPU is then calculated as the ratio between the amount of dietary nitrogen retained in the body of the subject and the amount of nitrogen consumed, corrected for nondietary endogenous fecal and urinary nitrogenous losses. The parameter BV is determined in a similar manner as NPU; however, it takes the determination a step further. Whereas NPU is expressed simply in terms of how much nitrogen the subject "consumed," the BV is expressed in terms of how much nitrogen was "consumed and digested" by the subject, again corrected for nondietary endogenous fecal and urinary nitrogenous losses. Protein utilization efficiency data for various microalgae fed to humans and a wide range of target animal subjects reported in the literature are shown in Table 19.6. It should be noted that the data presented includes only those studies that have

Table 19.6: Published parameters of protein utilization for various microalgae fed to target test subjects.

Microalgae genus	Test subjects	PER[a]	PPV[b]	NPU[c]	BV[d]	NRE[e]	Reference(s)
Anabaena	Indian major carp	1.6	–	23	–	–	Mishra et al. (2017)
Arthrospira	European sea bass	1.1–1.3	–	–	–	–	Hasanein et al. (2018)
	Indian major carp	1.4–1.7	–	–	–	–	Nandeesha et al. (2001)
		2.2	–	46	–	–	Mishra et al. (2017)
	Lebranche mullet	1.1–1.5	–	–	–	–	Rosas et al. (2019)
	Mozambique tilapia	0.9–2.9	–	–	–	–	Olvera-Novoa et al. (1998)
	Nile tilapia	1.1	–	–	–	–	Hussein et al. (2013)
	Parrot fish	0.9–1.0	–	–	–	–	Kim et al. (2013)
	Poultry	–	50–65	56–75	–	–	Neumann et al. (2018a)
		–	39–53	47–61	–	–	Velten et al. (2018)
	Rainbow trout	2.4	–	–	–	–	Güroy and Ergünb (2015)
	Rats	–	–	36–50	48–65	–	Clément et al. (1967)
		2.2	–	–	68	–	Chung et al. (1978)
		–	–	53–62	68–82	–	Narasimha et al. (1982)
		1.8–1.9	–	–	–	–	Becker and Venkataraman (1984)
		1.8–2.1	–	53–65	68–78	–	Becker (2007)
		1.8–2.2	–	38–65	51–82	–	Becker (2013)
		1.8–2.6	–	53–92	–	–	

							Hoseini et al. (2013)
		1.6–2.7	–	–	–	–	Moreira et al. (2013)
		1.9	–	62	75	–	Gutiérrez-Salmeán et al. (2015)
	Red tilapia	1.6–1.8	–	–	–	–	Rincón et al. (2012)
	Rohu	0.4	–	–	–	–	Makvana et al. (2018)
	Swine	–	–	58–72	–	–	Neumann et al. (2018b)
	Siberian sturgeon	2.7–3.3	–	–	–	–	Palmegiano et al. (2005)
	Silver seabream	0.9–1.5	19–32	–	–	–	El-Sayed (1994)
Aurantiochytrium	Nile tilapia	–	–	–	–	38–39	Fernandes et al. (2019)
Chlorella	African catfish	2.1–2.5	–	–	–	–	Enyidi (2017)
	Common carp	0.8–1.0	–	–	–	–	Khani et al. (2017)
	European sea bass	1.1–1.3	–	–	–	–	Hasanein et al. (2018)
	Gibel carp	1.4–1.5	–	–	–	–	Xu et al. (2014)
	Mice	–	–	46–67	60–76	–	Neumann et al. (2018c)
	Pacific white shrimp	0.9–1.4	–	14–23	–	–	Shapawi et al. (2017)
	Poultry	0.3–1.5	–	–	–	–	Leveille et al. (1962)
		–	–	–	42–80	–	Lipstein and Hurwitz (1983)
	Rats	1.8	–	–	–	–	Burlew (1953)
		0.9	–	–	–	–	Leveille et al. (1962)
		1.7–2.9	–	–	–	–	Lubitz (1963)
		0.7–2.0	–	50–57	71–76	–	

Continued

Table 19.6: Published parameters of protein utilization for various microalgae fed to target test subjects—cont'd

Microalgae genus	Test subjects	PER[a]	PPV[b]	NPU[c]	BV[d]	NRE[e]	Reference(s)
							Erchul and Isenberg (1968)
		0.8–1.3	–	–	–	–	Cheeke et al. (1977)
		1.9	–	–	–	–	Thananunkul et al. (1977)
		1.1	–	–	–	–	Herrero et al. (1993)
		2.0	–	70	78	–	Herrero et al. (1993)
		1.0–2.1	17–36	48–67	94–101	–	Janczyk et al. (2005)
		1.1–2.0	–	31–68	53–77	–	Becker (2007)
		1.0–2.1	–	48–64	93–101	–	Janczyk et al. (2007)
		0.7–2.0	–	31–70	53–80	–	Becker (2013)
	Red drum	0.9–1.7	–	–	–	–	Patterson and Gatlin (2013)
Coelastrum	Rats	1.6	–	53	68	–	Pabst (1974)
		1.9	–	63	–	–	Saleh et al. (1985)
		1.7–2.1	–	57–68	76	–	Ciferri (1983)
		1.9	–	67	75	–	Saleh et al. (1985)
		1.9	–	67	75	–	Herrero et al. (1993)
Desmodesmus	Atlantic salmon	2.3–2.4	–	–	–	–	Kiron et al. (2016)
Dunaliella	Rats	1.2	–	–	–	–	Mokady and Cogan (1988)
		2.1	–	–	–	–	Herrero et al. (1993)
Entomoneis	Atlantic salmon	2.4–2.6	–	49–52	–	–	Norambuena et al. (2015)

Haematococcus	Pacific white shrimp	1.4	–	–	–	–	Ju et al. (2012)
	Yellow perch	1.6–1.8	–	–	–	30–31	Jiang et al. (2018)
Micractinium	Poultry	2.0	–	–	–	–	Mokady et al. (1978)
		–	–	–	42–80	–	Lipstein and Hurwitz (1983)
Microcystis	Rats	1.1	–	–	–	–	de la Fuente et al. (1977)
Nannochloropsis	European seabass	1.5	–	–	–	27–28	Haas et al. (2016)
	Gilthead seabream	1.3–1.4	–	–	–	–	Jorge (2016)
	Mice	–	–	49–54	59–61	–	Neumann et al. (2018c)
	Nile tilapia	2.9	–	–	–	–	Gbadamosi and Lupatsch (2018)
		1.7–2.1	–	–	–	–	Sarker et al. (2018)
	Red drum	1.2–2.9	–	–	–	–	Patterson and Gatlin (2013)
	Senegalese sole	1.4	–	–	–	–	Vizcaíno et al. (2018)
Nanofrustulum	Atlantic salmon	1.9–2.1	–	–	–	–	Kiron et al. (2012)
	Common carp	1.7	–	–	–	–	Kiron et al. (2012)
	Whiteleg shrimp	1.6–1.7	–	–	–	–	Kiron et al. (2012)
Navicula	Red drum	1.9–2.3	–	–	–	33–38	Patterson and Gatlin (2013)
Nostoc	Indian major carp	2.4	–	24	–	–	Mishra et al. (2017)
Pavlova	European seabass	1.5	–	–	–	27–31	Haas et al. (2016)
Phaeodactylum	Atlantic salmon	–	–	–	–	39	Sørensen et al. (2016)

Continued

Table 19.6: Published parameters of protein utilization for various microalgae fed to target test subjects—cont'd

Microalgae genus	Test subjects	PER[a]	PPV[b]	NPU[c]	BV[d]	NRE[e]	Reference(s)
	Mice	–	–	55–65	69–77	–	Neumann et al. (2018c)
Scenedesmus	Gilthead sea bream	0.9–1.1	–	–	–	–	Vizcaíno et al. (2014)
	Rainbow trout	2.2–2.4	46–49	–	–	–	Tomás-Almenar et al. (2018)
	Rats	1.7	–	35–44	49–56	–	Cook (1962)
		1.6–1.8	–	–	–	–	Cook et al. (1963)
		2.6	–	–	–	–	Saleh et al. (1985)
		1.1–2.0	–	52–67	72–81	–	Ciferri (1983)
		1.1–2.2	–	52–66	72–81	–	Becker (1984)
		2.1	–	67	76	–	Herrero et al. (1993)
		1.1–2.1	–	31–67	60–81	–	Becker (2013)
	Senegalese sole	1.4	–	–	–	–	Vizcaíno et al. (2018)
Schizochytrium	Atlantic salmon	2.7–2.8	–	–	–	–	Kousoulaki et al. (2015)
Spongiococcum	Poultry	0.4	–	–	–	–	Leveille et al. (1962)
	Rats	0.3–1.2	–	–	–	–	Leveille et al. (1962)
Tetraselmis	Atlantic salmon	1.9–2.1	–	–	–	–	Kiron et al. (2012)
	Common carp	1.8–2.0	–	–	–	–	Kiron et al. (2012)
	Rats	1.1	–	–	–	–	Herrero et al. (1993)
	Whiteleg shrimp	1.6	–	–	–	–	Kiron et al. (2012)
Thraustochytrium	Atlantic salmon	–	53	–	–	–	Carter et al. (2003)

	Rainbow trout	–	–	–	–	37–41	Betiku et al. (2016)
Tisochrysis	European sea bass	1.2	–	–	–	–	Tibaldi et al. (2015)
	Gilthead seabream	1.5–2.0	–	–	–	–	Palmegiano et al. (2009)
	Rats	1.1	–	–	–	–	Herrero et al. (1993)
	Senegalese sole	1.4	–	–	–	–	Vizcaíno et al. (2018)
Uronema	Rats	1.6	–	45	55	–	Pabst (1974)
		1.4	–	46	55	–	Ciferri (1983)
Westleopsis	Indian major carp	2.7	–	36	–	–	Mishra et al. (2017)

[a]Protein efficiency ratio (g of body weight gain per g of protein intake).
[b]Protein productive value (%).
[c]Net protein utilization (%).
[d]Biological value (%).
[e]Nitrogen retention efficiency (%).

reported the aforementioned protein quality parameters widely used (e.g., PER, PPV, NPU, BV, and NRE) and it is clear that the PER, NPU, and BV values dominate in the published literature. However, there are countless published microalgae feeding studies with animals where these particular parameters were not measured. These studies generally favored the use of other parameters such as weight gain (WG), specific growth rate (SGR), average daily gain (ADG), thermal growth coefficient (TGC), feed conversion ratio (FCR), and/or feed efficiency (FE). However, since these parameters are not "entirely" reflective of protein utilization, they have been excluded from this review. Variations in reported protein quality parameters for microalgae are predominantly related to particularities of the microalgae under investigation (e.g., species and strain, cultivation and harvesting strategy, postharvest drying and other processing conditions). Heterogeneous results are also likely to occur because of variable experimental parameters such as formulation and composition of the experimental test diets used to deliver the microalgal proteins, environmental parameters, sampling and bioanalytical practices, and data and statistical procedures, among others.

19.5 Challenges and opportunities

One of the early challenges encountered in microalgal protein utilization was the lack of adequate quantification of amino acid compositions across various species of microalgae, as highlighted by Brown (1991). Thus, it is difficult truly to compare the nutritional quality between the same microalgal species grown under different conditions and also between highly diverse species (James et al., 1989). One important observation is that the nutritional quality of particular microalgal species is determined not by the total protein content, but by the concentrations of specific essential amino acids of the protein fractions (Brown et al., 1989).

There are other potential challenges along the value chain, from the acquisition of raw materials to the utilization of the microalgal protein products. For food proteins and bioactive peptides, generally, there is a focus on discovering diverse sources, expanding applications in food and feed industry, and high-throughput categorization techniques (Hayes, 2018; Hayes et al., 2007). Microalgae have the potential to fill the protein gap for nutritional and health applications, considering their diverse protein reservoir and ease of production. An area for future development of microalgal proteins and bioactive peptides is their acceptability as functional ingredients in processed foods. Currently, there is a lack of comprehensive and integrated processing and testing facility for validating the biochemical, microbial, and toxicological profiles of microalgal protein products (Barka and Blecker, 2016). Additionally, utilization of microalgal food proteins and peptides is impeded by poorly developed strategies for the preparation and processing of microalgae biomass, and limited R&D by the food industry in developing microalgal protein-based products (Caporgno and Mathys, 2018). Recently, manufacturing challenges and bottlenecks in algal protein utilization have been comprehensively reviewed (Bleakley and Hayes, 2017). Major issues in this sector include lack

of accessibility to large-scale production facilities, variability in the growth season of particular species, sophistication of setups for microalgae protein isolation, and subsequent downstream processing of microalgal biomass, proteins, or protein hydrolysates for bioactive peptide production (Bleakley and Hayes, 2017; Joubert and Fleurence, 2008). Notably, protein characteristics can fluctuate significantly during production and processing, which can influence the bioaccessibility and bioavailability of indispensable (essential) amino acids (Boisen and Eggum, 1991), and bioactive peptides derived from microalgal proteins. Microalgal protein digestibility represents probably one of the biggest challenges in terms of nutritional protein quality (Bleakley and Hayes, 2017; and references therein). Although there is a poor understanding of the assimilation of microalgal proteins by human consumers, some species possess comparable digestibility to those of macroalgae and conventional protein sources (Becker, 2007).

Another obstacle in the use of microalgae for the production of dietary proteins and bioactive peptides is scalability, since microalgal protein manufacturing is still in its infancy (Harnedy and FitzGerald, 2013). In this regard, commercial-grade production may be facilitated by incorporation of emerging processes such as ultrasound and membrane technologies (Sheng et al., 2012). The aspect of food security cannot be ignored either. In addition to amassing nutrients, some microalgae species could also bioaccumulate deleterious heavy metals and toxins (Schiavon et al., 2017). Consequently, stringent region-specific regulatory and statutory guidelines need to be enforced. This could offer significant challenges for protein and bioactive peptide production from microalgal sources, although a recent study confirms that microalgae do not surpass the permissible quantities of heavy metals (van der Spiegel et al., 2013). However, industrial large-scale commercial algaculture is likely to involve the utilization of industrial flue gas and municipal or agro-industrial wastewater as inexpensive cultivation inputs, and this will have to be a priority consideration for nutritional and safety evaluation. In addition to these factors, one of the most critical considerations is to keep the costs of microalgae processing and protein/peptide production within an acceptable range (Bleakley and Hayes, 2017). According to Wells et al. (2017), the major challenges associated with microalgal protein utilization as food may be summed up as: (1) lack of a full picture about the nutritional/protein quality across different microalgal species, which varies depending on production sites and harvest periods; (2) identification and quantification of only the appropriate protein fractions and assessment of their bioavailability; and (3) a reasonable understanding of the interactions between the functional components of microalgal proteins with the host metabolic system. Special emphasis has been made on bioaccessibility and bioavailability as the most pressing issues, including the complex interplay between chemistry, manufacturing and processing techniques, and interference of other food components of the microalgal proteins and peptides. Finally, microalgal proteins should be explored for advanced functionalities in food product development, e.g., for food structuring and as delivery vehicles for bioactive compounds. These studies on microalgal proteins are expected to enhance

understanding of their structure and functionality and their utilization in food and feed products to close the protein gap sustainably in the future.

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CHAPTER 20

Bioactive polysaccharides from microalgae

Nastasia Prybylski[a,d], Claire Toucheteau[b], Hicham El Alaoui[a], Nicolas Bridiau[b], Thierry Maugard[b], Slim Abdelkafi[c], Imen Fendri[c], Cédric Delattre[d], Pascal Dubessay[d], Guillaume Pierre[d], Philippe Michaud[d]

[a]Université Clermont Auvergne, CNRS, LMGE, Clermont-Ferrand, France [b]Université de La Rochelle, UMR CNRS 7266, LIENSs, Equipe Approches Moléculaires Environnement-Santé, Département de Biotechnologies, La Rochelle, France [c]Unité de Biotechnologie des Algues, Biological Engineering Department, National School of Engineers of Sfax, University of Sfax, Sfax, Tunisia [d]Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut Pascal, Clermont-Ferrand, France

Chapter outline

20.1 Introduction 533
20.2 Structural diversity of polysaccharides from microalgae 535
20.3 Processes production, extraction, and purification of EPS from microalgae and cyanobacteria 540
20.4 Polysaccharides from microalgae as antiinfectious agents 551
20.4.1 Antiviral activity of PS from microalgae and cyanobacteria 551
20.4.2 Antibacterial activity of PS from microalgae and cyanobacteria 553
20.4.3 Antifungal activities of PS from microalgae and cyanobacteria 554
20.5 Bioactivity and structure-activity relationships 555
20.5.1 Antioxidant activity and free radical scavenging 556
20.5.2 Anticancer activity 557
20.5.3 Immunomodulatory activity 558
20.5.4 Antiinflammatory activity 559
20.5.5 Anticoagulant activity 560
20.5.6 Antitussive activity 561
20.5.7 Antilipidemic and antiglycemic activities 561
20.5.8 Antiaging activity 562
20.6 Future perspectives 562
References 563

20.1 Introduction

Microalgae and cyanobacteria constitute a diverse group of photoautotrophic single cell-organisms present in various ecosystems including airborne, terrestrial, and most of all aquatic environments (Tesson et al., 2016). Cyanobacteria are often called microalgae or blue

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microalgae even if they are photosynthetic prokaryote microorganisms. In this chapter, the term "microalgae" will be used to designate the sole eukaryotic photosynthetic microorganisms. Microalgae and cyanobacteria are capable of living in severe conditions using solar energy, water, and inorganic nutrients to reduce CO_2 into complex organic compounds with efficiencies exceeding those of terrestrial plants. This specific metabolism is a real opportunity to produce bioenergies (biofuel, bioethanol, CH_4, H_2, etc.) without competition with food production for arable soils exploitation. Moreover, even if microalgae are mainly photoautotrophic organisms, they have the ability to use organic carbon, dividing their metabolism into three types: photoautotrophic, heterotrophic, and mixotrophic.

The classification of microalgae is under permanent development and can be found in algae database resources (e.g., www.algaebase.org). Briefly, from a taxonomic point of view, microalgae (or eukaryotic microalgae) stemmed from two successive and independent endosymbioses leading to Rhodophyta, Chlorophyta, and Glaucophyta (first endosymbiosis) and to Euglenophyta, Chlorarachniophyta, Cryptophyta, Heterokonta (or Ochrophyta), Haptophyta, and Dinophyta for the second one. A third endosymbiosis between Heterokonta and Dinophyta created Bacillariophyta (Cooper and Smith, 2015). Microalgae emerged later than cyanobacteria, which were the first microorganisms able to perform complete photosynthesis on Earth. It has been estimated that about 800,000 species exist (including cyanobacteria), of which 35,000 species have been described in detail (Guiry, 2012; Gaignard et al., 2019). Among all these putative or described strains of microalgae and cyanobacteria, only 100–200 species have been studied in academic laboratories for the production of valuable metabolites, of which only a dozen species are exploited by industry.

In a context of fossil fuels depletion and anthropogenic climate change, the culture of microalgae and their refining to produce biofuels has recently sparked the interest of scientists. Even if the potential of microalgae in this field is evident, numerous technological advances have to be realized to attain an economical model. Indeed, approaches of biological engineering and not only of biology are necessary for the sustainable photoproduction intensification, the recycling of culture media, the harvesting of microalgal biomass, and their refining. However, the frenetic activity of researchers to improve the biofuel production by microalgae through numerous research projects has led to the development of technologies and notably to production of biomass in photoautotrophy at large scale. This opportunity opens the way to the exploitation of microalgae for production of some high-value metabolites such as pigments, peptides, enzymes, polyunsaturated fatty acids, and polysaccharides (Pignolet et al., 2013). Among them, polysaccharides (PS) and particularly extracellular polysaccharides (ECP) or exopolysaccharides (EPS) are of major interest as they have been exploited not only for their rheological but above all for their biological activities.

EPS from microalgae and cyanobacteria have been poorly described in the literature compared to those from terrestrial plants, seaweeds, fungi, or nonphotosynthetic microorganisms

(Delattre et al., 2016; Gaignard et al., 2019). They are mainly heteropolysaccharides without repeating units in their structures and are often associated with nonsugar components such as sulfates. These original structures, composed sometimes of rare monosaccharides as main components, are of high molecular weights, giving them a hydrocolloid character. Clearly, the hydrocolloid market is closed to EPS of microalgae and cyanobacteria, as the costs to obtain them are too high to allow competition with texturing agents from plants and macroalgae. However, the nutraceutical, cosmetic, and therapeutic fields, which always need new high-value products, are open for original biopolymers. This chapter focuses on the next developments in the characterization of biological activities of PS from microalgae and cyanobacteria.

20.2 Structural diversity of polysaccharides from microalgae

According to the IUPAC-IUB nomenclature, glycans can be classified in two categories according to their degree of polymerization (DP): oligosaccharides ($2 < DP < 10$) and polysaccharides ($DP > 10$). In practice, the border between poly- and oligosaccharides is more complex, and low molecular weight polysaccharides (BPMs) are often considered as oligosaccharides. Polysaccharides are condensed polymers consisting of long chains of monosaccharide units. Their great structural variability comes from the large number of available monosaccharide units (mainly hexoses and pentoses) and the possibility of making glycosidic linkages between the anomeric hydroxyl group of an ose and any of the hydroxyl groups of another monosaccharide. A simple monosaccharide such as glucose can bind with its anomeric carbon (α or β) with the five hydroxyls of a second glucose, giving rise to 11 different structural isomers. The structure of glycans thus strongly depends not only on their composition but also on the type of glycoside bonds, which introduces a considerable structural and conformational flexibility. Overall, the complexity of glycans can be distinguished in five items: (i) the flexibility of the macromolecular chain—a parameter influenced by the nature of the glycoside bonds involved; (ii) the nature of the carbon skeleton (homopolysaccharide or heteropolysaccharide consisting of neutral oses and/or acids); (iii) the molecular weight and mass distribution; (iv) the type of functional groups carried by the macromolecular chain (substitution)—groups defined by their position and rate of incorporation; and (v) the presence of other covalently linked molecules such as proteins (proteoglycans).

Why itemize something so obvious? Because the direct consequence of this structural variability is a great diversity in the composition and role of carbohydrates in living organisms. Obviously, this applies to microalgae, for which polysaccharides play key roles ranging from C-storing to cell-structuring functions, but also as biological activators, adhesion supports, carbon metabolism, or water-content regulators. To this first layer of biochemical complexity

(otherwise called the famous "structure/function relationship") is added the wide diversity and significance of marine algae (Singh and Saxena, 2015). Even by limiting the point of view to only microalgae, the phylogenetic diversity of nonpolysaccharide and polysaccharide producers can be considered as a bottomless chasm. This overall understanding highlights the millions of possibilities and an unlimited universe in the compositions and structural variability of polysaccharides from microalgae. Hopefully, nature has preserved the best and/or more appropriate (useful) configurations for specific biotopes, although various genes (or remains) coding for nonexpressed glycotransferases can be found by genomic methodologies, which are essential for a phylogenetic approach. In this way, Rossi and De Philippis (2016) showed the possibility to explore cyanobacteria and microalgae EPS producers by identifying various genes and putative proteins involved in EPS metabolism and processing. Another point, which is still poorly recognized, is the importance of the extraction and purification procedures as well as the analytical methods used for determining the structure of microalgal polysaccharides. These procedures always affect the quality, quantity, and type of studied polysaccharides. Depending on their bad (misunderstood) uses, structural changes of polysaccharides can be falsely observed and errors attributed to culture conditions, specific species, etc. (Delattre et al., 2016).

Polysaccharides from microalgae can be distinguished into three main groups: intracellular, structural, and extracellular glycans. Newcomers should keep in mind that the last category is more difficult to apprehend because of misuses and/or abusive uses in the literature for describing extracellular polysaccharides. EPS, ECP, and polysaccharidic exudates belong to this class, which also includes cell-bound polysaccharides (BPS) and released polysaccharides (RPS). To go further, BPS can be subdivided into sheath, capsule, and slime, and RPS are often defined as colloidal EPS but also low molecular weight exudates (Delattre et al., 2016).

Regarding the literature, few microalgae EPS are fully detailed in terms of structure, probably because of: (i) the number of various monosaccharides (sometimes up to seven) and uronic acids; (ii) the lack of repeating units; (iii) the presence of nonsugar substituents; and (iv) the rheological behavior in culture media, making their proper recovery without structural modifications difficult (e.g., decrease in molar mass). Tables 20.1–20.5 give an exhaustive overview of the main structural data available in the literature for EPS extracted from various microalgae phyla, i.e., Charophyta (Klebsormidiophyceae, Zygnematophyceae), Chlorophyta (Chlorodendrophyceae, Chlorophyceae, Trebouxyophyceae), cyanobacteria, Myozozoa (Dinophyceae), Ochrophyta (Bacillariophyceae, Coscinodiscophyceae, Mediophyceae, Pinguiophyceae, Raphidophyceae), and Rhodophyta (Porphyridiophyceae, Rhodellophyceae).

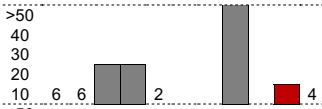
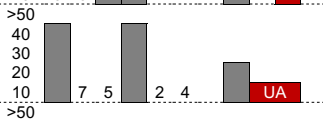
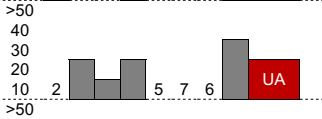
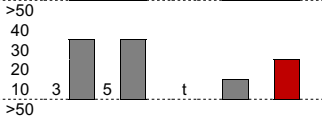
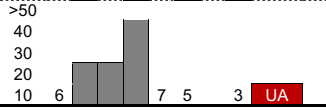
EPS from Charophyta exhibit a tryptic profile of Gal, Fuc, and Xyl, depending on the species (Table 20.1). Fucan compositions are the most reported, and specific structural features, such as (1,3) and (1,4)-linked Fuc*p*, seem to be recurrent. The content in uronic acids is also not

Table 20.1: Overview of EPS heterogeneity from microalgae belonging to Charophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO_4^{2-}, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace.

Species	%	Ara	Gal	Glc	Fuc	Man	Rha	Rib	Xyl	GalA	GlcA	Others	Main structural features	Nonsugar groups	References
Charophyta															
Closterium sp.	>50 40 30 20 10		5	5		1	1						(1,4)-Linked Fuc (3,4)-Linked GlcA	–	Domozych et al. (1993)
Cosmarium sp. 1	>50 40 30 20 10						4	3			UA		Predominance of 2-Fuc residues (2,3,4)-Linked Fuc (2,3)-Linked Ara	Prot, SO_4^{2-}	Kiemle et al. (2007)
Cosmarium sp. 2	>50 40 30 20 10						6	1			nd		–	nd	Kiemle et al. (2007)
Hyalotheca dissiliens	Presence	X	X		X		X		X			X	(1,4)-Linked GlcA Branchpoints in *C*-3 position Complex linkage of Fuc units	–	Vieira and Paulsen (1994)
Klebsormidium flaccidum 446C	>50 40 30 20 10	2	2	3	4						UA		Rhamnan?	SO_4^{2-} (t)	Barberousse et al. (2006)
Klebsormidium flaccidum 748A	>50 40 30 20 10										UA		Mannan?	SO_4^{2-}	Barberousse et al. (2006)
Klebsormidium flaccidum 749B	>50 40 30 20 10		7	5							UA		Mannan?	–	Barberousse et al. (2006)
Micrasterias denticulata							–						–	–	Oertel et al. (2004)
Netrium digitus	>50 40 30 20 10					4					UA		–	SO_4^{2-}	Kiemle et al. (2007)
Netrium oblongum	>50 40 30 20 10	7		7	7	3					UA		(1,2)-Linked Ara*p* Fragments non consistent with the MS composition	Prot, SO_4^{2-}	Kiemle et al. (2007)
Netrium interruptum	>50 40 30 20 10		4			3	5	5			UA		(3,4)-Linked Fuc T-Xyl	SO_4^{2-}	Kiemle et al. (2007)
Netrium interruptum UTEX 2509	>50 40 30 20 10			4		4	3				nd		–	nd	Kiemle et al. (2007)
Penium cylindrus	>50 40 30 20 10		7	3		3	4				UA		–	nd	Kiemle et al. (2007)

Continued

Table 20.1: Overview of EPS heterogeneity from microalgae belonging to Charophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

Penium margaritaceum		(3,4)-Linked Glc*p* A (1,3) and (1,4)-Linked Fuc*p* (3,4)-Linked Fuc*p*, T- Xyl*p*	Me	Domozych et al. (2005)
Penium spirostriolatum		–	SO_4^{2-}	Kiemle et al. (2007)
Pleurotaenium trabecula		(1,3)-Linked Fuc (3,4)-Linked Fuc (1,2)-Linked Xyl*p*	Prot, SO_4^{2-}	Kiemle et al. (2007)
Spondylosium panduriforme		(1,3) and (1,4)-Linked Fuc*p* (1,3)-Linked Gal (1,4)-Linked GlcA	Me	Paulsen and Vieira (1994)
Staurastrum iversenii		–	Prot	Freire-Nordi et al. (2006)
Tetmemorus brebissonii		(3,4)-Linked Fuc (2,3)-Linked Ara*p* (3)-Linked Gal	Prot, SO_4^{2-}	Kiemle et al. (2007)

negligible (up to 30%). EPS from Chlorophyta are often mainly composed of Gal (*Chlorella*) or Glc (*Chlamydomonas*) with various amounts of uronic acids (Table 20.2) in contrast to EPS from Ochrophyta, which are rich in Glc and Fuc (Table 20.4). Cyanobacteria mainly produce Glc-rich EPS, and GlcN and GalN were reported in some species (Table 20.3). Their EPS are often substituted by acetyl, methyl, pyruvyl, and sulfate groups, and proteins could be associated to the structure (proteoglycan). EPS from Rhodophyta are characterized by high levels of Xyl, the presence of GlcA in the main backbone, and significant amounts of sulfates. Depending on the species, the main data reported in the literature highlighted galacto- and rhamnoxylan backbones (Table 20.5). Some structural features are reported concerning their backbones, such as for *Porphyridium* sp., with a repetition of β-D-Gal*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 4)-α-L-Gal*p*, which is itself branched in the *O*-2 position of L-Gal*p* by β-D-Xyl*p* residues (Gloaguen et al., 2004). Overall, the poor knowledge of EPS microalgae structures could be attributed to the apparent lack of repeating units or presence of numerous nonsugar groups, as previously stated. However, a simple exhaustive list of reported structural data (Tables 20.1–20.5) seems to draw some profile patterns. Combining ecological, biochemical, genomic, and transcriptomic approaches should provide significant advances in the understanding of EPS microalgae production, for both industrial uses and evolution comprehension.

Table 20.2: Overview of EPS heterogeneity from microalgae belonging to Chlorophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO_4^{2-}, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace.

Species	%	Ara	Gal	Glc	Fuc	Man	Rha	Rib	Xyl	GalA	GlcA	Others	Main structural features	Nonsugar groups	References
Chlorophyta															
Ankistrodesmus densus	>50 40 30 20 10		3	5									Fucosyl backbone Mannan backbone	Me	Paulsen et al. (1998)
Botryococcus braunii							–						–	–	Casadevall et al. (1985)
Bracteacoccus sp. 679E	>50 40 30 20 10		5								UA		nd	SO_4^{2-}	Barberousse et al. (2006)
Chlamydomonas augustae	>50 40 30 20 10		5				2		2				(1,3,4)-D-glucan Araf lateral chains	Prot	Allard and Tazi (1993)
Chlamydomonas corrosa	Molar ratio	1	**1.7**	t						t			(1,3)-D-Arabinan Galp lateral chains?	Prot	Allard and Tazi (1993)
Chlamydomonas humicola	>50 40 30 20 10	1.10	0.36	1.10			0.27		**2.97**		1.00		Xylan Proteoglycan?	Prot	Flaibani et al. (1989)
Chlamydomonas mexicana	>50 40 30 20 10					4	3	2			UA		–	–	Barclay and Lewin (1985)
Chlamydomonas peterfii	Molar ratio	0.70	**2.40**	0.12							1.00		Galactan Proteoglycan?	Prot	Flaibani et al. (1989)
Chlamydomonas reinhardtii	>50 40 30 20 10	X	X	X			X	X	X	X			–	Pyr	Bafana (2013)
Chlamydomonas sajao	Molar ratio	0.49	**2.54**	0.07			0.08		0.16		1.00		Galactan Proteoglycan?	Prot	Flaibani et al. (1989)
Chlorella autotrophica							–						–	–	Fábregas et al. (1999)
Chlorella ellipsoidea	>50 40 30 20 10				5						UA		Galactan?	–	Barberousse et al. (2006)
Chlorella mirabilis 678F	>50 40 30 20 10			5	7						UA		Galactan?	SO_4^{2-}	Barberousse et al. (2006)

Continued

Table 20.2: Overview of EPS heterogeneity from microalgae belonging to Chlorophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

Chlorella mirabilis 7410G	>50 40 30 20 10 3 6 5 4 UA	Galactan?	SO_4^{2-} (t)	Barberousse et al. (2006)
Chlorella pyrenoidosa Chick S-39	>50 40 30 20 10 X X X X X X X X	–	–	Maksimova et al. (2004)
Chlorella sp.	>50 40 30 20 10 X X X X	–	–	Moore and Tischer (1964)
Chlorella stigmatophora	Presence X X	–	SO_4^{2-}	Kaplan et al. (1987)
Desmococcus olivaceus	>50 40 30 20 10 7 5 5 6 6	–	Prot	Hokputsa et al. (2003)
Dunaliella salina	>50 40 30 20 10 X X X X	–	–	Mishra and Jha (2009)
Dunaliella tertiolecta UTEX LB 999	>50 40 30 20 10 X	α-(1,4)-D-Glucan	Prot	Goo et al. (2013)
Nephrocytium lunatum	>50 40 30 20 10 2 3 1 7 6 2 3	Mannan?	Ac	Freire-Nordi et al. (2003)

20.3 Processes production, extraction, and purification of EPS from microalgae and cyanobacteria

In photoautrotophic conditions, the growth of microalgae (and cyanobacteria) is directly bound to photosynthesis activity, and then to light irradiance, even if several optimal values are strain-dependent (Delattre et al., 2016). The EPS produced by microalgae is also a light-dependent mechanism (Gaignard et al., 2019) and is highly enhanced by continuous light and great light intensities, as exhibited for *Arthrospira platensis* (Trabelsi et al., 2009b), *Gloeocapsa gelatinosa* (Raungsomboon et al., 2006), *Porphyridium* (Merchuk et al., 1998), *Cyanospira capsulata* (De Philippis et al., 1998), or *Synechococcus* BG0011 (Phlips et al., 1989). For cultures conducted in a dark/light regime, the lighting period was greater with a maximum release of EPS, confirming that the EPS production is a light-dependent mechanism. Light irradiance and/or dark-light cycles have no impact on the polysaccharides composition (nature and amounts of monosaccharides, glycosidic linkages type, etc.) of cyanobacteria such as

Table 20.3: Overview of EPS heterogeneity from microalgae belonging to Cyanobacteria. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO_4^{2-}, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace.

Species	%	Ara	Gal	Glc	Fuc	Man	Rha	Rib	Xyl	Gal/A	Glc/A	Others	Main structural features	Nonsugar groups	References
Cyanobacteria															
Anabaena augstmalis VRUC 163	>50 40 30 20 10		1	**10**	5	7			1		2	6	–	–	Di Pippo et al. (2013)
Anabaena cylindrica 10C	Molar ratio		1	**3.6**	1	1	1		3.1				–	–	Lama et al. (1996)
Anabaena flos-aquae	Presence			X			X		X		X		–	–	Moore and Tischer (1964)
Anabaena sp.							–						–	–	Moreno et al. (1998)
Anabaena spiroides	>50 40 30 20 10		4	1	1	1	2		1		2	2	–	–	Gouvêa et al. (2005)
Anabaena sphaerica	Molar ratio	1	**4**	3		3							–	–	Nicolaus et al. (1999)
Anabaena torulosa	Molar ratio	4.3	1	2.4	2.7		6		**73.4**			4.7	–	–	Nicolaus et al. (1999)
Anacystis nidulans	Molar ratio		14	**66**		20							–	–	Sangar and Dugan (1972)
Aphanocapsa halophytia	Molar ratio		3		**53**	15	2		3				–	Prot, SO_4^{2-}	Sudo et al. (1995)
Aphanothece halophytica GR02	Molar ratio	1		**2.87**	2.08	1.57					16%		T-Glc, 1,3-Linked Glc 1,3- and 1,4-Linked Fuc, 1,3-Linked GlcA, 1,3-Linked Ara 1,2,4- and 1,3,6-Linked Man	–	Li et al. (2001)
Arthrospira maxima	Molar ratio		0.3	1	0.2		1		**1.8**	0.07	0.2		–	Prot	Nie et al. (2002)
Arthrospira platensis	>50 40 30 20 10	1				t	4				1		Glycoprotein (55% dw)?	Prot, SO_4^{2-}	Trabelsi et al. (2009)

Continued

Table 20.3: Overview of EPS heterogeneity from microalgae belonging to Cyanobacteria. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

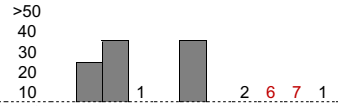
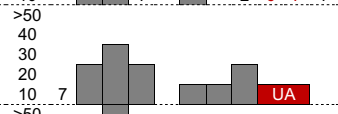
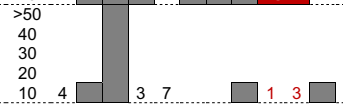
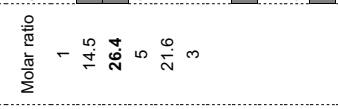
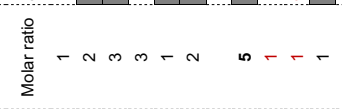
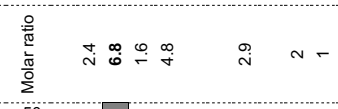
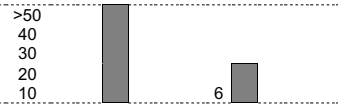
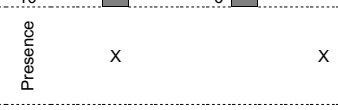
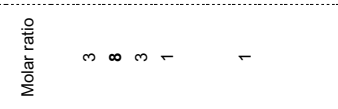
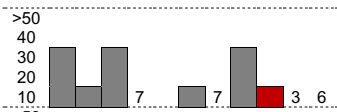
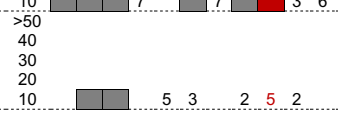
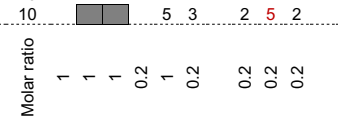
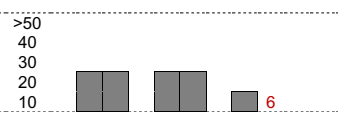
Arthrospira platensis		–	–	Depraetere et al. (2015)
Calothrix pulvinata 745A		–	SO_4^{2-} (t)	Barberousse et al. (2006)
Calothrix sp. VRUC 166		–	–	Di Pippo et al. (2013)
Chlorogloeopsis sp. 6912		–	–	Nicolaus et al. (1999)
Chroococcus minutes		–	Prot	Fischer et al. (1997)
Chroococcus submarinus BM		–	Prot, SO_4^{2-}	Richert et al. (2005)
Cyanothece sp.		–	Prot	De Philippis et al. (1993)
Cyanothece sp. ATCC 51142		–	Prot, SO_4^{2-}	Parikh and Madamwar (2006)
Cyanothece sp. ATCC 51142		2-C-Me-Glc, Ido-2-C-carboxylic acid, 2-deoxy-Ido	Me, Prot, SO_4^{2-}	Shah et al. (2000)
Fischerella muscicola		–	–	Nicolaus et al. (1999)
Gleocapsa kuetzingigiana		–	–	Rossi et al. (2012)
Gleothece sp. PCC 6909		–	Prot, SO_4^{2-}	Tease et al. (1991)
Johannesbaptistia pellucida GC		–	Prot, SO_4^{2-}	Richert et al. (2005)
Leptolyngbya foveolarum 671B		–	SO_4^{2-} (t)	Barberousse et al. (2006)

Table 20.3: Overview of EPS heterogeneity from microalgae belonging to Cyanobacteria. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

Leptolyngbya tenuis 679B	>50 40 30 20 10 — 6 — 6 UA	–	SO_4^{2-} (t)	Barberousse et al. (2006)
Leptolyngbya sp.	>50 40 30 20 10 — 1 1 — 5 — t 6	–	nd	Rossi et al. (2012)
Lyngbya confervioides	Presence — X X X X X t X X X	–	nd	Gloaguen et al. (1995)
Mastigocladus laminosus	Presence — X X X X X X X X	Backbone: 4)-α-Glc*p*A-(1,2)-α-Gal*p*A-(1,2)-β-Man*p*-(1,4)-β-Gal*p*-(1,2)-α-Rha*p*-(1 Many (complex) lateral chains	nd	Gloaguen et al. (1999)
Microcoleus vaginatus	>50 40 30 20 10 — 3 2 3 5 6 5	–	Prot	Ge et al. (2014)
Microcoleus vaginatus	>50 40 30 20 10 — 4 — 6 5 3 6	Mannan/glucan (1,4)-Rha*p*, (1,3,5)-Ara*f*	Prot	Hu et al. (2003)
Microcystis aeruginosa	Molar ratio — 1 1.3 **5** t	–	nd	Forni et al. (1997)
Microcystis aeruginosa-flos-aquae	Molar ratio — **20** 1 t	–	–	Forni et al. (1997)
Microcystis viridis	Molar ratio — **15** 1 2 t t 3	–	nd	Forni et al. (1997)
Nostoc carneum	>50 40 30 20 10 — — — —	Xylomannan?	Prot	Parikh and Madamwar (2006)
Nostoc commune	Molar ratio — 3 **5** 2 **5** t	–	Prot	Sakamoto et al. (2011)
Nostoc commune DRH-1	Molar ratio — 1 **2** 1 1 t	(1,4)-Linked xylogalactoglucan D-Ribufuranose (pendant group) Nosturonic acid (pendant group)	nd	Helm et al. (2000)
Nostoc entophytum	–	–	–	Najdenski et al. (2013)
Nostoc insulare 54.79	>50 40 30 20 10 — — — — X —	→1)-Glc*p*-(3→1)-Glc*p*-[(3→1)-3-*O*-Me-Ara*f*](4→1)-Glc*p*A-(4→	Ac, Prot, Pyr	Volk et al. (2006)

Continued

Table 20.3: Overview of EPS heterogeneity from microalgae belonging to Cyanobacteria. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

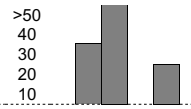
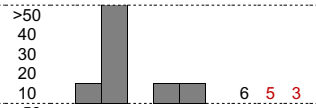
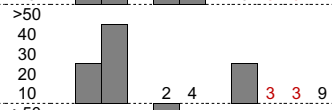
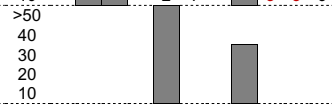
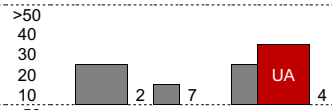
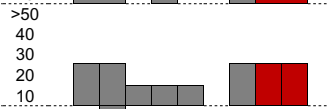
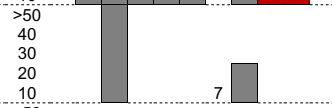
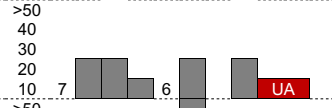
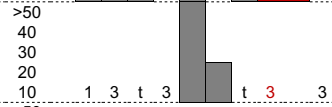
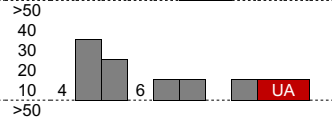
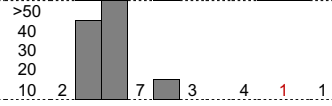
Nostoc flagelliforme		–	–	Huang et al. (1998)
Nostoc muscoru	-	–	–	Najdenski et al. (2013)
Nostoc sp.		–	Prot	Ge et al. (2014)
Nostoc sp.		–	Prot	Hokputsa et al. (2003)
Nostoc sp.		Xylomannan?	Prot	Parikh and Madamwar (2006)
Nostoc verrucosum		–	Prot	Sakamoto et al. (2011)
Oscillatoria amphibia		–	SO_4^{2-}	Gloaguen et al. (1995)
Oscillatoria corallinae		–	SO_4^{2-}	Gloaguen et al. (1995)
Oscillaria sp.		–	Prot	Parikh and Madamwar (2006)
Phormidium ambiguum 692B		–	SO_4^{2-} (t)	Barberousse et al. (2006)
Phormidium autumnale VRUC 164		–	–	Di Pippo et al. (2013)
Phormidium corium 444A		–	SO_4^{2-} (t)	Barberousse et al. (2006)
Phormidium ectocarpi K5		–	–	Gloaguen et al. (1995)
Phormidium cf. *foveolarum* MEU		–	Prot?	Gloaguen et al. (1995)

Table 20.3: Overview of EPS heterogeneity from microalgae belonging to Cyanobacteria. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

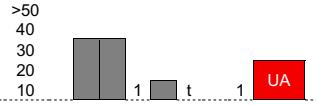
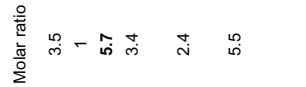
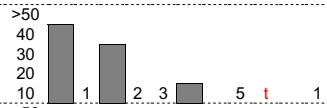
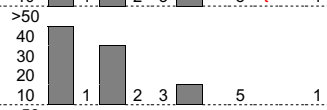
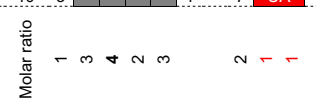
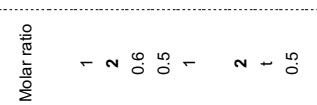
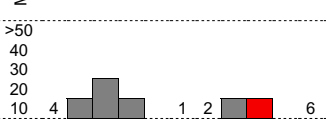
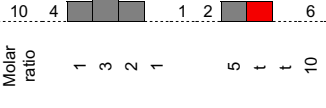
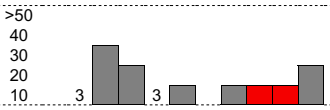
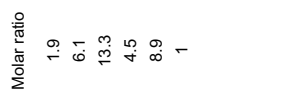
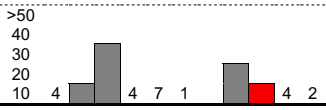
Phormidium minutum D5		–	–	Gloaguen et al. (1995)
Phormidium sp.		–	–	Nicolaus et al. (1999)
Phormidium tenue		–	Prot	Hokputsa et al. (2003)
Phormidium tenue		(1,3)- and (1,3,4)-Ara*f* T-Glc*p*, (1,3)- and (1,4)-Glc*p* (1,3)-Rha*p*	Prot	Hu et al. (2003)
Phormidium usteri		–	SO_4^{2-} (t)	Barberousse et al. (2006)
Plectonema battersii GF		–	Prot, SO_4^{2-}	Richert et al. (2005)
Plectonema golenkinianum FF			Prot	Richert et al. (2005)
Plectonema sp.		–	nd	Rossi et al. (2012)
Rhabdoderma rubrum CH			Prot, SO_4^{2-}	Richert et al. (2005)
Scytonema hofmanni		–	–	Nicolaus et al. (1999)
Scytonema javanicum		Unclear structural data Glc/Man/Gal not separated	Prot	Hu et al. (2003)
Synechococcus sp.	-	–	–	Phlips et al. (1989)
Synechocystis aqualitis VRUC 165		–	–	Di Pippo et al. (2013)
Synechocystis sp.	-	–	–	Najdenski et al. (2013)
Tolypothrix tenuis		–	–	Nicolaus et al. (1999)
Trichormus variabilis VRUC168		–	–	Di Pippo et al. (2013)

Table 20.4: Overview of EPS heterogeneity from microalgae belonging to Myozoza and Ochrophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO_4^{2-}, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace.

Species	%	Ara	Gal	Glc	Fuc	Man	Rha	Rib	Xyl	GalA	GlcA	Others	Main structural features	Nonsugar groups	References
Myozoza															
Cochlodinium polykrikoides	Presence		X	X		X				X			–	Prot, SO_4^{2-}	Hasui et al. (1995)
Crypthecodinium cohnii	>50 40 30 20 10				3	7		6				2	–	–	Liu et al. (2015)
Gyrodinium impudicum	>50 40 30 20 10									3			Sulfated galactan	SO_4^{2-}	Yim et al (2004)
Ochrophyta															
Achnanthes coffeaeformis	>50 40 30 20 10												T-, 4-GlcA units T-, 4-, 3,6-Glc units 4-, 4,6-Man residues	Prot, SO_4^{2-}	Wustman et al. (1997)
Achnanthes longipes	>50 40 30 20 10	3				3							2,3-/3,6-Gal residues T-, 2-GlcA units T-Fuc, D-Gal and D-Man units	SO_4^{2-}	Wustman et al. (1997)
Amphora holsatica	>50 40 30 20 10					3							–	nd	Leandro et al. (2003)
Amphora rostrata	>50 40 30 20 10	4		3					2	UA			–	Prot, Pyr, SO_4^{2-}	Khandeparker and Bhosle (2001)
Aulacoseira granulata	>50 40 30 20 10											7	–	–	Gouvêa et al. (2005)
Chaetoceros affinis	Presence	X	X	X	X	X	X						–	SO_4^{2-}	Myklestad et al. (1972)
Chaetoceros curvisetus	>50 40 30 20 10		1		**3.5**		0.3						Fucan Fuc*p* and Fuc*f* (branch points) Rha linked in *O*-2 positions	Me, SO_4^{2-}	Paulsen et al. (1975)
Chaetoceros decipiens	>50 40 30 20 10			3					3				T-, 2-Rha, T-, 2,3,4-Fuc units 2-Gal*p*, 4-Gal*p* (backbone) 2-, 6-, 2,4-, 2,3-Man (branching)	SO_4^{2-}	Urbani et al. (2012)
Coscinodiscus nobilis	Presence			X	X	X				X			Sulfated glucuronomannan	SO_4^{2-}	Percival et al. (1980)

Table 20.4: Overview of EPS heterogeneity from microalgae belonging to Myozoza and Ochrophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

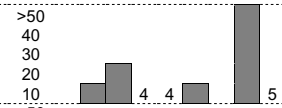
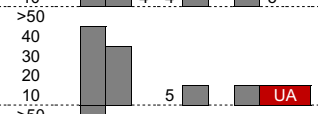
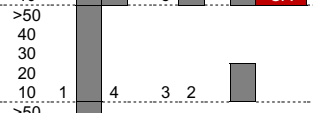
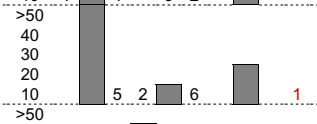
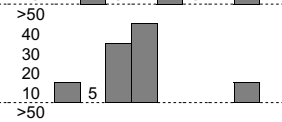
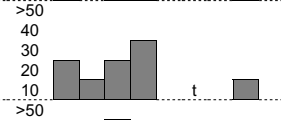
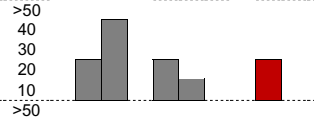
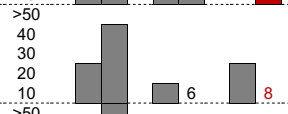
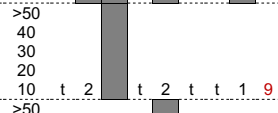
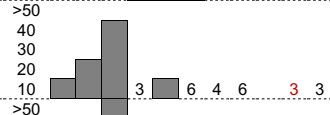
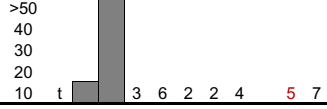
Cylindrotheca closterium		–	Prot	Staats et al. (1999)
Cylindrotheca fusiformis		–	SO_4^{2-}	Magaletti et al. (2004)
Cymbella cistula		4-; 4,6-; 3,4-Linked/substituted Gal T-; 4*f*/5*p*- and 3*p*-linked/substituted Xyl	SO_4^{2-}	Wustman et al. (1997)
Cymbella mexicana		4-Gal units 4*f*/5*p*-Xyl units	SO_4^{2-}	Wustman et al. (1997)
Melosira nummuloides		–	nd	Leandro et al. (2003)
Navicula directa		–	nd	Leandro et al. (2003)
Navicula jeffreyi		–	–	Klein et al. (2014)
Navicula salinarum		–	Prot, SO_4^{2-}	Staats et al. (1999)
Navicula subinflata			Me, Prot, Pyr, SO_4^{2-}	Bhosle et al. (1995)
Thalassiosira sp.		1,4-, 1,4,6-Man (backbone) 1,3-Rha and 1,3-Fuc units Various T- residues	Ac	Giroldo et al. (2003)
Phaeodactylum tricornutum CCMP 632 (ovoid)		3-Glc (backbone) List of many fragments (>35)	Me	Willis et al. (2013)
Phaeodactylum tricornutum CCMP 632 (fusiform)		3-Glc (backbone) List of many fragments (>35)	Me	Willis et al. (2013)

Table 20.5: Overview of EPS heterogeneity from microalgae belonging to Rhodophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO_4^{2-}, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace.

Species	%	Ara	Gal	Glc	Fuc	Man	Rha	Rib	Xyl	GalA	GlcA	Others	Main structural features	Nonsugar groups	References
Rhodophyta															
Flintiella sanguinaria	>50 40 30 20 10	2		6									–	Ac, Me, Prot	Gaignard et al. (2018)
Porphyridium aerugineum	>50 40 30 20 10										UA		GlcA-(1,3)-Gal D-Glc-(1,4)-Gal, D-Glc-D-Xyl D-Xyl-(1,3)-D-Xyl	Me, Prot, SO_4^{2-}	Percival and Foyle (1979)
Porphyridium cruentum	>50 40 30 20 10						4		7		UA		–	Prot, SO_4^{2-}	de Jesus Raposo et al. (2014)
Porphyridium cruentum	>50 40 30 20 10										UA	5	GlcA-(1,3)-Gal D-Glc-(1,4)-Gal, D-Glc-D-Xyl D-Xyl-(1,3)-D-Xyl	Me, Prot, SO_4^{2-}	Percival and Foyle (1979)
Porphyridium marinum CCAP 1380/10	>50 40 30 20 10				t						UA		–	SO_4^{2-}	Roussel et al. (2015)
Porphyridium purpureum CCAP 1380/1A	>50 40 30 20 10				t						UA		–	SO_4^{2-}	Roussel et al. (2015)
Porphyridium sp.	>50 40 30 20 10	1				1	1				5	8	–	Me, Prot, SO_4^{2-}	Geresh et al. (1992)
Porphyridium sp.	>50 40 30 20 10		X	X					X		X		β-D-Gal*p*-(1,3)-β-D-Glc*p*-(1,3)-β-D-Xyl*p*-(1,4)-β-D-Xyl*p*-(1,4)-α-L-Gal*p* β-D-Xyl*p* branched in *O*-2 of L-Gal*p*	Prot	Gloaguen et al. (2004)
Porphyridium sp. UTEX 6371	>50 40 30 20 10										nd		–	nd	Geresh et al. (2002)
Porphyridium sp. UTEX 637	Molar ratio		1	1					3		0.5		2 or 4)-β-D-Xyl*p*-(l,3)]$_m$-α-D-Glc*p*-(1,3)-α-D-Glc*p*A-(1,3)-L-Gal*p*-(1	SO_4^{2-}	Geresh et al. (2009)
Rhodella grisea	>50 40 30 20 10	1		4		t					UA		Proteoglycan	Me, Prot	Capek et al. (2008)
Rhodella maculata CCAP 1388/2	>50 40 30 20 10	2		1		5					UA		–	Prot, SO_4^{2-}	Roussel et al. (2015)

Table 20.5: Overview of EPS heterogeneity from microalgae belonging to Rhodophyta. *Ac*, acetyl groups; *Me*, methyl groups; *nd*, not defined; *Prot*, proteins; *Pyr*, pyruvate groups; SO42−, sulfate groups; *UA*, uronic acids; *X*, presence; *t*, trace—cont'd

Rhodella reticulata	Presence: X X X X X X X	α-D-Glc*p*A-(1,3)-α,β-L-Gal*p* 3-, 4-*O*-methylpentose	Me	Geresh et al. (1990)
Rhodella violacea LMGEIP 001	>50 40 30 20 10: 7 6	–	Prot, SO_4^{2-}	Villay et al. (2013)
Rhodella violacea CCAP 1388/5	>50 40 30 20 10: 2 1 5 UA	–	Prot, SO_4^{2-}	Roussel et al. (2015)

C. capsulata (Ge et al., 2014b). High values of light irradiances exhibit high EPS quantities, and the differences registered by several authors could reflect only a greater biomass production, as cellular productivities are generally omitted. These observations are in agreement with the production of EPS by *Rhodella violacea*, which was investigated at several values of irradiance. Indeed, greater EPS production was found at an irradiance of saturation just previous to the photosynthesis inhibition (photoinhibition) (Villay et al., 2013). In a similar way, EPS yields of cyanobacteria *Nostoc* sp. and *Microcoleus vaginatus* increased with the rise of light intensity without affecting their monosaccharide composition (Ge et al., 2014a,b).

However, the light spectral composition could affect the photosynthetic activity and the polysaccharides production. For example, blue (spectral irradiance ranging from 400 to 500 nm) and red (spectral irradiance ranging from 600 to 700 nm) lights, used at low irradiances (from 20 to 40 µmol photons m^2 s^{-1}), raised the photosynthetic activity and then the EPS production by *Porphyridium cruentum* (You and Barnett, 2004). Red light induced the photoinhibition of *Nostoc flagelliforme* growth, and stimulated its EPS production. ECP can act as a photoprotective layer surrounding the microalgae cells, as for *Nostoc commune*. Their biosynthesis occurred simultaneously with the photoprotective pigments when submitted to the UVB radiations (Ehling-Schulz et al., 1997). On the other hand, a continuous cultivation of *A. platensis* for 5 months using red light (620 nm, 135 µmol photons m^2 s^{-1}) has shown no significant differences in growth rates and EPS production compared to white light (Farges et al., 2009).

The complex chemo-diversity of EPS from cyanobacteria and microalgae combined with the development of green production and purification processes makes them very attractive for bioprospection of new and original sources of polysaccharides. Each kind of polysaccharides requires specific processes for its extraction and purification (Delattre et al., 2016). As shown in Fig. 20.1, EPS are traditionally extracted from microalgae cultivation media when the best PS concentrations are validated (irradiance, salinity, pH, temperature, aeration, etc.). In the first step, PS are recovered and separated using centrifugation or microfiltration to remove

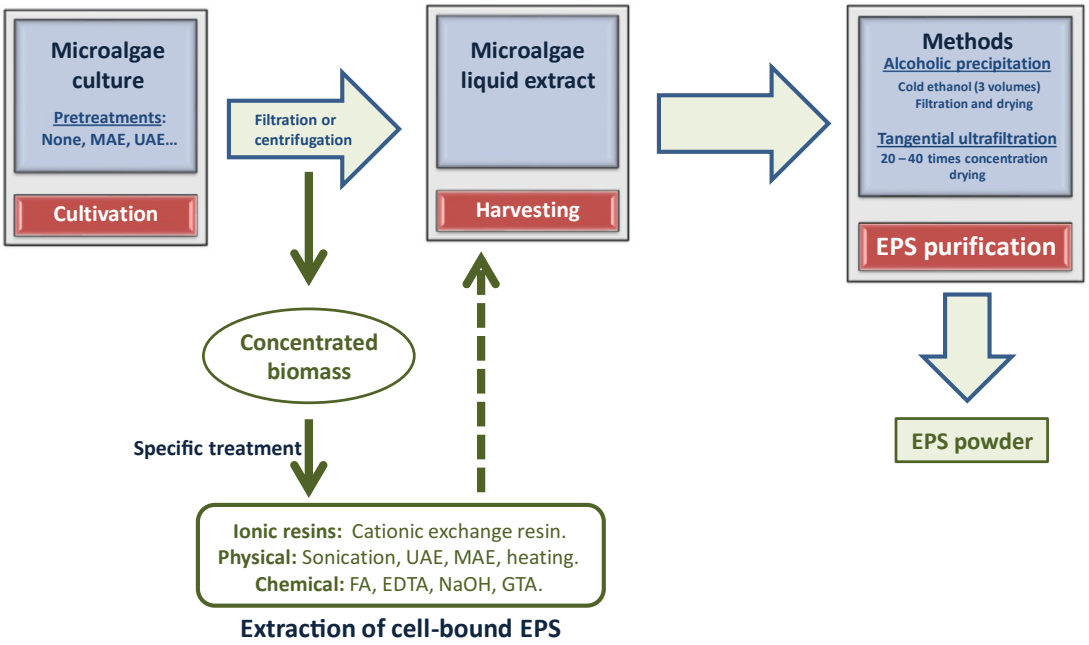


Fig. 20.1
The main strategies developed to extract and purify polysaccharides from microalgae. *UEA*, ultrasound-assisted extraction; *MAE*, microwave-assisted extraction.

microalgae (Delattre et al., 2016; Patel et al., 2013; Li et al., 2001; Zhang et al., 2008; Ye et al., 2005). Secondly, EPS are concentrated from filtrate/supernatant (under vacuum or by using ultrafiltration process) and selectively fractionated by precipitation with 2–3 volumes of cold alcohol such isopropanol or ethanol (Delattre et al., 2016; Patel et al., 2013; Usov, 2011; Gloaguen et al., 2004). They are then dried and crushed to obtain fine powder. In alternative polysaccharide purification procedures, some studies have described the use of specific treatments (tangential ultrafiltration, trichloroacetic acid, etc.) to remove contaminant biomolecules such as phenolic compounds, pigments, or even salts (Patel et al., 2013; Li et al., 2001; Moore and Tischer, 1964). When marine microalgae cultivations need high salinity conditions, alcoholic precipitation may result in coprecipitation of EPS with salt and, consequently, additional expensive alcohol fractionations are still necessary to remove salts efficiently (Gaignard et al., 2019; Delattre et al., 2016; Patel et al., 2013). Therefore, it is essential to provide tangential ultrafiltration or dialysis methods in order to purify polysaccharides of interest highly from marine microalgae (Delattre et al., 2016; Eteshola et al., 1998).

In a very interesting comparative study realized on EPS from *P. cruentum*, Patel et al. (2013) demonstrated the efficient purification of PS using tangential ultrafiltration achieved with polyethersulfone filtration membrane having a molecular weight cut off of 300 kDa in contrast to conventional alcoholic precipitation. Furthermore, the works of Zhang and Santshchi (2009) and Li et al. (2001) suggest pilot-scale microfiltration and tangential ultrafiltration procedures to

purify EPS highly from *Haematococcus pluvialis*, *Chlorella pyrenoidosa*, *Chaetoceros muelleri*, *Nostoc* sp., and *Spirulina platensis*. Nonetheless, alcoholic precipitations and tangential ultrafiltration conventional methods used to extract and purify PS are not efficient enough to recover all microalgae EPS since, according to the literature, more than 50% of these PS stay bonded to cells during extraction (Gaignard et al., 2019; Delattre et al., 2016). As shown in Fig. 20.1, many alternative extraction forms were developed to extract and concentrate these PS. Among them, physical methods such as sonication, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) treatments were used to unbond BPS (Gaignard et al., 2019; Delattre et al., 2016; Kurd and Samavati, 2015; Kadam et al., 2013; Budarin et al., 2012; Herrero et al., 2006). Other more specific chemical and/or ionic methods have been recognized for their effectiveness in isolation of BPS from microalgae, such as ethylene diamine tetracetic acid (EDTA), hydroxide sodium (NaOH), formaldehyde (FA), glutaraldehyde (GTA) hot water, and ionic resin treatments (Gaignard et al., 2019; Delattre et al., 2016; Comte et al., 2006; Frølund et al., 1996; Brown and Lester, 1980).

20.4 Polysaccharides from microalgae as antiinfectious agents

20.4.1 Antiviral activity of PS from microalgae and cyanobacteria

Virus infections are a worldwide issue threatening human and animal health. In the last few years, numerous viral infectious diseases have emerged or reemerged mainly due to an increase of drug resistance. The nature of the viral agent makes the search for antiviral agents particularly difficult. Indeed, these simple life-forms depend completely on the metabolic system and energy of the host cell. Thus, an antiviral drug must on the one hand inhibit the virus in the host cell and on the other hand have no impact on the metabolism of the host. Many antiviral drugs have serious side effects (Poole and Kimberley, 2017), making it essential to discover novel sources of therapeutic compounds. PS are natural macromolecules participating in numerous biological processes such as cell adhesion, cell-to-cell communication, immune response, and infection of bacteria, parasites, and viruses (Dwek, 1996). Studies on the antiviral effects of marine PS are increasing and becoming a new trend in antiviral drugs as they possess a low toxicity and probably a multistep mechanism, meaning lesser selective pressure for the emergence of resistant strains (Yu et al., 2018; Witvrow and DeClercq, 1997; Damonte et al., 2004). In general, PS possess the same antiviral mechanism against enveloped viruses. Ionic interactions between the anionic groups of the PS and glycoprotein's basic amino acids form a complex, limiting the viral adsorption (Raposo et al., 2014).

Naviculan was isolated from the diatom *Navicula directa*. This sulfated PS consists of Fuc, Xyl, Gal, Man, Rha, and other trace amounts of sugar moieties (Lee et al., 2006). It possesses a broad antiviral spectrum against enveloped viruses such as herpes simplex virus (HSV) type 1 and 2, influenza (IFV) A virus, and human immunodeficiency virus (HIV). It is known

that HSV and IFV infections start by binding to carbohydrates such as heparan sulfate or sialylate residues, localized on the host cell surface. Naviculan interferes with very early stages of viral replication such as viral binding and penetration into host cells. Naviculan also interferes with cell-to-cell fusion between HIV glycoprotein 160 and CD4 expressing cells (Lee et al., 2006). *Arthrospora platensis* produces a sulfated PS known as calcium spirulan, which has shown antiviral activity (Hayashi et al., 1996; Lee et al., 2001). It is composed of Rha, Rib, Man, Fru, Gal, Xyl, Glc, GlcA, GalA, sulfate, and calcium. It inhibits the replication of several enveloped viruses such as HSV type 1, human cytomegalovirus, measles virus, mumps virus, IFV A virus, and HIV-1 by inhibiting the penetration of viruses into host cells (Hayashi et al., 1996; Reichert et al., 2017).

Another study investigated antivaccinia virus activities of EPS from *Porphyridium purpureum* and *A. platensis* (TK V3). It was shown that these PS inhibit the binding of viruses to cells. A higher antiviral effect was observed when the antiviral treatment and the infection occurred at the same time (Radonić et al., 2010). The use of a cell wall-sulfated PS from *Porphyridium* sp. showed significant antiviral activity against HSV type 1 and 2 and the Varicella zoster virus (VZV) (Huheihel et al., 2002). In this study, it was shown that PS from *Porphyridium* sp. prevents various steps during the life cycle of the HSV and VZV viruses. A strong interaction between HSV-1 particles and *Porphyridium* sp. PS is most likely responsible for the significant inhibition of viral infection. This study also suggests that *Porphyridium* PS has a pleiotropic mode of action during the infection cycle of HSV and probably of VZV. Another study showed that the red microalgal PS from *Porphyridium* sp., *Porphyridium aerugineum*, and *Rhodella reticulata* exhibit antiviral activity against the replication and the transforming ability of two retroviruses: the Moloney murine sarcoma virus and the Moloney murine leukemia virus (Talyshinsky et al., 2002). When cells were treated with PS before or at the time of infection, a more important inhibitory activity was observed. Part of the inhibitory effect of PS is due to blocking some of the viral receptors, therefore interfering with the penetration of the virus into the host. However, the reversibility of the inhibition suggests that the antiviral activity occurred only on a certain event after proviral integration. The PS of *Porphyridium* sp. seems to possess a pleiotropic mode of action throughout the infection cycle of the Moloney murine sarcoma virus. The application of PS from *Porphyridium* sp. seems to be an interesting option for viral therapy. Indeed, treatment with molecules that possess a pleiotropic mode of action is less likely to result in the development of resistant mutants. The step affected by the use of these PS remains unknown (Talyshinksky et al., 2002; Huheihel et al., 2002).

Fabregas et al. (1999) showed that endocellular extracts from *P. cruentum*, *Chlorella autotrophica*, *Isochrysis galbana* var Tiso, *Ellipsoidon* sp., and *Dunaliella tertiolecta* were able to inhibit rhabdovirus of viral hemorrhagic septicemia (VHSV) of salmonid fish replication. The extracts from *P. cruentum*, *C. autotrophica*, and *Ellipsoidon* sp. were also capable of inhibiting African swine fever virus (ASVF). Most of the endocellular extracts inhibiting VHSV replication also inhibited the ASVF one, indicating common activity against both enveloped viruses. Additionally, exocellular extracts from these microalgae were also

analyzed, and the results obtained with endocellular extracts were confirmed, except for those from *Isochrysis galbana* var Tiso with both viruses, and from *C. autotrophica* and *D. tertiolecta* with ASFV (Fabregas et al., 1999).

The *Gyrodinium impudicum* strain KG03 produces an EPS known as p-KGO3, composed of Gal conjugated with uronic acid and sulfate groups (Ahmadi et al., 2015). Yim et al. (2004) studied its activity against the Encephalomyocarditis virus (EMCV). EMCV infects pigs, and can provoke acute focal myocarditis with sudden death in young pigs. p-KGO3 was able either to inhibit completely or to slow down the cytopathic effect in EMCV-infected cells depending on its concentration. Another study reported that p-KGO3 had a great inhibitory activity on the influenza A virus infection but not on all influenza B ones in vitro (Kim et al., 2012). Authors showed that there was no virus replication when p-KGO3 was added during the infection process, meaning that the PS targets were the viral adsorption and internalization steps. Therefore, p-KGO3 is capable of preventing the attachment of the virus to host cells, and can also block its cellular internalization and early stages of replications (Kim et al., 2012). *Cochlodinium polykrikoides* produces EPS known as A1 and A2 PS. They are composed of Glc, Gal, Man, and uronic acids with sulfate groups (Ahmadi et al., 2015; Hasui et al., 1995). A1 and A2 were able to inhibit the cytopathogenic effects of HIV-1 in MT-4 cells, influenza virus types A and B in MDCK cells, and respiratory syncytial virus types A and B in Hep-2 cells. However, A1 was shown to be effective against herpes virus type 1 and A2 against parainfluenza virus type 2 in the same cell line HMV-2.

N. flagelliforme, an edible blue-green alga, synthetizes an acidic PS called Nostoflan. It is mainly composed of (→4)-β-D-Glc*p*-(1→4)-D-Xyl*p*-(1→ and →4)-[β-D-GlcA*p*-(1→6)-]-β-D-Glc*p*-(1→4)-D-Galp-(1→). Nostoflan exerts an inhibitory effect on the virus binding process but not on the penetration occurring after its binding (Kanekiyo et al., 2005).

20.4.2 Antibacterial activity of PS from microalgae and cyanobacteria

Large-scale screening among marine microalgae, focusing on the identification of antibacterial agents against pathogenic bacteria, has highlighted the potential of EPS (Hu et al., 2015; Mudimu et al., 2014). The antibacterial mechanism of PS remains unclear, but may involve interactions with bacterial cell wall and cytoplasmic membrane, leading to increasing of membrane permeability, protein leakage, and binding with bacterial DNA (Amorim et al., 2012; Pierre et al., 2011; He et al., 2010). The affinity of some bacterial species for sulfated glycoconjugates exposed on the host cell surface suggests the potential action of sulfated EPS and PS from microalgae as antiadhesive therapy (Ofek et al., 1978; Hook et al., 1984). The reality is, however, not so obvious, as it has been observed for some sulfated EPS from *Tetraselmis* sp., *Neochloris oleobundans*, and *Phaeodactylum tricornutum* 50% inhibition of *Helicobacter pylori* attachment to HeLa cells, whereas sulfated PS from other microalgae species (e.g., *Chlorella capsulata*, *Isochrysis* sp.) had an opposite action on adhesion (Guzman-Morillo et al., 2000).

This divergent action of sulfated PS indicates that other factors such as structure and composition may play significant roles in antibacterial activity. The red microalga *Porphyridium* sp. has been intensively studied for the production of a sulfated PS (Arad and Levy-Ontman, 2010). The anionic charge of this sulfated galactoxylan suggested a role of plate-form for metal incorporation, conferring on it its antibacterial activity. An argument in favor of this hypothesis was provided by the study of Netanel et al. (2016), which highlighted the improvement of antibacterial activity combining Zn ions and polysaccharide. A sulfated galactan, obtained from an Israeli strain of *P. cruentum*, was shown to inhibit *Salmonella enteritidis* growth markedly, reducing CFU to 19%, whereas *Staphylococcus aureus* and *Escherichia coli* were less sensitive to the PS (Raposo et al., 2014). These different activities could be attributed to the structure and composition of the bacterial cell wall, to the lack of a single structure in the bacteria, or to the potential modifications induced by the bacteria toward the chemical structure of the PS (Michael et al., 2002).

The exploitation of antiadhesive properties of some microalgae PS has led to the study of the activity of PS extracted from *Chlorella* and *Spirulina* on the ability of *H. pylori*-associated gastroduodenal diseases to bind to porcine gastric mucin (Loke et al., 2007). It was found that 35 µg of the PS from *Spirulina* reduced the *H. pylori* adhering to mucin by about 90%, whereas 80 µg of the PS from *Chlorella* was required to achieve this effect, without killing *H. pylori* or affecting gastric epithelial cells. The mechanism involved was not determined, but would be independent of the outer membrane adhesins. To conclude, bioactive PS against bacterial infections are well-documented for PS extracted from macroalgae such as fucoidan and laminarin, which have been successfully exploited in drug delivery to inhibit the growth of *S. aureus* and *E. coli*, or to prevent *H. pylori* biofilm adhesion in gastric mucosa (Shannon and Abu-Ghannam, 2016). PS from microalgae or cyanobacteria have been less explored as antibacterial agents compared to studies describing their biological activities as antiviral agents, antioxidants, immunomodulatory system, antiinflammatory properties, or in health (Raposo et al., 2014).

20.4.3 Antifungal activities of PS from microalgae and cyanobacteria

The limited amount of antifungal molecules associated to their high toxicity and the emergence of resistance mechanisms drive researchers to find newer and safer agents (Scorzoni et al., 2017). *Tetraselmis* species are known to produce water-soluble PS (WSPs). These molecules have shown antifungal activities, as they demonstrate abilities to inhibit the growth of *Candida albicans* and *Penicillium italicum*, two fungi involved in human and plant diseases, respectively (Amna Kashif et al., 2018). Indeed, the growth of *C. albicans* was inhibited by 70%–80% using two base (alkali)-extracted WSPs from two different *Tetraselmis* strains. Antifungal activities of EPS have also been demonstrated in the case of the fungi-related parasites called Microsporidia involved in human and animal health (Texier et al., 2010). The potential of

11 sulfated PS (among them, microalgal ones) was tested to evaluate their antimicrosporidian activity. Two PS extracted from *Porphyridium* spp. showed an in vitro antiparasitic activity of about 95% on a microsporidian model, *Encephalitozoon cuniculi* (Roussel et al., 2015). The EPS from *Porphyridium marinum* was then tested in adult honeybees infected by the microsporidian *Nosema ceranae*, causing an intestinal disease called nosemosis. It allowed a reduction of bee mortality (20%) and parasite load (34%) in laboratory experiments (Roussel et al., 2015). Semifield experiments were also undertaken and indicated that the colonies treated with this EPS showed a decrease of parasite prevalence of about 35%. The amount of parasites was divided by three in foraging bees originating from polysaccharide-treated colonies, reducing parasite pressure and allowing a better vitality of colonies (Roussel, personal communication). The results obtained suggest that microalgal-sulfated polysaccharides could be used to control nosemosis in apiaries.

20.5 Bioactivity and structure-activity relationships

In this section, the known bioactivities of PS and EPS from microalgae, including antioxidant, antiinflammatory, anticancer, and immunomodulatory properties, will be discussed (Table 20.6).

Table 20.6: Biological activities of PS and EPS from marine microalgae.

Species	Activity	EPS or PS	Reference
Porphyridium cruentum	Antioxidant	EPS	Tannin-Spitz et al. (2005) and Sun et al. (2009)
Graesiella sp.		EPS	Trabelsi et al. (2016)
Rhodella reticulate		EPS	Chen et al. (2010)
Chlorella vulgaris, *Scenedesmus quadricauda*		PS	Mohamed (2008)
Tetraselmis species		PS	Dogra et al. (2017) and Amna Kashif et al. (2018)
Arthrospira platensis (formerly *Spirulina platensis*)		PS	Abd El Baky et al. (2014) and Kurd and Samavati (2015)
Porphyridium cruentum	Anticancer	EPS	Gardeva et al. (2009)
Gymnodinium sp.		EPS	Sogawa et al. (1998) and Umemura et al. (2003)
Tribonema sp.		PS	Chen et al. (2019)
Graesiella sp.		EPS	Trabelsi et al. (2016)
Arthrospira platensis (formerly *Spirulina platensis*)		PS	Abd El Baky et al. (2013)
Phaeodactylum tricornutum		PS	Yang et al. (2019)
Porphyridium cruentum	Immunomodulatory	EPS	Sun et al. (2012)
Thraustochytriidae sp.		EPS	Park et al. (2017) and Yim et al. (2005)

Continued

Table 20.6: Biological activities of PS and EPS from marine microalgae—cont'd

Species	Activity	EPS or PS	Reference
Gyrodinium impudicum		EPS	Yim et al. (2005)
Tribonema sp.		PS	Chen et al. (2019)
Chlorella stigmatophora, *Phaeodactylum tricornutum*		PS	Guzmán et al. (2003)
Pavlova viridis		PS	
Haematococcus pluvialis		PS	Liu et al. (2018)
Arthrospira platensis (formerly *Spirulina platensis*)		PS	Løbner et al. (2008)
Porphyridium cruentum	Antiinflammatory	EPS	Matsui et al. (2003)
Chlorella stigmatophora, *Phaeodactylum tricornutum*		PS	Guzmán et al. (2003)
Coccomyxa gloeobotrydiformis		PS	Dai et al. (2018)
Crypthecodinium cohnii		EPS	Ma et al. (2017)
Arthrospira platensis (formerly *Spirulina platensis*)	Anticoagulant	Ca-PS	Hayakawa et al. (1996) and Yamamoto et al. (2003)
Rhodella grisea	Antitussive	EPS	Nosáľová et al. (2012)
Wollea saccata		EPS	Šutovská et al. (2017)
Porphyridium cruentum	Antilipidemic, antiglycemic	EPS	Dvir et al. (2000) and Dvir et al. (2009)
Porphyridium cruentum	Antiaging	EPS	Diaz Bayona et al. (2012)

20.5.1 Antioxidant activity and free radical scavenging

In their natural environment, microalgae are exposed to different oxidative stresses, including ultraviolet radiation and toxins produced by cyanobacteria (Codd et al., 2005). They have therefore developed protective systems against reactive oxygen species (ROS), generated during normal oxygen metabolism (Pulz and Gross, 2004). According to Mohamed (2008), *Chlorella vulgaris* and *Scenedesmus quadricauda* grow and flourish in the presence of toxins through an increase of their EPS and PS contents. Antioxidant activity involves complex mechanisms that strongly depend on the structural features of microalgae PS. Some of them seem essential, such as sulfate content, sulfation pattern, Mw, and monosaccharide composition. According to Tannin-Spitz et al. (2005), the antioxidant activity of EPS from *P. cruentum* is due to a combination of factors such as its sulfate content (4.5%) and/or the presence of a glycoprotein of 66 kDa. This glycoprotein, noncovalently associated to the negatively charged mucilaginous PS complex, consists of a polypeptide of 58 kDa and a glycan moiety of approximately 8 kDa containing *N*-linked terminal mannose (Shrestha et al., 2004). After denaturation of this glycoprotein, the antioxidant activity of this EPS decreases by 71%.

The relationship between the antioxidant activity and Mw of *P. cruentum* EPS was also assessed using hermetical microwave degradation to produce PS of various sizes (Sun et al.,

2009). The smallest EPS (6 kDa) showed the best antioxidant activity, compared to the other two (60 and 256 kDa). Moreover, this enhanced antioxidant potential was confirmed as the 6 kDa EPS more efficiently inhibited lipid peroxidation induced by Fe^{2+}/ascorbic acid in liver homogenates from healthy male Kunming mice. Conversely, the superoxide anion radical scavenging activity of EPS from *R. reticulata* decreased after ultrasonic depolymerization (Chen et al., 2010), revealing that Mw is not the only essential feature involved in antioxidant activity. Sulfate content and pattern must also be considered. Indeed, the content of exposed sulfate esters of the 6 kDa *P. cruentum* EPS (17%) was higher than those of the other two (14%) (Sun et al., 2009). Furthermore, despite its high sulfate content of 11%, EPS from *Graesiella* sp. did not show any positive effect on superoxide radical scavenging (Costa et al., 2010), but exhibited high potential as a total antioxidant, ferric chelator, and hydroxyl radical scavenger (Trabelsi et al., 2016).

The monosaccharide composition may also play a key role as it can differ widely between different microalgae species, even from the same genus. A particular study demonstrated this role very recently, comparing the purified PS from *Tetraselmis* species KCTC 12236 BP, composed of several moieties, Rha, Gal, Glc, Man, and Xyl for the most part, and PS from *Tetraselmis* species KCTC 12432BP, mainly composed of Gal, Glc, and to a lesser extent Rha (Dogra et al., 2017). Only the former had an antioxidant potential, which highlighted the strong influence of the primary structure on the antioxidant activity. In addition to structural features, the method used to produce and isolate microalgae PS may also play a role. Indeed, these authors also showed that the antioxidant activity of PS from *Tetraselmis* species KCTC 12236 BP extracted in neutral, acidic, or basic aqueous solution was not similar. Its scavenging activity was even twofold higher after basic extraction (Amna Kashif et al., 2018). On the other hand, Abd El Baky et al. (2013) demonstrated that the antioxidant activity of PS from *A. platensis* was correlated to culture conditions.

20.5.2 Anticancer activity

Anticancer treatments, in particular chemotherapy and radiotherapy, are known to cause many side effects. Therefore, there is a compelling need to identify novel effective and nontoxic anticancer compounds from natural sources, and PS from microalgae may be interesting candidates. The anticancer activity of microalgae PS is mainly assessed using antiproliferative assays on tumor cell lines such as leukemic, liver cancer (HepG2), human colorectal adenocarcinoma (Caco-2), primary Graffi tumor, or breast cancer (MCF-7) cell lines. EPS from *Graesiella* sp. exhibits antiproliferative effects on HepG2 and Caco-2 cell lines, but the underlying mechanism remains unclear (Trabelsi et al., 2016). EPS from *Gymnodinium* sp., which consists of a D-galactan sulfate associated with L-(+)-lactic acid, has been revealed to be cytotoxic against several human leukemic cell lines by inducing apoptosis without impacting the cell cycle and mitosis (Sogawa et al., 1998), through the inhibition of DNA topoisomerases I and II—two nuclear enzymes regulating DNA topology (Umemura et al., 2003).

Very similar results have since been described with other microalgae PS. For example, the proliferation of HepG2, a liver cancer cell line, was shown to be reduced via the increase in cell apoptosis induced by PS from *Tribonema* sp. and *P. tricornutum* (Chen et al., 2019; Yang et al., 2019). According to Gardeva et al. (2009), *P. cruentum* EPS reduces proliferation of primary Graffi tumor cells while it promotes proliferation of bone marrow-derived cells. These results have been confirmed in vivo as *P. cruentum* EPS was proved to decrease transplantability and delay the growth of Graffi myeloid tumors in hamsters, leading to prolonging of survival by about 10–16 days. Moreover, *A. platensis* PS exhibited strong anticancer activity against proliferation of HepG2 (−86%) and MCF-7 (−88%) tumor cells (Abd El Baky et al., 2013).

20.5.3 Immunomodulatory activity

Immunomodulating agents act on the innate and adaptive immune responses. Activated macrophages produce increased levels of nitric oxide (NO) and release proinflammatory cytokines. Moreover, immunomodulatory activity is closely related to anticancer activity. Ultrasonic depolymerization of *P. cruentum* EPS was applied by Sun et al. (2012) to produce EPS of various Mw, ranging from 6.553 to 1002 kDa, and assess their effect on NO production by Raw 264.7 macrophages. Low-Mw EPS dose-dependently increased NO production. Moreover, all depolymerized *P. cruentum* EPS were shown to increase the proliferation of splenocytes, which are the primary cells of the adaptive immune system. Again, low-Mw EPS were the most active. Other PS and EPS have been proven to modulate cell proliferation and/or expression levels of cytokines. For example, *Thraustochytriidae* sp.-derived mutant GA produced EPS under gamma radiation that promoted the increase of B cell growth (not T cells), contributing to reinforce the adaptive immune system. It also reduced the release of interleukin-6 (IL-6) and interferon-γ (IFN-γ) by T cells, but had no effect on tumor necrosis factor-α (TNF-α). IL-6 stimulates the inflammatory and auto-immune processes in many diseases such as atherosclerosis, diabetes, and rheumatoid arthritis (Zhang and An, 2007). This EPS is therefore a candidate for treatment of diseases associated with excessive IL-6 release (Park et al., 2017).

EPS from the red-tide microalga *G. impudicum* strain KG03, mainly composed of Gal, uronic acid, and sulfate groups, increased in vivo not only the nonspecific immune functions mediated by natural killer cells and macrophages but also the levels of IgM and IgG antibodies, as well as cytokines IL-1β, IL-6, and TNF-α, via a single dose given by gavage to male C57BL/6 mice (Yim et al., 2005). According to Guzmán et al. (2003), PS from *Chlorella stigmatophora* and *P. tricornutum* have different effects on the phagocytic activity of macrophages. Indeed, in vitro and in vivo studies have demonstrated that *C. stigmatophora* PS exerts an immunosuppressive activity by reducing phagocytic activity, while *P. tricornutum* PS increases phagocytic activity and is immunostimulant. Other polysaccharides modulate cell proliferation only. PS from *Pavlova viridis* was submitted to free radical depolymerization using the H_2O_2/ascorbate system assisted by ultrasonic waves, to produce two low-Mw polysaccharides, PPS1 and PPS2, with Mw of 386.99 and 54.99 kDa, respectively, and a similar sulfate content within

the range 15%–18% (Sun et al., 2016). At 50 mg/L, all three polysaccharides significantly stimulated the proliferation of Raw 264.7 macrophages and stimulated their phagocytic activity and NO production, but PPS2 was the most effective. The strong activity of PPS2 is likely to be due to the combination of its low Mw and high uronic acid content (8.5%), which were 2.7- and 1.6-fold higher than those of PPS0 (3.5%) and PPS1 (5.9%), respectively. PS from *Tribonema* sp. has also been shown to increase Raw 264.7 macrophage proliferation and the release of IL-6, IL-10, and TNF-α, at low concentrations ranging from 25 to 200 μg/mL (Chen et al., 2019). Finally, PS from *H. pluvialis*, and Immulina, a nutritional supplement based on a high-Mw PS from *A. platensis*, have been proved to increase splenocyte and B lymphocyte proliferation at various concentrations (Liu et al., 2018), and to increase *C. albicans*-induced $CD4^+$ T-helper cell proliferation in 11 healthy male volunteers for the latter (Løbner et al., 2008).

20.5.4 Antiinflammatory activity

Inflammation is a response of the immune system to counteract potentially deleterious agents, including invading viruses, bacteria, and other pathogens. An excessive or deregulated inflammatory response may result in the nonrecovery of tissue health and the onset of a chronic condition (Dai et al., 2018). PS from microalgae are new potential antiinflammatory compounds. A convincing example is the EPS from *P. cruentum*, which both inhibited the migration of polymorphonuclear leukocytes, which are the immune system cells involved in adhesion and chemotaxis during the inflammatory process, and partially ensured their adhesion on endothelial cells (Matsui et al., 2003). The same authors performed a clinical study using balsam of Peru, a natural mixture of resins able to induce inflammatory erythema, which demonstrated that *P. cruentum* EPS inhibits inflammation and that this effect is correlated with the viscosity of the polymer. Guzmán et al. (2003) showed at the same time that low concentrations of PS from *C. stigmatophora* and *P. tricornutum* had significant antiinflammatory effects on rat paw edemas, with IC_{50} values lower than that of the reference drug indomethacin.

Very recently, other PS and EPS were shown to reduce the inflammation induced by lipopolysaccharide (LPS), a potent activator of inflammatory cytokine and chemokine production by macrophages and monocytes. According to Dai et al. (2018), PS from *Coccomyxa gloeobotrydiformis* is able to inhibit the LPS-induced production of NO and prostaglandin E_2 (PGE_2) by reducing the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in Raw 264.7 macrophages. It also suppresses the activation of LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inhibits the phosphorylation of mitogen-activated protein kinases (MAPK) such as p38 or c-jun N-terminal kinase (JNK) induced by LPS. In addition, this PS activates the nuclear factor erythroid 2-releated factor/heme oxygenase-1 (Nrf2/HO-1) signaling pathway that inhibits inflammatory responses. EPS from *Crypthecodinium cohnii*, composed of a main backbone of (1,6)-linked Man*p* (61%), (1,6)-linked Glc*p* (23%), and (1/3,6)-linked Gal*p* (23%) and Rha*p*

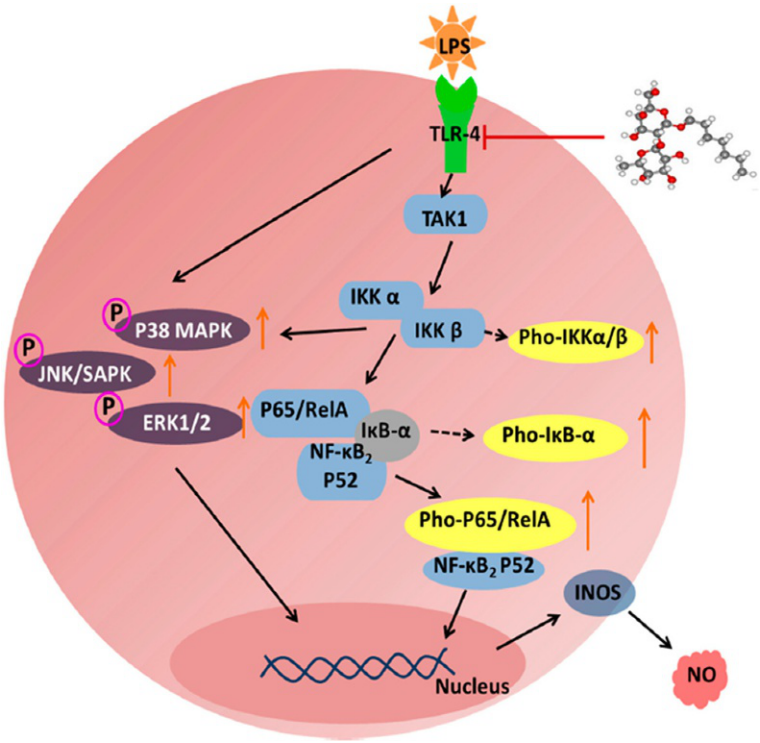


Fig. 20.2
Schematic diagram of the targets proposed by Ma et al. (2017) for the antiinflammatory effects of *C. cohnii* EPS in Raw 264.7 macrophages stimulated by LPS, potentially leading to the inhibition of the proinflammatory cytokines and related mediators.

(3%) as branch and terminal residues, respectively, was also shown to dose-dependently reduce the LPS-induced production of NO and proinflammatory cytokines IL-1β, IFN-γ, and TNF-α by Raw 264.7 macrophages, without any cytotoxicity (Ma et al., 2017). This study demonstrated that *C. cohnii* EPS was able to inhibit three LPS-induced metabolic pathways (Fig. 20.2): (i) phosphorylation of p38, ERK1/2, and JNK; (ii) expression of toll-like receptor 4 (TLR4) and transforming growth factor beta-activated kinase 1 (TAK1); and (iii) NF-κB pathway activation, through the downregulation of κB inhibitor (IκB) expression, IκB-α degradation, and NF-κB-P65 nuclear translocation. The authors proposed a possible mechanism involving a competitive suppression of the binding of LPS to TLR4 by *C. cohnii* EPS to explain its activity.

20.5.5 Anticoagulant activity

Unlike PS from macroalgae that have been extensively studied during the past 30 years for their anticoagulant activities (Mestechkina et al., 2010), only calcium-spirulan is known for its anticoagulant activity. Different studies have demonstrated that Ca-SP induces a 10,000-fold

increase in antithrombin activity of heparin cofactor II, which is a plasma serine protease inhibitor well-known to reduce coagulation through inhibition of thrombin when associated with heparin or dermatan sulfate. The presence of calcium or another cation (Na-SP) is needed for spirulan activity. From a mechanistic point of view, the study of Hayakawa et al. (1996) involving different mutations in the N-terminal acidic domain of heparin cofactor II has revealed that, unlike heparin or dermatan sulfate, Ca-SP does not interact with this domain, but only with the anion-bonding exosite I. Ca-SP and Na-SP have also been shown to influence the blood coagulation-fibrinolytic activity of human fetal lung fibroblasts or vascular endothelial cells. It was found that SP indirectly enhances the activity of tissue- and urokinase-type plasminogen activators (t-PA and u-PA) that convert the proenzyme plasminogen into plasmin; its active form degrades fibrin through induction of urokinase-type plasminogen activator secretion and inhibition of plasminogen activator inhibitor type 1 (PAI-1) secretion (Yamamoto et al., 2003). It is noteworthy that the cation is not required in this case, while the sulfate groups are essential for inhibition of endothelial PAI-1 secretion by Na-SP. The study by Yamamoto et al. indeed showed that desulfated SP was unable to inhibit PAI-1 secretion and even stimulated it.

20.5.6 Antitussive activity

In the last decades, natural compounds such as alkaloids, flavonoids, and carbohydrates have enriched the portfolio of antitussive agents. *Rhodella grisea*, a unicellular freshwater red microalga, releases an extracellular mucilaginous proteoglycan mainly composed of Xyl (methylated or not) and glucuronic acid. An in vivo study conducted on nonanesthetized cats has demonstrated that *R. grisea* proteoglycan has a cough-relieving effect on a mechanically induced laryngopharyngeal cough, but not on the tracheobronchial part of airway mucosa (Nosáľová et al., 2012). More recently, EPS from a freshwater cyanobacterium, *Wollea saccata*, was reported to suppress the cough reflex induced by chemical tussigen, but with a lower effect than that of codeine, the strongest antitussive agent, while its bronchodilatory effect was similar or even greater than the effect of the reference drug, salbutamol. Moreover, pharmacological studies did not report any toxicity or side effects in animals following administration of this EPS (Šutovská et al., 2017).

20.5.7 Antilipidemic and antiglycemic activities

Only *P. cruentum* EPS has been studied for its antilipidemic potential. This EPS was shown by Dvir et al. (2000) to decrease weight, as well as plasma cholesterol and cholecystokinin levels, in a male Sprague-Dawley rat model. The authors suggested that metabolic changes could explain morphological ones, and confirmed this by demonstrating that total serum triglyceride and hepatic cholesterol levels were reduced while high-density lipoprotein/low-density lipoprotein (HDL/LDL) ratio and fecal excretion of neutral steroids and bile acids were increased in rats receiving the *P. cruentum* EPS supplemented diet. In addition, this hypocholesterolemic effect could also be due to the high viscosity of *P. cruentum* EPS, which would modify nutriment absorption in the gut (Dvir et al., 2009). Finally, *P. cruentum* EPS

exhibits a possible antiglycemic effect because it has been shown to lower insulin and/or glucose levels in diabetic rodents, without inducing any change in pancreatic island cells, fibrosis, or hemorrhagic necrosis (de Jesus Raposo et al., 2015).

20.5.8 Antiaging activity

Microalgae are generally found under the form of extracts in cosmetic studies and preparations, and are essentially used for their antioxidant properties. However, a study assessing *P. cruentum* EPS reported the antiaging potential of this biopolymer for cosmeceutical applications. Indeed, this sulfated high-molecular-weight EPS was proved to inhibit hyaluronidase and elastase, two key enzymes catalyzing the hydrolysis of hyaluronic acid and elastin, respectively, when used at concentrations ranging from 0.25 to 2.5 mg/mL. However, it did not inhibit the bacterial collagenase from *Clostridium histolyticum* (Diaz Bayona et al., 2012). These extracellular matrix-degrading enzymes are involved in the aging-associated alteration of derma that causes skin modifications, such as wrinkle formation and loss in skin elasticity leading to its sagging. They are therefore privileged targets for skin care applications.

20.6 Future perspectives

Identifying bioactive components from new renewable natural resources is a major challenge, in particular from microalgae. These microorganisms are capable of synthesizing many various molecules, such as pigments, proteins, lipids, and PS. PS from microalgae and cyanobacteria are of particular interest because they are generally sulfated, exhibit a high molecular weight and viscosity, and have a high constitutive monosaccharide diversity. PS can be extracted from microalgae or released by microalgae in their culture media, leading to EPS. During the last decade, these "old plants" appeared to be the subject of renewed interest back to the production of original metabolites and notably PS. As discussed earlier in this chapter, the biological activities of these biopolymers are real and various, and some authors have attempted to explain them by structure function relationships. The main example is the correlation of sulfate contents of some of these polysaccharides with biological activities. Therefore, it is very surprising that this important literature is not really correlated with the arrival on the market of new PS from microalgae, notably in the therapeutic field. Indeed, even if a recent extract from *P. tricornutum* with high PS content (Sensityl) has been recently marketed by the Givaudan company as a new cosmetic agent for skin treatments, PS from *Porphyridium* species remain the sole commercial success, but exclusively in the cosmetic arena (Gaignard et al., 2019).

Without claiming to be exhaustive, EPS of *Porphyridium* are exploited by several companies including Frutarom (http://www.frutarom.com), Algosource Technology (http://www.algosource.com), Microperi Blue Growth (http://www.micoperibg.com), Greensea (http://greensea.fr/en), and others. However, all these companies exploit a niche market that is

extremely versatile: that of cosmetic products. The size and the high renewal need of this market could, at least superficially, give some opportunities to other PS and EPS from microalgae and cyanobacteria. This naïve verdict does not take into account the existence of the Inventory of Existing Cosmetic Ingredients established by the China Food and Drug Administration (CFDA). This inventory included, in 2015, 8783 existing cosmetic ingredients that have already been used in cosmetics in China. Cosmetic ingredients that are not listed are regarded as new cosmetic ingredients and must be approved by the CFDA before they can be used in cosmetics in China. This complex administrative issue makes suppliers of cosmetic agents very prudent in the development of new products from species of microorganisms not present on this list.

Even if the costs of production of PS and EPS from microalgae and cyanobacteria are always prohibitive compared to those of terrestrial plant or seaweed polysaccharides to access large markets, such as that of hydrocolloids, they should be able to compete with some animal PS, such as glycosaminoglycans, as therapeutic compounds. However, this market is currently closed to these biopolymers as their structures remain unclear and limited to their sole monosaccharide compositions. The applications for marketing authorization of a medicinal product are very restrictive and require specific data on structure and biological efficiency for all new molecules. The development of modern tools of structural characterization of poly- and oligosaccharides, and notably that of high resolution LC-MS/MS, could resolve part of this problem, opening the way for therapeutic agent developments.

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CHAPTER 21

Sterols from microalgae

Mariane Bittencourt Fagundes, Raquel Guidetti Vendruscolo, Roger Wagner

Department of Food Science and Technology, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Chapter outline

21.1 Introduction 573
21.2 Structural aspects of sterols 574
21.3 Sterol biosynthesis 576
21.4 Sterols 581
21.5 Sterols in cyanobacteria 582
21.6 Environmental influence on microalgae sterols production 583
21.7 Sterols analysis 587
21.8 Industrial sterol production 589
21.9 Conclusion 590
References **591**

21.1 Introduction

Microalgae are well-known as a source of molecules with high bioactivity; these promising microorganisms can be used in order to increase health humans conditions (Jacob-Lopes et al., 2019). In this regard, there are many classes of bioactive compounds that could be isolated, such as carotenoids, phycobiliproteins, fatty acids, amino acids, and other important bioactives (Maroneze et al., 2019; Francisco et al., 2010; Vendruscolo et al., 2019; Pan-utai and Iamtham, 2019).However, there are a lot of compounds that need better investigation, being one of this the sterols. These molecules play an important activity in concern to microalgae cell structure, because they help in the membrane permeability (Clair and London, 2019).

Microalgae strain can present a wide range of sterols, from cholesterol to β-sitosterol, as major compounds (Fagundes et al., 2019a). Therefore, modifications in culture conditions could also change the sterol profile. This study's findings could be explored as a substantial new research area, due to the importance of sterols as antioxidant, anticarcinogenic, and antiinflammatory compounds (Volkman, 2016). This chapter discusses some aspects of microalgae sterol

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bioactivity, analytical tools for sterol determination, and sterols' potential in terms of industrial production. Based on the above, we focus on microalgae as a potential source of sterols, showing the aspects of their chemical structure, bioactivity potential, biosynthesis, and production, as well as the possible aspects of improving the concentration in microalgae strains.

21.2 Structural aspects of sterols

The ocean could be considered a new potential source of bioactive molecules; it is well known that marine chemistry currently needs to be explored further. Because many healthy human problems can be overcome by the discovery of new chemical compounds from these microorganisms, among these molecules, the sterols can be better studied. Sterols primordial structure is a cyclopentaneperhydrophenanthrene, known as a sterane ring with two angular methyl groups. The sterol structure is similar to steroids, considered their subgroup, and the major difference is the presence of a hydroxyl group (OH) at the 3-position from the A-ring. These compounds are also considered amphipathic molecules, and their primordial structure can be observed in Fig. 21.1.

Structurally, sterols have divisions built by three cyclohexanes (A, B, and C) and one cyclopentane (D). Characteristics with regards to the A section is the most important chemical part, due to the presence of the hydroxyl group (OH), which is responsible for all hydrogen-bond interactions, being active hydrogen for other chemical interaction. B is more related to structure planarity, and C is associated with the orientation of the side chain at C20. The other groups are responsible for the tilt and shape; however, depending on the chain bonded in D-cyclopentane, they can help in cell-chemical interactions (Nes, 2011).

A great diversity of sterols are found, and these variations undoubtedly influence the sterols' bioactivity and the bioaccessibility of these structures. It is important to note that sterols' configuration directly affects the cellular membrane, because the specificity of β cholesterol's 3-hydroxyl configurations is responsible for the interactions with chiral targets. Tsuchiya and Mizogami (2017) showed that the chiral sterol contained in the prokaryotic cell membranes differs from that in eukaryotic microorganisms, and also that normally these compounds in

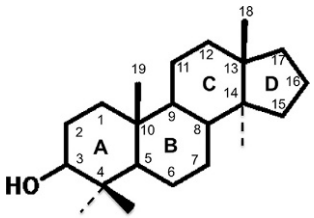

Fig. 21.1
Sterol basic chemical structure.

prokaryotic cells are replaced by hopanoids, which are similar to pentacyclic compounds. However, this is not a total substitution, because sterols can be found in some cyanobacteria (Belin et al., 2018).

Microalgae present some characteristics in sterols' structure, which is associated with the carbon 24 side-chain, with modifications in this side chain it could distinguish the microalga species. Volkman (2003) describes how 24 β-methyl sterols are located in many less-advanced organisms, including fungi and protozoa. On the other hand, the 24 α-ethyl sterols are more common in advanced organisms, being considered similar to plant metabolism. Usually, sterol configuration can present after the carbon 24 α a methyl, ethyl, or propyl groups, however, the propyl formation is more difficult to acquire them the other ones.

However, in terms of molecular phytosterols' structure, these molecules have a chain increased at carbon 24 compared to cholesterol, and this feature may provide an increase in van der Waals forces. The explanation of membrane chemicals can aid understanding of the physical cellular aspects—for example, in cell disruption studies, assisting in the comprehension of how to acquire these intracellular bioactive compounds from microalgae. In this sense, as mentioned, sterols are also essential for cell protection, being a constant group of the compound to be isolated, studied, and elucidated.

Clair and London (2019) showed the specific cell endocytosis, which is the absorption of molecules by the membrane. This situation occurs mostly according to the domains found that can be characterized as symmetric or asymmetric vesicles. In this research it can be observed differences in the membrane endocytosis, and this is associated with the membrane symmetry. Cholesterol stabilize ordered domain more than cholesten-3-one, while for symmetric vesicles the less ordered domain was epicholesterol. On this point, the endocytosis levels are closely associated with the ability of sterols to form ordered domains, and this theme has been a new approach in membrane studies.

With this in mind, it is essential to comprehend the class of sterol found, not only due to their high value, but also because these molecules are important to understand the microalgae phylogenetic organization (Leblond et al., 2010). For example, the phylum Chlorophyta (green algae) presents similarities to plant metabolism; however, there are too many species in this phylum that make difficult the sterols' determination, with the classes of 24 α, and 24 β sterols being the most common forms found (Volkman, 2016; Patterson, 1974). Abdel-Aal et al. (2015) determined the following compounds in the sterol composition of *Spirogyra longata*: stigmasterol, β-sitosterol, campesterol, ergosterol, brassicasterol, Δ7-stigmasterol, and Δ7-avenasterol. However, when analyzing Chrysophyta (gold algae), such as *Ochromonas danica* and *Ochromonas malhamensis*, these strains present mostly in their composition the 24 β, such as poriferasterol and cholesterol. Gershengorn et al. (1968) also described the same compounds in this strain. In the same line, the strain *Synura petersenii* has proven to have huge production of cholesterol (Collins and Kalnins, 1969), being the major compound described for

this phylum. The phylum Rhodophyta is known as red algae and also presents cholesterol as a major sterol, being similar to the Chrysophyta phylum (Tsuda et al., 1958). Other molecules from cholesterol metabolism such as cholestanol were observed that were found in *Gracilaria salicornia* and *Hypnea flagelliformis* (Nasir et al., 2011).

Regarding Dinophyta, dinoflagellates phylum, the major sterols present are 4-methyl, and 4-desmethyl sterols; for example, dinosterol can be found, which is considered the most important molecule from this phylum (Volkman, 2016). The phylum Euglenophyta presents ergosterol as a major compound in its composition, a typical molecule found as a fungal biomarker (Watanabe et al., 2017). Another well-known phylum is Phaeophyta; these microorganisms are known as brown algae, and present as major metabolite fucosterol (Patterson, 1971).

In the field of sterols, there are discussions among many researchers about cyanobacteria classification, because they are prokaryotic microorganisms (Volkman, 2018), and there are reports suggesting that these strains are not sterol producers (Levin and Bloch, 1964). However, the number of studies on microalgae sterol production has increased.

21.3 Sterol biosynthesis

Sterols can be synthesized by distinct pathways, as microalgae are very versatile, and capable of working with different pathways. For their production, two pathways could be used: the first one allows the formation of squalene, which is considered a key precursor for sterol formation; the other leads to the formation of specific sterols. These two pathways are shown in Figs. 21.2 and 21.3.

Microalgae cells need sources of chemical energy for the activation of metabolic pathways; the energy could be obtained by the chemical process of photosynthesis or by chemical phosphorylation in a heterotrophic culture (Perez-Garcia et al., 2011; Williams and Laurens, 2010). Usually, all microalgae by their nature are photosynthetic. However, some strains are capable of metabolizing complex or simple exogenous carbon sources (Perez-Garcia et al., 2011). In this pathway, after the obtainment of energy, the major building blocks for squalene production are glyceraldehyde-3-phosphate (GAP), pyruvate, and acetyl coenzyme A (acetyl-CoA).

Considering the photosynthetic medium, the building-block molecules are obtained in two phases (light and dark), initially in the light phase, which starts at the thylakoids lamella of the chloroplast. In the thylakoids are the photosystems, and its act capturing sunlight and assist the electrons transfer. The photosystems consist of proteins, pigments, and electron carriers. After different photons are obtained, light starts an oxidative phosphorylation, producing adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADPH); after this, the water photolysis electrons are released. These processes will be used to replace the other electrons

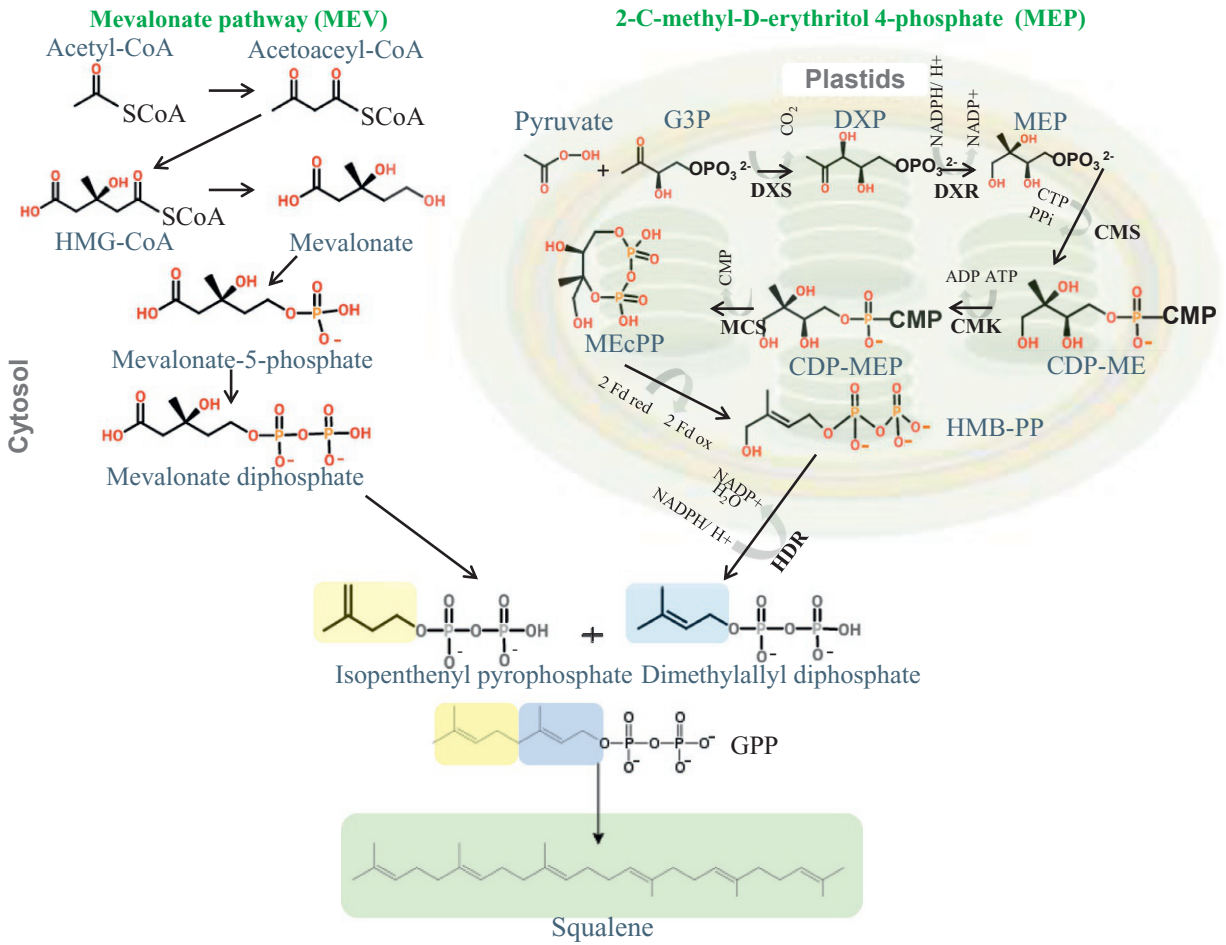

Fig. 21.2

1-Sterols biosynthesis: mevalonate (MEV) pathway and 2-C-methyl-D-erythritol-4-phosphate (MEP). Acetyl coenzyme A (acetyl-coA); 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA); 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA sintase); 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase); mevalonic acid (MEV); mevalonate kinase (MEV kinase); mevalonate phosphate (MEV phosphate); mevalonate pyrophosphate kinase (MEV pyrophosphate kinase); mevalonate pyrophosphate (MVA pyrophosphate); mevalonate pyrophosphate decarboxylase (MVA pyrophosphate decarboxylase); isopenthyl pyrophosphate (IPP); dimethylallyl pyrophosphate (DMAPP); glyceraldehyde-3-phosphate (G3P); 1-deoxy-D-xylulose-5-phosphate (DXP); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR); 2-C-methyl D-erythritol 4-phosphate (MEP); 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS); 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CDP-ME); 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (CDP-MEP); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS); (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (MecPP); (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (HDS); 4-hydroxy-3-methylbut-2-enyl diphosphate (HMB-PP); 1-deoxy-d-xylulose 5-phosphate synthase (DXS); 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR).

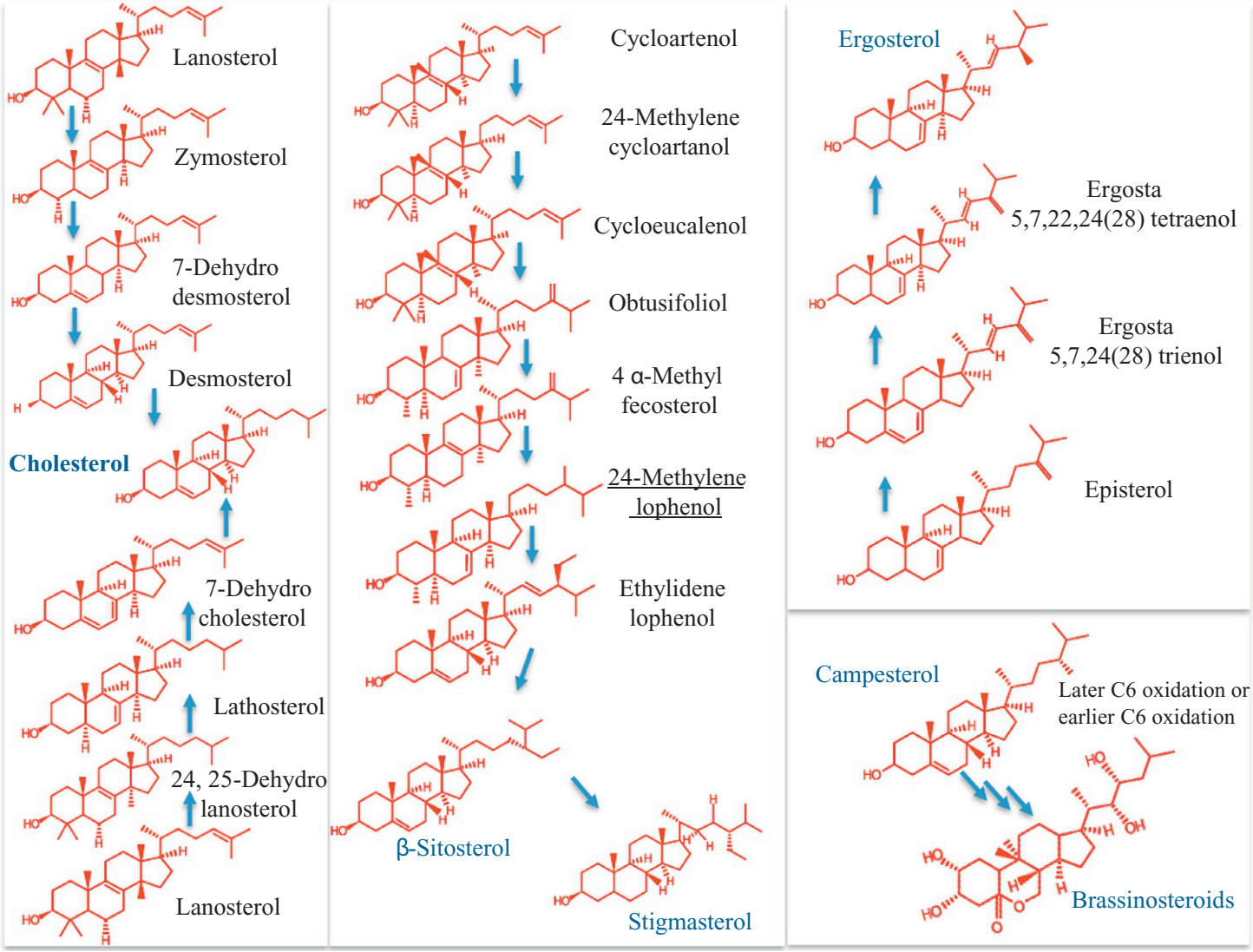

Fig. 21.3
Different sterols pathways found in microalgae metabolism.

lost by the chlorophyll in photosystem II and later to produce the oxygen molecule. The second phase is characterized by the Calvin cycle, in which several reactions lead to the formation of GAP, and two of these molecules originate glucose phosphate (Williams and Laurens, 2010). In contrast, the heterotrophic metabolism occurs in the total absence of luminosity, being known as the pentose-phosphate pathway (PPP), and also the Embden-Meyerhof pathway. At first, in these metabolisms occurs the reduction of complex carbohydrate structures to their simplest forms, which are assimilated through the metabolic process—for example, the glucose structure. After the glucose transformation, oxidative phosphorylation reactions are initiated, resulting in glucose-6-phosphate, the PPP route, in which ribulose-5-phosphate forms, and the formation of this metabolite leads to the production of glyceraldehyde-3-phosphate (Perez-Garcia et al., 2011). The sequence occurs by two possible pathways, and according to Lohr et al. (2012), it can be activated depending on the algae's evolutionary history. The route possibilities are the mevalonic acid pathway (MEV) and the methylerythritol phosphate pathway (MEP) (Fig. 21.2). The first route occurs in the cellular cytosol, whereas the second production route takes place mostly in the plastids (Gohil et al., 2019). MEV begins by condensation of acetyl-CoA with 3-hydroxy-3-methyl-glutaryl-CoA producing hydroxymethylglutaryl coenzyme A. Next, the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) forms the mevalonate (MEV) (Miziorko, 2011; Eisenreich et al., 2004). In MEP occurs the condensation of glyceraldehyde-3-phosphate and pyruvate; production of these leads to the formation of deoxy-D-xylulose-5-phosphate, followed by the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which produces 2-C-methyl-D-erythritol-4-phosphate (MEP).

Additionally, after the formation of MEV and MEP, the following reactions lead to the same molecule, which is considered a meeting point of both metabolic routes, the isopentenyl pyrophosphate, which is converted to farnesyl pyrophosphate by the enzyme farnesyl pyrophosphate synthase (FPPS), leading to a conversion by squalene synthase (SQS) enzyme to squalene. Then, in the presence of oxygen squalene epoxidase (SQE), the enzyme converts squalene to 2,3-oxidosqualene (Lohr et al., 2012; Bhattacharjee et al., 2001).

The sterols pathway end products, e.g., cholesterol, stigmasterol, and others is going to depend on the microalgae species, and also the environmental conditions, being the microalgae system possible metabolic transformations are shown in Fig. 21.4.

In this sense, to produce phytosterols such as stigmasterol and β-sitosterol, the enzyme cycloartenol synthase (CAS) converts 2,3-oxidosqualene to cycloartenol, which by the enzyme Δ24-sterol methyltransferase (SMT) is converted to 24-methylenecycloartenol, followed by the formation of obtusifoliol. This compound is converted to Δ8,14-sterol by the obtusifoliol-14-demethylase enzyme (ODM). Following other conversions this leads to the formation of 4-α-methylfecosterol to 24-methylene-fecosterol, to 24-methylene lupeol, to cicloestradiene, to avenasterol, and finally to β-sitosterol, and stigmasterol is synthesized from β-sitosterol by cytochrome P450 CYP710A1 through denaturation of carbon 22 (Griebel and Zeier, 2010).

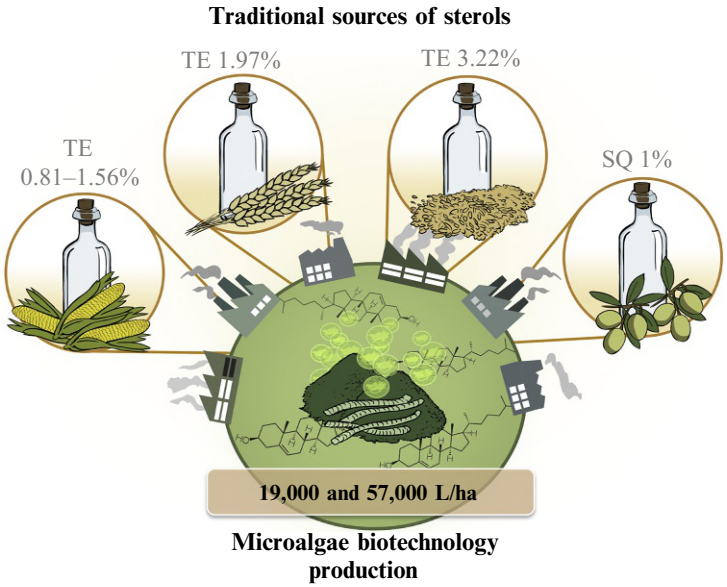

Fig. 21.4

Biotechnology production compared with traditional sources sterols and squalene, *TE*, total sterols; *SQ*, squalene; maize oil (0.81%–1.56%), germ oil (1.97%), rice bran oil (3.22%), and olive oil (1%).

In the cholesterol pathway, after 2,3-oxidosqualene, the formation of lanosterol occurs by squalene monooxygenase enzyme (SQLE) to 4,4,14-trimethylcholesta-8(9)-en-3β-ol. The conversions are as follows: 4,4-dimethylcholesta-8 (9)14-dien-3β-ol, then 4,4-dimethylcholesta-8(9)-en-3β-ol, followed by the formation of 4-α-methylcholesta-8(9)-en-3β-ol, to cholesta-8(9)-en-3β-ol, and then to cholesta-7-en-3b-ol. Finally, the cholesta-5,7-dien-3b-ol is formed as the last intermediate, and then is converted to cholesterol (cholesta-5-en-3β-ol) (Cerqueira et al., 2016).

For ergosterol, according to Brumfield et al. (2010), *Chlamydomonas reinhardtii* presents a difference compared to fungal ergosterol's metabolism; fungi metabolism starts with the lanosterol conversion. The difference pointed out by the authors is that this pathway starts with cycloartenol, followed by obtusifoliol. In this step we have the metabolism similar to the phytosterols, so, as can be observed in Fig. 21.3, the 24-methylene lophenol is converted to episterol, and a sequence of reactions then leads to ergosterol (Fagundes et al., 2019a; Brumfield et al., 2010).

Eukaryotes microorganisms present many reactions well studied in terms of sterols pathways, being methylations, unsaturations, and isomerizations, however, for prokaryotes, is not the same (Wei et al., 2016). According to Villanueva et al. (2014) there is some pathways already elucidated, e.g., ergosterol conversion to 5,7-ergostatrienol, 5,7,28-(24)-ergostatrien-3-ol, 5,7,22,28-(24)-ergostatetraen-3-ol, and ultimately to the end

product ergosta-5,7,22-trien-3-ol (ergosterol) (Fig. 21.3). So, prokaryotic microorganisms, can activate either lanosterol or cycloartenol pathways as described in a previous work with the strain *Phormidium autumnale* (Fagundes et al., 2019a).

Brassinosterols are another important end product pathway, and could be synthesized by many microalgae, being an example of the strain *Chlorella vulgaris* as described by Bajguz (2019). The possible paths involved are the C6-oxidation or earlier C6-oxidation; these two paths start with campesterol and lead to 6-deoxocathasterone to 6-deoxoteasterone, and subsequently to 6-deoxo-3-dehydroteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone. The next compound is castasterone, which is found before brassinosterol; this is characterized as the later C6 oxidation. The last path also starts with campesterol, but the ensuing transformation sequences are as follows: 6-oxocampesterol, cathasterone, teasterone, 3-hydroteasterone, typhasterol, and cathasterone to brassinolide.

21.4 Sterols

The synthesis of sterols can be different among microalgae as mentioned before, since a class exists that characterizes each phylum. In addition, culture modifications can also lead to different sterol responses and composition (Volkman, 2003).

The study about green algae is not new. Patterson (Patterson, 1974) in 1974 characterized some green algae; among them in *Cladophorales* large amounts of cholesterol, 24-methylene cholesterol and 28-isofucosterol were identified, while for *Spirogyra* sp., clionasterol and poriferasterol were found. In the same study, *Chlorella* species were studied, and in their composition, the class of 24β sterols was found.

Li et al. (2017) identified by 1D and 2D nuclear magnetic resonance spectroscopy new structures of sterols compounds as (24*R*)-5,28-stigmastadiene-3β,24-diol-7-one, (24*S*)-5,28-stigmastadiene-3β,24-diol-7-one, and (24*R*) and (24*S*)-vinylcholesta-3β,5α,6β,24-tetraol from the strain *Ulva australis*. Other new sterols were found due to continuous research in this field, such as isofucoterol, (24*R*,28*S*)- and (24*S*,28*R*)-epoxy-24-ethylcholesterol, and (24*S*)-stigmastadiene-3β,24-diol, in the same strain.

Sterols from 13 species of algae belong to the phyla: Dinophyceae, Bacillariophyceae, Ulvophyceae, and Pelagophyceae were reported by Geng et al. (2017). In this work, the authors found Ulvophycea sterols, such as 28-isofucosterol and cholesterol. The Pelagophyceae presented six sterols, including cholesterol, (24*E*)-24-propylidenecholesterol, (24*Z*)-24-propylidenecholesterol, (22*E*)-stigmasta-5,22-dien-3β-ol, campest-5-en-3β-ol, and stigmast-5-en3β-ol. For Dinophyceae, in a dinoflagellate biomass, dinosterol, cholesterol, (22*E*)-ergosta-5,22-dien-3β-ol, and (22*E*)-stigmasta-5,22-dien-3β-ol were identified. In contrast, in diatoms cholesterol, (22*E*)-ergosta-5,22-dien-3β-ol, and campest-5-en3β-ol were found.

Mouritsen et al. (2017) stated that microalgae could produce only fucosterol and desmosterol; however, this cannot be confirmed, because there is a complexity of sterols that could be produced.

In other research, the sterols ergosterol, fucosterol, cholesterol, campesterol, stigmasterol, and β-sitosterol were analyzed in 21 algae from Chlorophyta, Rhodophyta, Phaeophyta, and Spermatophyte phyla (Mikami et al., 2018). In this study, the variations were huge, with a major concentration 4135.7 $\mu g\, g^{-1}$ dry biomass of cholesterol in *Gracilaria vermiculophylla* (Rhodophyta), followed by 731.1 $\mu g\, g^{-1}$ dry biomass of fucosterol, and 661.0 $\mu g\, g^{-1}$ dry biomass of β-sitosterol from *Sargassum fusiforme* (Phaeophyta). For ergosterol, 93.4 $\mu g\, g^{-1}$ of dry biomass was found in *Grateloupia asiatica* (Rhodophyta), and 86.9 $\mu g\, g^{-1}$ dry biomass in *G. vermiculophylla* (Rhodophyta) and *Undaria pinnatifida* (Phaeophyta). On the other hand, campesterol was observed in only one algae, *Zostera marina* L. (Spermatophyta), with a concentration of 125.9 $\mu g\, g^{-1}$ dry biomass; the same stigmasterol presented 60.7 $\mu g\, g^{-1}$ dry tissue in *G. asiatica* (Rhodophyta). The brown algae *Ectocarpus siliculosus* presented concentrations of fucosterol, cholesterol, and ergosterol, which ranged in their concentration according to the specimens (Mikami et al., 2018).

21.5 Sterols in cyanobacteria

In the literature, many studies have described how cyanobacteria cannot produce sterols, providing a huge discussion among researchers. According to Levin and Bloch (1964), the class of microalgae that is well-known as blue-green algae could not produce these biomolecules, but this knowledge is considered out of date. In 1968, only some years after the publication of an article describing the absence of sterol in cyanobacteria, the authors De Souza and Nes (De Souza and Nes, 1968) published a study reporting seven unsaturated sterols produced by cyanobacteria *Phormidium luridurn*. Reitz and Hamilton (1976) described the profile from two strains: *Anacystis nidulans* and *Fremyella diplosiphon*.

In addition, some authors define the strain *Synechocystis* sp. (Kaneko and Tabata, 1997) as a protein gene producer responsible for sterol synthesis. Forin et al. (1972) showed the principal class of sterols as 24-ethyl.

The cyanobacteria *Spirulina platensis* Geitler also presented similar profiles, producing several sterols, such as cycloartenol, 24-methylene-cycloartanol, cycloeucalenol, obtusifoliol, 4α-methyl-Δ8-ergostenol, Δ7-cholestenol, cholesterol, Δ7-ergostenol, Δ5-ergostenol, isofucosterol, Δ7-chondrillastenol, clionasterol, and poriferasterol. Therefore, after these findings, Loeschcke et al. (2017) proved the presence of the enzyme 2,3-oxidosqualene; this enzyme signifies a principal step in the transformation of squalene to sterols. The expression of this enzyme was also observed in the cyanobacteria *Synechocystis* sp. Despite all

this evolution, many cyanobacteria need to be elucidated concerning this enzyme expression (Volkman, 2003).

21.6 Environmental influence on microalgae sterols production

According to Volkman (2016), environmental variations influence sterol production by microalgae significantly, such as temperature and nutrients. Concerning sterol metabolic pathways, the first important metabolite acquired is squalene; as described earlier, this compound presents many bioactive proprieties, so many studies have provided information about the accumulation of this compound. Nevertheless, when squalene accumulation occurs in the cell sterols are not produced.

The temperature affects the plant sterols' metabolism, because the ethyl groups branched at C24 are produced in order to protect the cell from thermal shocks (Beck et al., 2007). With this in mind, squalene could also be produced by varying the temperature in *Thraustochytrid aurantiochytrium*, to acquire it by biotechnology production. Nakazawa et al. (2012) studied the temperatures 10°C, 15°C, 20°C, 25°C, 30°C, and 35°C in the culture system, and the best response acquired from this study was 25°C. Additionally, they evaluated the diverse glucose concentrations, in the range of 2%–6%, and the addition of seawater was investigated, varying from 25% to 50%. A squalene concentration of 171 $mg\,g^{-1}$ dry biomass was obtained using 2% glucose and 50% seawater concentrations in the medium at 25°C.

Zhang et al. (2017), studying the strain *Schizochytrium limacinum*, evaluated the influence of butanol addition in the cultivation system. The use of this molecule was capable of increasing the squalene content from 0.65 to 20.09 $mg\,g^{-1}$. In the same line, Naziri et al. (2011) performed an optimization by using the strain *Aurantiochytrium mangrovei* cultured with distinct concentrations of terbinafine and methyl jasmonate, and the authors reported that the major concentration obtained was 10.02 $mg\,g^{-1}$ of dried biomass. In addition, in other studies using distinct terbinafine concentrations in the same strain, the authors observed an increase of 60% in squalene content (Fan et al., 2016).

In terms of sterol production, few studies describe the influence of external nutrients on the microalgae profile. Fagundes et al. (2019a) showed the differences between exogenous carbon sources in a heterotrophic cultivation system and their influence on sterol profile. In this research, exogenous carbon source glucose, sucrose, and wastewater were explored. Glucose presented in squalene in a concentration of 1440.4 $\mu g\,g^{-1}$ of dry biomass, and ergosterol 1033.3 $\mu g\,g^{-1}$ of dry biomass; for sucrose a concentration of 225.4 $\mu g\,g^{-1}$ of dry biomass of squalene was found, and by using wastewater it was capable of producing a diversity of sterols, being 425.6 $\mu g\,g^{-1}$ of dry biomass of squalene, 820.6 $\mu g\,g^{-1}$ of dry biomass of cholesterol, and 455.3 $\mu g\,g^{-1}$ of stigmasterol, and β-sitosterol in a concentration of 279.0 $\mu g\,g^{-1}$ of dry biomass. In the same study, it was also possible to characterize many intermediary pathway sterols.

In the same line, considering the increase in sterol production, Ahmed and Schenk (2017) reported the increase in sterol content by using UV-C radiation on the strain *Pavlova lutheri*. In this study, higher total sterol contents ($20.3\,mg\,g^{-1}$) were found in an irradiation of $100\,mJ\,cm^{-2}$. In addition, the authors evaluated a treatment by using H_2O_2; the concentrations explored were: 1, 5, 10, 50, 100, and $500\,\mu mol\,L^{-1}$, and the major concentration was obtained by using $100\,\mu L\,L^{-1}$, being the total sterol $400\,\mu g\,g^{-1}$ of dry biomass.

21.7 Sterols analysis

In complex matrices such as microalgae, analyses of sterols and their precursor squalene may involve several steps, generally extraction, saponification and identification, and quantification by chromatographic techniques (Lv et al., 2015). The extraction of free and esterified sterols is usually carried out as lipid extraction due to the polarity of the compounds, but it is worth noting that some sterols such as glycosylated sterols (steryl glycosides and acylated steryl glycosides) may be neglected because they have superior polarity (Feng et al., 2015).

The extraction with reflux of organic solvents, known as the Soxhlet method, is a reference in lipid extractions and other nonpolar substances such as squalene, free sterols, and esterified sterols, and uses solvents such as petroleum ether (Abdallah et al., 2015), methanol-chloroform mixture (Hidalgo et al., 2015), hexane (Kozłowska et al., 2016), and dichloromethane (Martins et al., 2016). Although it is simple and efficient, the Soxhlet method presents long periods of extraction, consumes high volumes of toxic and flammable solvents, and involves high-energy usage for distillation and recovery of organic solvents (Mubarak et al., 2015).

The partitioning by the solvents methanol, chloroform, and water used in the method of Folch (1957) and Bligh and Dyer (1959) has shown great relevance in extracting the nonpolar fraction of microorganisms to biological tissues (Breil et al., 2017). Optimal extraction occurs by homogenizing the sample with the mentioned solvents to form a single-phase solution. Afterwards, a bi-phasic system is intentionally induced, and the nonpolar substances are solubilized in the chloroform phase and the polar compounds in the methanol-water phase (Odeleye et al., 2019). Although is not used higher temperatures in the conventional methods, as in Soxhlet method, Folch and Bligh and Dyer they are responsible to expend large volumes of organic solvents, with this in mind, current works bring as an alternative the miniaturization of these methods (Vendruscolo et al., 2018).

Supercritical fluid mainly using carbon dioxide (CO_2) has been used for extraction of lipids and sterols (Attard et al., 2018; Li et al., 2016); its advantages include efficiency, environmental friendliness by being free of organic solvents, and maintaining thermolabile compounds. Studies evaluated the squalene and β-sitosterol extraction using supercritical fluids of CO_2 and Liquefied Petroleum Gas (LPG); although the LPG has extractive capacity of nonpolar compounds, the highest concentrations of the sterol and its precursor were obtained with the use

of CO_2 (Scapin et al., 2017a,b). Despite its advantages, when compared with extraction with organic solvents, supercritical fluid extraction is a process of high cost and operational complexity (Mubarak et al., 2015).

In microalgae, free and bound sterols are found. However, normally is used a saponification step after the lipid extraction to obtain free sterols from esterified sterols is recommended, as well as to eliminate interferers saponifiable such as triacylglycerols (Vendruscolo et al., 2019). Potassium hydroxide in methanol or ethanol is the most common saponification agent, with concentrations ranging from 0.35 to 2.7 M (Albuquerque et al., 2016). The saponification reaction with alkaline reagent can be carried out at room temperature or under heating at temperatures that can reach close to 100°C; usually higher temperatures require shorter reaction times (Uddin et al., 2018; Menéndez-Carreño et al., 2016). Some studies, including those on microalgae, have performed saponification directly on the sample, without the need for extraction first as mentioned previously, reducing steps and analysis time (Fagundes et al., 2019a; Pereira et al., 2016).

After the saponification, the free forms of the esterified sterols are obtained; however, the acetal bond between the sterol hydroxyl group and the sugar cannot be hydrolyzed in alkaline conditions as in the case of glycosylated sterols (Yu et al., 2018). In addition, in lipid extractions prior to saponification, due to superior polarity the glycosylated sterols (steryl glycosides and acylated steryl glycosides) are not extracted (Feng et al., 2015). Thus, for determination of the glycosylated sterols, acid or enzymatic hydrolysis is recommended to cleave the glycosidic bond (Munger et al., 2015). Acid hydrolysis can degrade compounds and isomerize some sterols; enzymatic hydrolysis is recommended to obtain true concentrations of glycosylated sterols (Moreau et al., 2018). Munger et al. (2015) performed acidic hydrolysis with 6 M HCl at 85°C for 30 min. and enzymatic hydrolysis with commercial mixture of exo- and endoinulinases at 40°C for 18 h in different samples; the total glycosylated sterol values were at least 65% higher in the enzymatic hydrolysis.

Gas chromatography (GC) coupled with mass spectrometry is the most common technique for analysis of sterols and squalene (Yuan et al., 2017). Before GC analysis, derivatization reactions can be performed to obtain products with better chromatographic conditions—for example, improving volatility, sensitivity, selectivity, and thermal stability (Poojary and Passamonti, 2016). Acylating agents and silylants are used to derivatize sterols (Gachumi and El-Aneed, 2017). The acylation reaction consists of introducing an acyl group into an organic molecule with active hydrogen using reagents such as acyl halides, acid anhydrides, or reactive acyl derivatives such as acylated imidazoles (Segura et al., 1998). The acyl halides are highly reactive and during the reaction hydrogen acid is formed; a basic compound is then required for neutralization. Therefore, it is recommended to eliminate excess reagent in order not to damage the chromatographic system (Gachumi and El-Aneed, 2017).

The silylation reaction is the most prevalent among derivatization reactions. A second-order nucleophilic attack occurs (SN2), where active hydrogen of an acid, alcohol, thiol, amine,

amide, aldehyde, and enolizable ketone is replaced with a trimethylsilyl group (Miyagawa and Bamba, 2019). Several reagents are used as silylating agents, including trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA), *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), and *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA). The obtained products can be injected directly into the chromatographic system; no purification step is required as in the acylation (Schummer et al., 2009).

Gas chromatography with a flame ionization detector (GC-FID) is also widely used in the analysis of sterols, and is described in several protocols of the American Oil Chemists' Society (AOCS) (Alberici et al., 2016). As the FID is nondiscriminatory and the identification is performed by a reference retention time, misunderstandings and co-elution of compounds with similar affinity to the chromatographic column may occur. In these cases, it is possible to use columns with different phases or to confirm the identity by GC/MS through spectrum comparison of the substances, although this detector is less indicated for quantifications than the FID (Duong et al., 2016).

GC/MS may also not be robust enough in cases of complex samples with multiple sterols because these compounds and interfering may have the same ionic fragments. Thus, higher sensitivity and resolution can be obtained with comprehensive two-dimensional gas chromatography (GC × GC) (Xu et al., 2015). The GC × GC has the objective to submit chromatographic bands where there is co-elution of compounds for further separation, in a continuous and sequential mode (Tranchida et al., 2015). Two chromatographic columns with stationary phases of different polarities are used; they are separated by an interface called a modulator, so the column effluent of the first dimension enters the column of the second dimension (Muscalu and Górecki, 2018). As in GC, GC × GC can be used in different detectors, usually FID and MS for sterol analysis, and also flight-of-time mass spectrometry (ToF/MS) (Xu et al., 2014, 2015, 2018). The ToF/MS presents greater mass accuracy and higher acquisition rates compared to single quadrupole MS, so it is preferable in GC × GC, but it is expensive, which limits accessibility (Keppler et al., 2018). Although GC with FID and MS was the most used separation technique for quantification and identification of sterols and squalene, it has the following disadvantages: complex sample preparation, requirement of high temperatures, and destructive detection technique (Feng et al., 2015). Thus, some methods have been developed using high performance liquid chromatography (HPLC), which has the main advantage of analyzing these compounds in both their free and bound forms without the steps of hydrolysis and derivatization used in GC; only the steps of extraction, saponification, or direct saponification, as already discussed, are necessary. In general, the methods utilize normal-phase HPLC for determination of sterol classes, and reverse-phase HPLC for separation of free sterols and conjugated sterols analysis (Moreau et al., 2018).

Different detectors can be used for determination of squalene and sterols by HPLC. As these compounds exhibit absorption in the ultraviolet (UV) region, some works were carried out

using a UV detector or diode array detector (Feng et al., 2015; Yuan et al., 2017; Delgado-Zamarreno et al., 2016; Novak et al., 2018). Identifications using a UV and DAD detector are based on reference retention times obtained from commercial standards or by mass spectra using the HPLC-MS (Villegas et al., 2018). In HPLC with UV or DAD, there are limitations of sensitivity and also selectivity—for example, squalene and sterols absorb UV light in the 190–210 nm range, which allows absorption by other substances (Moreau et al., 2018). Different detectors based on mass spectrometry were used to detect and identify squalene and sterols in order to solve problems of sensitivity, selectivity, and particularly cases of co-elutions: tandem mass spectrometry (MS/MS) (Flakelar et al., 2017; Jauković et al., 2017; Pereira et al., 2016) and quadrupole time-of-flight mass spectrometry (QToF) (Millan et al., 2016).

Methods used more recently in sample preparation for analysis of squalene and sterols in microalgae were presented and discussed, as well as the analytical techniques used for their detection, separation, and identification. Table 21.1 lists the studies that carried out these analyses on microalgae samples.

21.8 Bioactivity of sterols

Bioactivity is defined by obtaining specific effects after exposure to a particular substance; these effects may include tissue uptake, metabolism, or physiological response (Karaś et al., 2017). The bioactivity can be assessed from methodologies in vivo (animal or human studies), ex vivo (gastrointestinal organs in laboratory conditions), and in vitro (simulated gastrointestinal digestion, artificial membranes, cell cultures, isolated and reconstituted cell membranes, using chambers). However, only in vivo assays can provide accurate bioactivity responses of a specific compound (Carbonell-Capella et al., 2014).

Just as sterols do, the precursor squalene has some human health benefits. Reduced cholesterol levels, protection against coronary heart disease, antioxidant properties, potential anticarcinogenic activity, and tumor reduction were effects that were observed in different sources of this compound (Chhikara et al., 2018). However, the literature has reported the great potential for sustainable production of squalene from microalgae, but the bioactivity of this compound obtained from the microalga *P. autumnale* was not evaluated (Fagundes et al., 2019a,b). Fernando et al. (2018) evaluated the bioactivity of squalene isolated from the alga *Caulerpa racemosa*, where it was possible to observe potent antioxidant activity and a wide range of antiinflammatory function in a cell assay.

Sterols are indicated as compounds with high bioactivity, and have already presented several effects in humans including antiinflammatory, antioxidant, anticancer, acting in immunomodulation to reduce the effects of neurological diseases like Parkinson's and Alzheimer's, antihypercholesterolemic, and antidiabetic (Luo et al., 2015; Khan et al., 2018;

Table 21.1 Recent studies analyzing squalene and/or sterols in microalgal biomass.

Strain	Compounds	Objective	Sample preparation	Tool	Ref
Nannochloropsis oceanica	SQU/STE	Characterization/ biosynthetic pathway determination	SE/SAP	GC/MS	Lu et al. (2014)
4 Microalgae strains	STE	Lipid extraction method/ characterization	SAP/DER	GC/FID-GC/MS	Ryckebosch et al. (2014a)
8 Microalgae strains	STE	Nutritional value	SE/SAP/DER	GC/FID-GC/MS	Ryckebosch et al. (2014b)
Schizochytrium sp.	SQU/STE	Characterization/ effect on cholesterol reduction	SE/SAP/DER	GC/MS	Chen et al. (2014)
5 Microalgae strains	SQU/STE	Characterization	SE/SAP	GC/FID-GC/MS	Yao et al. (2015)
Schizochytrium aggregatum	STE	Oxidative stability of microalgae oil/ bioaccessibility/ antioxidant ability of digested	SE/SAP	GC/MS	Lv et al. (2015)
17 Microalgae strains	STE	Characterization	SE/SAP/DER	GC/FID-GC/MS	Martin-Creuzburg and Merkel (2016)
Pavlova lutheri	STE	Effects of UVeC radiation and hydrogen peroxide	DSAP/DER	GC/MS	Ahmed and Schenk (2017)
Phormidium autumnale	SQU	Production	SE/DER	GC/FID-GC/MS	Fagundes et al. (2019b)
Phormidium autumnale	SQU/STE	Distinct sources of carbon	DSAP	GC/FID-GC/MS	Fagundes et al. (2019a)

SQA, squalene; *STE*, sterols; *SE*, solvent extraction; *SAP*, saponification; *DER*, derivatization; *DSAP*, direct saponification; *GC/MS*, gas chromatography mass spectrometry; *GC-FID*, gas chromatography with flame ionization detector; *Ref*, reference.

Moreau et al., 2018). Some studies have verified the activities of sterols extracted from microalgae; Chen et al. (2014) compared the effect of β-sitosterol only with a *Schizochytrium* sp. sterol extract (lathosterol, ergosterol, stigmasterol, 24-ethylcholesta-5,7,22-trienol, stigmasta-7,24(24[1])-dien-3β-ol, and cholesterol) and squalene, in the reduction of total cholesterol in hamster plasma. Although less effective than β-sitosterol alone, sterol extract and squalene at the 0.06 and 0.30 $g\,kg^{-1}$ diet doses reduced the total plasma cholesterol by 19.5% and 34%, respectively, when compared to the control.

The lipid fraction of the microalgae *Schizochytrium aggregatum* was analyzed and presented in its composition 42% of polyunsaturated fatty acids (PUFA) and the sterols: cholesterol, campesterol, 24-methylene cholesterol, 24-methyl-cholest-7-en-3b-ol, ergosterol, stigmasterol, and Δ7,24-stigmastadienol. After characterization, the bioactivity property of this microalga was evaluated by in vitro gastrointestinal simulation, and the results showed good bioaccessibility and moderate antioxidant activity (Lv et al., 2015). The bioactivity of a sterol-rich fraction extracted from the microalga *Nannochloropsis oculata* was also evaluated in macrophages and cancer cells. This fraction, containing about 64% of total sterols, was shown to be viable in the development of drugs for the treatment of diseases associated with inflammatory processes and also cancer of promyelocytic leukemia (Sanjeewa et al., 2016).

21.9 Industrial sterol production

In terms of the first bioactive compound obtained, squalene is known for being traditionally extracted from the liver of deep-sea sharks. This compound can represent up to 80% of the oil extracted from that organ. However, the growth and slow reproductive cycle of these animals, in addition overfishing for different purposes allied to the growing environmental concern and restrictive laws, have reduced this practice to obtain squalene (Zhuang and Chappell, 2015). Vegetable sources may be alternatives, and amaranth oil and olive oil present about 7% and 1%, respectively, of squalene in its composition; however, the slow development of these plants, seasonality, and edaphoclimatic dependence are restrictive factors. Thus, biotechnology routes attract attention; different strains of the *Aurantiochytrium* microalga present 3.3%–31.8% of squalene in their oil and strains of *Schizochytrium* 1.1%–8.4%. These values are associated with a high productivity of biomass and lipids (minimum of 30% in dry mass) (Aasen et al., 2016).

The main sources of total sterols (TE) for human consumption are plant species, as shown in Fig. 21.4, such as maize oil (0.81%–1.56%), germ oil (1.97%), and rice bran oil (3.22%) (Khan et al., 2018). However, as already mentioned, microalgae biotechnology presents advantages in the potential production of these compounds. Thus, the oil of *Isochrysis galbana* presented 0.37%, *S. aggregatum* 0.52%, *Schizochytrium* sp. 0.66%, *Phaeodactylum tricornutum* 1.13%, *Nannochloropsis* sp. 2.47%, and *Nannochloropis gaditana* 2.52% (Ryckebosch et al., 2014a, 2014b; Chen et al., 2014; Lv et al., 2015). It is estimated that the annual production of

microalgal oil can reach between 7000 and 23,000 L/ha, representing 60–200 times more production than high-performance plant species (Luo et al., 2015).

At the end of microalgae cultivations, harvesting techniques are based on solid-liquid separations, the most common being filtration, flotation, centrifugation, and, although slower, gravity sedimentation. After obtaining the biomass, processes of thermal drying or lyophilization are required (Grima et al., 2003). Thermal drying can be artificial (spray-drying, drum drying), where the drying temperature must be controlled so that the compounds of interest are not degraded, or natural, by using solar energy, but this process depends on the weather conditions and can be extended for days (Khanra et al., 2018).

The extraction of squalene and sterols can be facilitated with a prior cell disruption. Some procedures are performed for this purpose, such as freezing and thawing cycles, chemical disruption (acid hydrolysis), enzymatic treatment, high-pressure homogenization, and bead-milling. Extraction with organic solvents is widely used due to its high recovery efficiency of intracellular metabolites; chloroform, hexane, and petroleum ether may be cited. However, with the current tendency of not using these organic solvents due to their toxicity, it is worth highlighting the supercritical fluid, mainly with CO_2 supercritical (Grima et al., 2003). For obtaining these compounds, saponification processes are required; as already mentioned in the sterol analysis section, the process is performed with an alkaline alcohol solution (Albuquerque et al., 2016). If the goal is to obtain fine chemicals, in the case of purified sterols, subsequent chromatography steps are required (Khanra et al., 2018).

The squalene and sterols can be consumed from the ingestion of dried biomass. In this sense, the process of separation of the culture medium and drying are performed to obtain this product, and these steps represent the major cost of production. In the case of the strain *Schizochytrium*, these costs represent about 38% of the market value, for example. However, the cost of production can be higher if the target products are microalgae oil or even squalene and sterols isolated. In these cases, depending on the purity degree, the cost can reach 90% of the sale value (Jacob-Lopes et al., 2019). Despite the great productive potential, the cost of obtaining fine chemicals from microalgal cultivation is still considered high, but this can be overcome by increasing the production scale (Caporgno and Mathys, 2018).

21.10 Conclusion

In this chapter we discussed aspects related to sterol structure, and production by diverse microalgae (eukaryotic and prokaryotic), plus reports about the occurrence in diverse phyla and the strategies for their production. This chapter presented new attempts for microalgae exploring sterols and their derivate squalene as a potential high-value chemical to be employed in diverse industry areas.

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CHAPTER 22

"Bioplastics from microalgae"—Polyhydroxyalkanoate production by cyanobacteria

Martin Koller

Office of Research Management and Service, c/o Institute of Chemistry, University of Graz, Graz, Austria; ARENA - Association for Resource Efficient and Sustainable Technologies, Graz, Austria

Chapter outline

22.1 Introduction 598

22.2 Polyhydroxyalkanoate (PHA) biopolyesters as a potential remedy of today's plastic scenario 600

22.2.1 General aspects about PHA biopolyesters 600

22.2.2 Raw materials available for economic and sustainable PHA biopolyester production 603

22.3 Cyanobacteria as PHA-producing cell factories 606

22.3.1 General aspects of PHA biosynthesis by cyanobacteria 606

22.3.2 Enzymatic background of PHA biosynthesis by cyanobacteria 610

22.4 Factors affecting cyanobacterial PHA composition, production, and other storage compounds 613

22.4.1 Composition of PHA produced by cyanobacteria 613

22.4.2 Production and intracellular degradation conditions for PHA in cyanobacteria 614

22.4.3 Other storage compounds produced by cyanobacteria in parallel with PHA biosynthesis 616

22.5 Comparison of autotrophic and mixotrophic cultivation of cyanobacteria 618

22.5.1 *Spirulina* sp. under different carbon-supply regimes 618

22.5.2 *Nostoc* sp. under different carbon-supply regimes 619

22.5.3 *Anabaena* sp. under different carbon-supply regimes 620

22.5.4 *Synechocystis* sp. under different carbon-supply regimes 620

22.5.5 *Aulosira* sp. 622

22.6 Extremophilic cyanobacterial PHA producers 623

22.6.1 PHA production by thermophilic cyanobacteria 623

22.6.2 PHA production by halophilic cyanobacteria 624

22.6.3 Cyanobacteria cultivated at extreme pH conditions 625

22.7 Mixed microbial culture cultivations 625

22.8 Genetic engineering—A tool to boost PHA biosynthesis by cyanobacteria 627

Handbook of Microalgae-Based Processes and Products. https://doi.org/10.1016/B978-0-12-818536-0.00022-1

22.9 Production scales and production techniques for cyanobacterial PHA production 632
22.10 Conclusion 636
References 636

22.1 Introduction

It is undisputed that the microbiological group of "microalgae" carries out fundamental biological tasks in aquatic environments as outstanding converters of CO_2 to molecular oxygen, and as the lion's share of the phytoplankton, which constitutes the basis of the food chain (Koller et al., 2012; Koller et al., 2014). However, the expression "microalgae" refers to a phylogenetically highly heterogeneous group of phototrophic microbes; frequently, especially in older literature, the term "microalgae" also includes the group of cyanobacteria (formerly referred to as "Cyanophyceae"). This clearly contradicts the 1978 recommendation of the International Committee on Systematic Bacteriology (ICSB) to differentiate rigorously cyanobacteria, which are prokaryotic species belonging to the biological domain of Bacteria, from "microalgae" *sensu stricto*, which are scientifically grouped into the domain of Eukarya. Older expressions for cyanobacteria, such as "blue (green) algae" should for this reason also be banished from current and future scientific literature (Stanier et al., 1978).

Generally, cyanobacteria occur in almost all aquatic or terrestrial habitats. Millions of years ago, cyanobacteria fundamentally transformed Earth's previously highly reducing atmosphere into an oxidizing one by generating molecular oxygen. This enabled the evolution of novel life-forms adapted to these oxidizing conditions. These new life-forms were characterized by their innovative strategies to protect themselves against oxidative stress caused by molecular oxygen and its harmful follow-up products like oxygen radicals or peroxides. Most of all, new respiration strategies were developed by these new species, which are based on energy generation by the use of oxygen as final electron acceptor, an approach that is utilized today by humans (Thorpe et al., 2004). Moreover, cyanobacteria contain many molecular nitrogen fixing (diazotrophic) representatives acting as model organisms for aerobic and anaerobic nitrogen fixation; this reaction is catalyzed by the enzymatic nitrogenase complex located in heterocysts, which are specialized forms of cyanobacterial cells (Kumar et al., 2010). Hence, as key players in the natural nitrogen cycle, cyanobacteria also contribute to the generation of reduced nitrogen compounds, which in turn supply various other species with essential nitrogen sources.

Among the unimaginable number and variety of phototrophic species, cyanobacteria attracted scientific and industrial consideration because of the extraordinary diversity of marketable bioproducts synthesized by them, such as well-known pigments like carotenoids or chlorophylls, which are typically present in phototrophic organisms. Another group of photosynthetic chromophores, namely the phycobilins phycoerythrin and phycocyanin, are

typical products of cyanobacteria (Pagels et al., 2019). These secondary pigments are responsible for the characteristic intense coloration of cyanobacterial cultivation cultures, and display high market potential for food technology as colorants of candies, ice cream, bakery and milk products, or pasta, or as colorants for cosmetics (Soheili et al., 2013). Moreover, it is well-described that addition of cyanobacterial biomass enhances the fermentation performance of lactic acid bacteria as starting cultures for probiotic milk products (Fadaei et al., 2013; Beheshtipour et al., 2013; Mazinani et al., 2015), and displays positive effects when preparing bakery products (Massoud et al., 2016).

In the high-price segment, phycobilins are used as chemical tags in immunofluorescence techniques (Tomitani et al., 1999). In addition, γ-linolenic (GLA), an essential fatty acid pivotal for prostaglandin biosynthesis, is a well-known product of the cyanobacterium *Spirulina* sp. (also known as *Arthrospira* sp.) (Golmakani et al., 2012). In addition, innumerable bioactive compounds with a spectrum of antimicrobial activity ranging from antibacterial, antialgal, antifungal, and antiviral, to antiprotozoan (e.g., antimalarian) are products of the secondary metabolism of cyanobacteria, as reviewed in detail by Singh et al. (2005). Importantly, these biological effects are highly specific against well-defined groups of organisms, which makes these products attractive candidates for new antibiotics (Singh et al., 2017).

In this context, one should especially highlight the cyanobacterial genus *Lyngbya*, which encompasses species producing antileukemia compounds (Mynderse et al., 1977) and numerous bioactives with antiproliferative and antimitotic effects useful for cancer therapy (Gerwick et al., 1994). A review by Swain et al. (2015) made the scientific community familiar with a total of 144 potential anticancer bioactives produced by *Lyngbya* sp. This number of potential anticancer compounds is steadily growing, as shown by more recent studies (Alvariño et al., 2016; Ding et al., 2018). Regarding cancer therapy with cyanobacterial products, it should be emphasized that many among these compounds, such as Apratoxin A from *Lyngbya majuscula*, looked promising when testing cancer cell lines *in vitro*, but did not hold promise in *in vivo* tests (Luesch et al., 2001). In other cases, *in vitro* activity of *Lyngbya* sp. products like jahanyne, an acetylene-containing lipopeptide, was evidenced by growth inhibition of human cancer cells and induction of apoptosis, but without confirmation of *in vivo* activity (Iwasaki et al., 2015). Only recently, a biorefinery process was suggested by Chandra et al. (2019) to fractionate different UV-protectants (mycosporine-like amino acids and lipids) from *Lyngbya* biomass. A novel trend is the accumulation of gold nanoparticles by cyanobacteria, which might be used to treat doxorubicin-induced heart failure or myocardial infarction (Bakir et al., 2018).

On the other hand, cyanobacteria are also microbial factories for various toxic compounds. Carmichael (1992) reviewed the knowledge available about cytotoxins, hepatotoxins, and liver tumor promotor production by these organisms, and introduced the collective expression

"cyanotoxins" for these secondary metabolites. Some cyanobacteria are also reported to produce dangerous neurotoxins, such as *Lyngbya wollei*; this strain was reported by Onodera et al. (1997) to generate six previously unknown saxitoxin neurotoxins. These toxins enter the food chain via mollusks, which can lead to mussel poisoning in humans while eating them.

Only in recent years has the range of products accessible via cyanobacteria been expanded by microbial PHA biopolyesters, opening up completely new application perspectives for these organisms in the currently emerging field of "white biotechnology." Simply put, this approach describes solar-driven bioplastic production from CO_2 as an abundant carbon source. Fig. 22.1 provides a focused overview of diverse bioproducts generated by cyanobacteria described above.

22.2 Polyhydroxyalkanoate (PHA) biopolyesters as a potential remedy of today's plastic scenario

22.2.1 General aspects about PHA biopolyesters

Biopolymers with material features similar to the properties that make established polymers from petrochemistry so popular are nowadays heavily discussed as prospective alternatives. In fact, there is hope that such biomaterials will sooner or later supplant well-established petro-plastics from the polymer market. Among the most auspicious candidates, thermoplastic starch (TPS), poly(lactic acid) (PLA), or, as the materials of interest in this chapter, polyhydroxyalkanoate (PHA) polyesters, which are produced by microbes, are the topic of present-day R&D undertakings. In this context, particularly PHA, a group of polyesters of hydroxyalkanoic acids, which are biosynthesized by numerous Gram-negative and Gram-positive bacteria and a steadily growing number of extremophilic haloarchaea as intracellular inclusion bodies ("PHA granules," "carbonosomes"), are nowadays in the spotlight (Koller et al., 2017; Kourmentza et al., 2017). *In vivo*, these PHA granules occur in a stabilized amorphous, flexible state (Sedlacek et al., 2019a). The production of PHA biopolyesters under controlled conditions occurs in bioreactors of diverse geometries (stirred tank bioreactors, tubular bioreactors, airlift bioreactors, bioreactor cascades), and is based on different cultivation regimes (batch, fed-batch, continuous cultivation), as exhaustively reviewed in the recent literature (Blunt et al., 2018; Koller, 2018a; Raza et al., 2019). The interdisciplinary research in PHA biopolyesters concerns scientists from different disciplines such as microbiology, enzymology, polymer science, process engineering, and, to an increasing extent, synthetic biology, bioinformatics, and genetic engineering. Fig. 22.2 shows the general chemical structure of microbial PHA polyesters.

PHA are the only group of "green plastics" *sensu stricto*: PHA biosynthesis starts from renewable resources (PHA are "biobased"); they are products of the secondary metabolism of prokaryotes (PHA are "biosynthesized"); they are degraded by the action of living organisms or

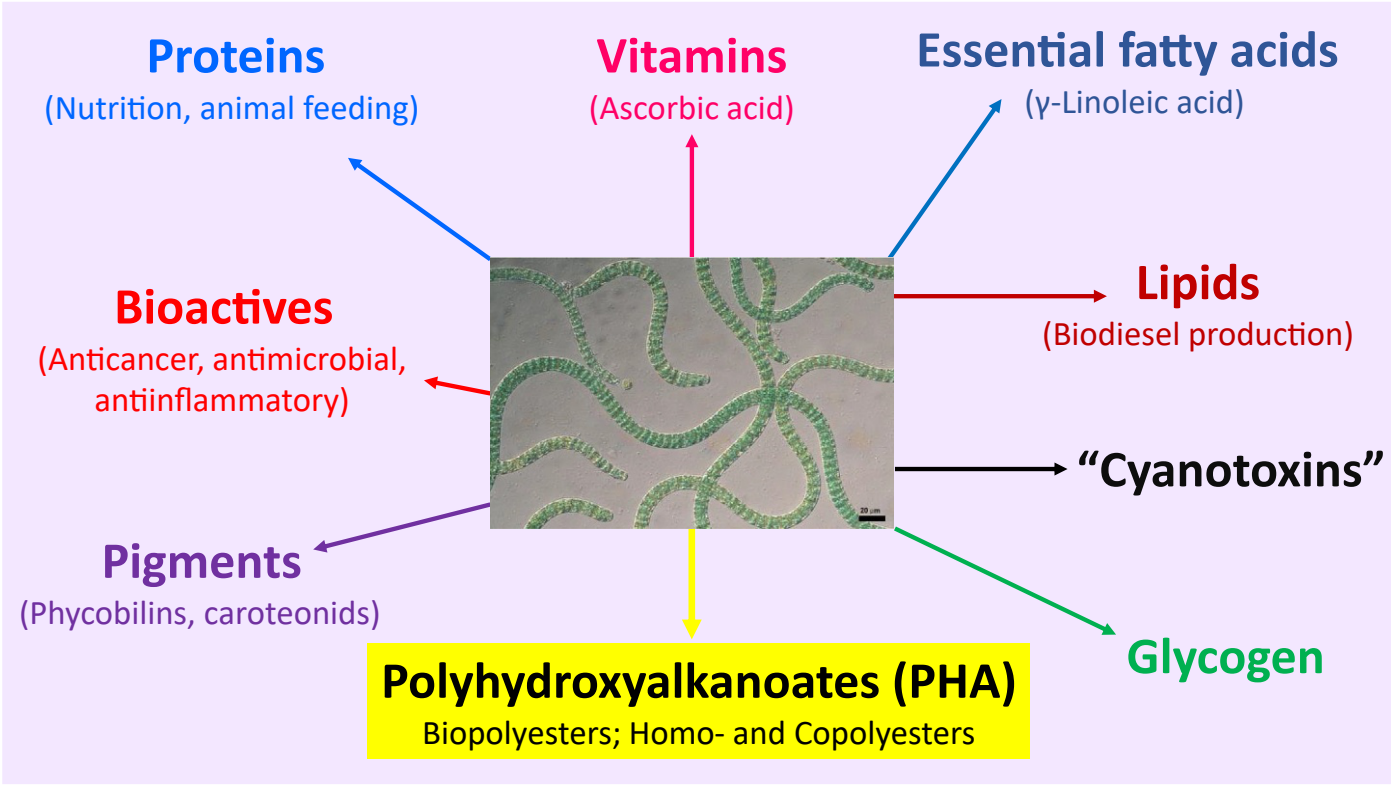


Fig. 22.1
Schematic illustration of important products biosynthesized by cyanobacteria.

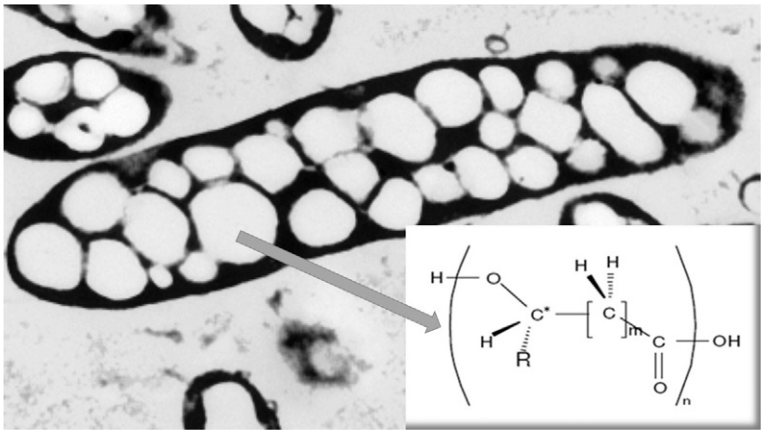


Fig. 22.2
Big picture: PHA-rich cells of *Cupriavidus necator*, magnification × 150,000. Bright PHA granules ("carbonosomes") clearly visible. Inserted picture: general formula of PHA biopolyesters. *R*, side chain of building block; *m*, backbone; *n*, degree of polymerization; *, chiral center of building block.

isolated enzymes (PHA are "biodegradable"); they undergo compositing according the respective standards definitions to water, CO_2, or CH_4, as the only products (PHA are "compostable"); and, finally, they do not exert any negative effects on living organisms or the environment (PHA are "biocompatible"). Because of their biobased nature, PHA production is independent on the availability of fossil resources. Their entire "cradle-to-grave" life cycle is part of nature's closed cycle of carbon, because their degradation does not raise the CO_2 level in the atmosphere, hence, it does not contribute to global warming and climate change, which is fundamentally different to the degradation of petro-plastics (Koller, 2019a).

In addition to their major role as storage compounds for carbon and energy, the protective function of PHA granules for cells containing them is currently in the process of elucidation (Obruca et al., 2018). In this context, the role of PHA to help living cells to withstand conditions of oxidative stress (Obruca et al., 2010), osmotic imbalance (Sedlacek et al., 2019b), repeated freezing/thawing cycles (Obruca et al., 2016a), exhaustive heavy metal loads (Chien et al., 2014), excessive UV-irradiation (Slaninova et al., 2018), or temperature up-shock (Obruca et al., 2016b) has been reported in relevant studies in the last few years.

What makes PHA so captivating for science and industry are their material properties, which are pre-definable during their biosynthesis, hence, *in statu nascendi* by the selection of the appropriate microbial production strain, adequate main carbon sources and co-substrates (precursors for specific PHA building blocks), and cultivation conditions like pH value, temperature, etc. Predominantly PHA's composition on the level of monomers is decisive for the material properties of a PHA sample. While the best-known example, the homopolyester

poly(3-hydroxybutyrate) (PHB), is highly crystalline and brittle, hence, of restricted applicability, *co*polyesters such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) or poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) display lower crystallinity, lower melting points, and a broader window of processability in molded state (Koller, 2018b). Generally, short-chain-length PHA (*scl*-PHA), consisting of hydroxyalkanoates with three to five carbon atoms, are distinguished from medium-chain-length PHA (*mcl*-PHA), which consists of hydroxyalkanoates with six to 14 carbon atoms. While *scl*-PHA, e.g., PHB and PHBHV, are typical thermoplastic materials (exception: the highly elastic homopolyester poly(4-hydroxybutyrate)), *mcl*-PHA are elastomeric resins with a low degree of crystallinity and low glass transition and melting temperature. Moreover, PHA are accessible to post-synthetic modification to fine-tune their properties (Raza et al., 2018). This paves the way for their application in several sectors of the current plastics market, encompassing the huge packing sector, the biomedical field, microelectronics, agriculture, bioremediation, and other applications (Koller et al., 2017; Kourmentza et al., 2017; Singh et al., 2019).

22.2.2 Raw materials available for economic and sustainable PHA biopolyester production

Regarding the biobased nature of PHA, renewable organic compounds such as different sugars (mainly mono- and disaccharides), polysaccharides (mainly starch), lipids (mainly triacylglycerides), alcohols (glycerol, for some organisms even methanol), or methane, which are compounds involved as substrates or products in the metabolism of living beings, are typical feedstocks to produce PHA and other biobased plastics. As will be shown later in this chapter, CO_2 is also a potential substrate for PHA production when resorting to cyanobacteria as production strains (Koller et al., 2017; Kourmentza et al., 2017). Fig. 22.3 illustrates the life cycle of PHA biopolyesters in comparison to the cycle of end-of-pipe full-carbon backbone plastics of petrochemical origin.

In order to make PHA production finally competitive, one has to take economic, ethical, and sustainability aspects into account. This is particularly true when choosing raw material to be used as carbon source for PHA production. It is well-known that many raw materials that have a nutritional value, such as sugars or edible oils, are particularly excellent substrates for PHA production processes due to fast microbial growth and high product formation rates achievable with them; numerous fermentation protocols were established for various substrate-production strain-combinations tested on laboratory or (semi)industrial scale. However, using resources of nutritional value to nourish PHA-producing bacteria is the topic of the current "plate-versus-plastics" debate, in analogy to the "plate-versus-tank" controversy, which criticizes the conversion of food resources to biofuels (Koller et al., 2017). Indeed, considering the enormous number of people starving on Earth, it can ethically not be acceptable, especially on a larger scale, to convert raw materials of nutritional value to bioplastics. Hence, solely the substitution of petro-plastics by biobased alternatives like PHA does not solve the plastic problem on a

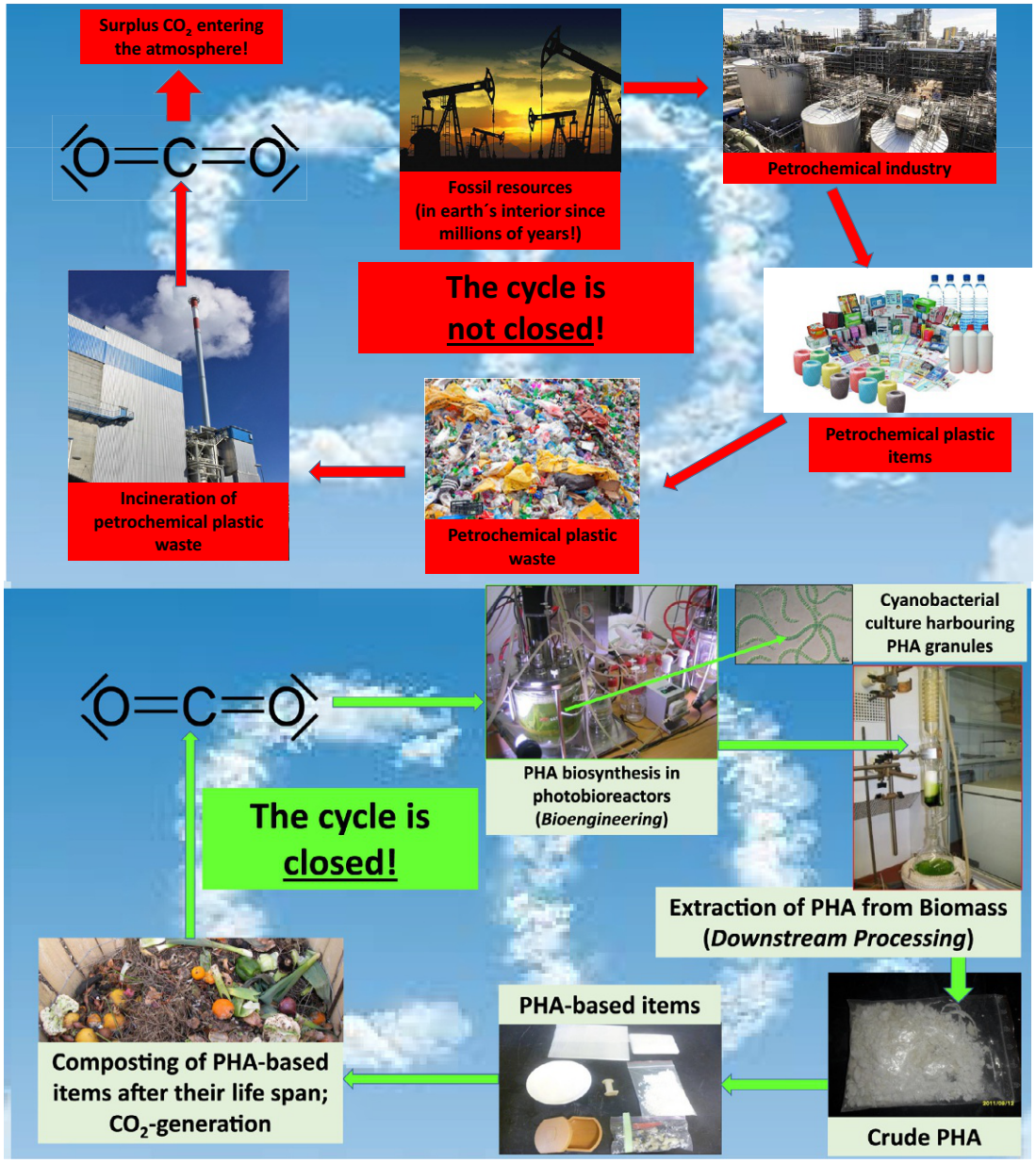


Fig. 22.3

Top: Life cycle of petrochemical plastics; CO_2 cycle is not closed. *Bottom*: Life cycle of PHA biopolyesters produced by cyanobacteria; CO_2 cycle is closed. *Based on Koller, M., 2019. Switching from petro-plastics to microbial polyhydroxyalkanoates (PHA): the biotechnological escape route of choice out of the plastic predicament? EuroBiotech J. 3 (1), 32–44; Koller, M., 2019. Linking food industry to "green plastics"–polyhydroxyalkanoate (PHA) biopolyesters from agro-industrial by-products for securing food safety. Appl. Food Biotechnol. 6 (1), 1–6; Koller, M., 2019. Polyhydroxyalkanoate biosynthesis at the edge of water activity-Haloarchaea as biopolyester factories. Bioengineering. 6 (2), 34.*

holistic basis; the entire process chain and the complete biopolymer life cycle from cradle to grave needs to be reviewed (Harding et al., 2007; Narodoslawsky et al., 2015). This means that a sustainable PHA production process needs to consider aspects of economics (cost-efficiency to compete with the low production prices of petro-plastics), the ecological footprint (e.g., energy source used to run the bioprocess), possibilities to optimize engineering (more efficient bioreactor facilities, enhanced downstream processing, etc.), and, last but not least, ethical perspectives (Koller et al., 2017).

One current strategy to solve the "plate-versus-plastics" dilemma is to link food and PHA production synergistically: huge quantities of carbon-rich waste streams currently produced in food and feed industry are available at zero costs (or often even cause costs for their disposal). These waste streams can be used as raw materials for biopolymer production (Brigham and Riedel, 2019; Koller, 2019b; Nielsen et al., 2017). To substantiate the viability and significance of this emerging approach, one should consider that in Europe, about 89 million tons of food waste is generated annually, the lion's share of this (about 80%) being attributed to the production process and the household sector (Brigham and Riedel, 2019). Replacing pure substrates (e.g., sugars) by such food- and agro-industrial waste has two major impacts: on the one hand, this strategy saves about half of the entire PHA production costs, which are heavily determined by the substrate costs; on the other hand, this approach enables food- and agro-industry to make their waste products something value-added, and, in parallel, to save disposal costs (Koller et al., 2017).

Different types of waste from agro- and food industry have already been tested as potential raw materials for PHA biosynthesis; however, most experiments were carried out only on a laboratory scale. These materials include hydrolyzed lignocelluloses (reviewed by Obruca et al., 2015) such as wheat straw (Cesário et al., 2014), rice straw (Ahn et al., 2016), bagasse (Lopes et al., 2014), corn stover digested by cellulases (Sawant et al., 2015), hydrolyzed (Davis et al., 2013) or saccharified grass (Kataria et al., 2018), or hydrolyzed wood (Kucera et al., 2017). Other substrates encompass molasses from the sugar industry (Akaraonye et al., 2012), steamed soybean wastewater (Hokamura et al., 2017), empty bunches of the palm fruit (Zhang et al., 2013), apple processing waste (Rebocho et al., 2019), waste glycerol as the main by-product of the biodiesel industry (Bhattacharya et al., 2016; Hermann-Krauss et al., 2013), low-quality saturated biodiesel fractions produced from animal lipids (Favaro et al., 2019; Koller and Braunegg, 2015; Koller et al., 2018), hydrolysate of reject paper fibers (Neelamegam et al., 2018), or waste streams from the paper production process (Weissgram et al., 2015).

In addition, the gastronomy sector generates waste cooking oil (Kourmentza et al., 2018; Kumar and Kim, 2019) or huge amounts of spent coffee grounds (Kovalcik et al., 2018a), which were also positively tested as raw materials for PHA production. Remarkably, even spent polyethylene, hence, a petrochemical plastic waste, was successfully converted to PHA after appropriate treatment (Johnston et al., 2017). In 200 L pilot-scale cultivation setups, surplus

whey from the dairy industry, which contains high loads of lactose, was successfully used for PHA production by *Haloferax mediterranei*, an extremophilic organism requiring excessive concentrations of sodium chloride (Koller et al., 2013), which, apart from cultivations using starchy raw materials by the same strain (Chen et al., 2006), up to now have been the only described large-scale PHA production processes based on carbon-rich waste streams. In the context of using complex mixtures of hydrolysis cocktails, especially of lignocellulosics, it is often required to remove generated inhibitors by adequate detoxification methods (Kovalcik et al., 2018b; Kucera et al., 2017).

In addition to all these discussed inexpensive heterotrophic carbon substrates, PHA production can also occur on an (photo)autotrophic basis, resorting to CO_2 as an amply available resource, which also accrues as a waste stream in the gaseous effluent of industrial plants, and by no means conflicts with the food and feed chain. Therefore, the subsequent sections of this chapter are dedicated to CO_2-based PHA biosynthesis using the most promising group of microbes to perform this task, namely cyanobacteria, while Fig. 22.4 illustrates the broad range of inexpensive substrates that can be used as carbon sources for PHA production by different prokaryotic species.

22.3 Cyanobacteria as PHA-producing cell factories

22.3.1 General aspects of PHA biosynthesis by cyanobacteria

As mentioned above, PHA production on a reasonable scale is reported merely for heterotrophic cultivation of well-known production strains such as the exhaustively described species *Cupriavidus necator*, *Azohydromonas lata*, *Pseudomonas* sp., *Aeromonas hydrophila*, *Burkholderia* sp., or *Hfx. mediterranei* (reviewed by Koller et al., 2017). In contrast, autotrophic and mixotrophic biosynthesis of PHA homo- and heteropolyesters using cyanobacteria is still at the stage of academic research. Nevertheless, such PHA production by cyanobacteria is currently studied and optimized by various scientific groups all over the world.These investigative activities include wild type as well as genetically modified (transformants) cyanobacteria. Available reviews on cyanobacterial PHA production focus on cultivation conditions and enzymatic and metabolic aspects (reviews by Drosg et al., 2015; Koller and Maršálek, 2015; Singh and Mallick, 2017), and, more recently, on aspects of process engineering (Costa et al., 2018; Costa et al., 2019; Kamravamanesh et al., 2018), design of photobioreactors for enhanced cultivation of these organisms (Koller et al., 2018), and genetic aspects (Katayama et al., 2018). Despite extensive research activities dedicated to genetics, metabolomics, and proteomics involved in PHA biosynthesis by cyanobacteria (Silvestrini et al., 2016), these processes are still awaiting industrial implementation. Considering the fact that PHA production by cyanobacteria links CO_2 removal with "bioplastic" generation, it is expected that this strategy will reach economic viability sooner or later. As a precondition, this

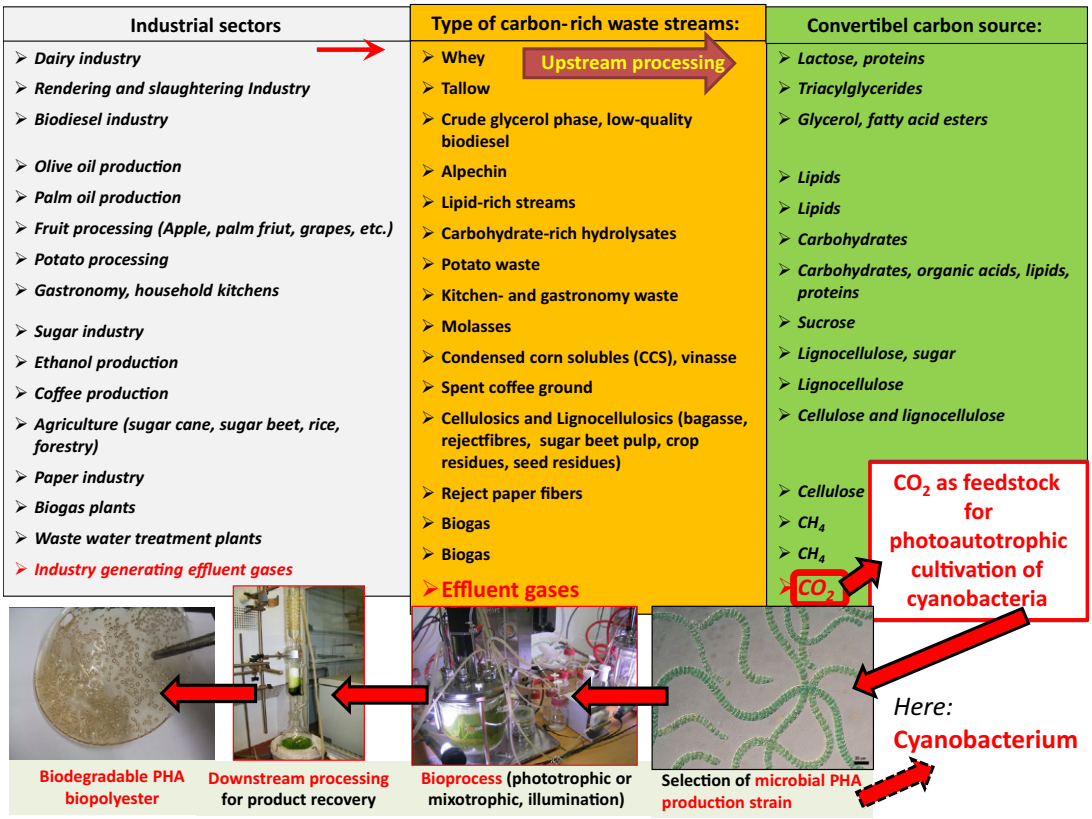


Fig. 22.4

Overview of inexpensive substrates available for PHA biopolyesters production; CO_2-rich effluent gases from industry, potential feedstocks for PHA production by cyanobacteria, are highlighted in *red* (dark gray in print version).

technology needs to be optimized for large-scale, high-throughput implementation (Kamravamanesh et al., 2018; Troschl et al., 2017).

For a long time, it was generally believed that cyanobacteria do not accumulate PHA biopolyesters at all, because these organisms possess a kind of "interrupted tricarboxylic acid cycle," where the two steps for conversion of 2-ketoglutarate via succinyl-CoA to succinic acid are replaced by the so-called "γ-aminobutyric acid shunt," which predominantly provides precursors for biosynthesis of pigments or amino acids (glutamate), while condensation of acetyl-CoA for PHA production seemed implausible in such an uncommon metabolic system (Xiong et al., 2014). In 1966, Carr published the very first report on PHA synthesis in the cyanobacterium *Chlorogloea fritschii*. This publication described the positive effect of supplying reduced carbon substrates, especially acetate, for PHA biosynthesis by this strain. Under purely autotrophic cultivation conditions, no PHA accumulation was observed in

C. fritschii. The polyester produced by this cyanobacterium was identified as PHB homopolyester, while other cyanobacteria like *Anabaena variabilis* investigated in this study did not display PHA accumulation at all (Carr, 1966). The importance of acetate as a pool of precursors for PHA production in cyanobacteria was later substantiated by Carr and Bradley, who studied the diazotrophic cyanobacterium *Anabaena cylindrica*. These authors supposed the occurrence of PHA granules in this organism predominantly in heterocysts, where nitrogenase activity takes place, which gave the first hints of the involvement of PHA in nitrogen fixation (Carr and Bradley, 1973).

Jensen and Sicko detected several inclusion bodies typical for prokaryotic cells using electron microscopic investigations of *C. fritschii*. Among these inclusions, the authors identified ribosomes, polyphosphate, and also somewhat electron-dense spherical granular inclusions with diameters of 100–800 nm, which were surrounded by a membrane of about 3 nm thickness. This first study of the fine structure of intracellular PHA carbonosomes in cyanobacteria showed high similarity to PHA granules produced by cyanobacteria and those produced by heterotrophic production strains (Jensen and Sicko, 1971).

Spirulina sp., which constitutes one of the most auspicious cyanobacterial candidates for production of a range of valued products like proteins or the pigments phycocyanin and phycoerythrin, was for the first time described as a PHA producer in 1982 by Campbell et al. PHB production under autotrophic conditions was detected by these authors in the strain *Spirulina platensis*. In this study, the maximum intracellular PHA fraction in biomass during exponential growth amounted to a rather modest 6 wt.-%. The product was isolated and characterized by electron microscopy, IR-spectroscopy, and characterization of its hydrolysis products. Despite the low productivity, this study constitutes the very first thorough report on pure autotrophic (no addition of any reduced carbon sources) PHA biosynthesis under elevated CO_2 concentration and constant light supply. It should be noted that Campbell et al. described PHA as an unwanted by-product of *Spirulina*, which reduces the nutritional value of its protein-rich biomass, the real target product of this study (Campbell et al., 1982).

In the meantime, PHA biosynthesis by cyanobacteria has been described for members of the orders Chroccocales (encompassing the genera *Chlorogleopsis*, *Gloeothece,* and *Spirulina*), Nostocales (genera *Nostoc*, *Aulosira*), Oscillatoriales (genus *Oscillatoria*), Pseudoanabenales (genus *Anabaena*), and Synechococcales (encompassing the genera *Synechococcos* and *Synechocystis*) (reviewed by Asada et al., 1999; Drosg et al., 2015; Koller and Maršálek, 2015; Costa et al., 2019). As an example, Bhati et al. (2010) studied 25 different cyanobacterial species out of 19 genera to assess their PHA biosynthesis potential under photoautrophic conditions; by analysis via UV-spectroscopy, 1H NMR spectroscopy, and GC-MS; 20 out of the 25 candidates indeed exposed PHA accumulation. Based on the five species, which did not show PHA accumulation, the authors concluded that PHA production by cyanobacteria might be a specific feature of individual species, but does not constitute a feature general of cyanobacteria in general.

From a techno-economic perspective, the use of cyanobacteria as oxygenic, photoautotrophic PHA production strains appears a viable approach to reduce costs on the one hand for carbon source (replacing organic substrates by CO_2) and, on the other hand, for oxygen supply; both are cost-determining factors in heterotrophic PHA production processes (Balaji et al., 2013; Samantaray et al., 2013; Sudesh et al., 2001). In this context, a study by Samantaray and Mallick (2012) estimated that the carbon requirement for PHA production by the cyanobacterium *Aulosira fertilissima* amounts to roughly 10% of the carbon requirement typically calculated for heterotrophic PHA-producing bacteria; hence, cost reduction in PHA production by cyanobacteria can be expected. For this reason, cyanobacteria are considered the group of phototrophic organisms that are most promising for light-driven PHA biopolyester production on a larger scale.

It should be underlined that only small amounts of PHA produced by cyanobacteria have been isolated up to now. Notably, as recently successfully demonstrated by Numata et al., it is not an insufficient activity of PHA biosynthesis enzymes, especially PHA synthases, in cyanobacteria, which hampers efficient PHA production by these organisms. This is in contrast to previous assumptions (Numata et al., 2015). It is rather the lack of photobioreactor (PBR) facilities specifically optimized for cyanobacterial cultivations, the understanding of the different intracellular ongoings under autotrophic, mixotrophic, and heterotrophic cultivation conditions still being in its infancy, insufficient engineering efforts to develop more sustainable and efficient downstreaming processes to recover PHA from cyanobacteria, and the as yet limited and incomplete knowledge about the biosynthetic background of cyanobacterial PHA production on the genome and proteome level impede the high-throughput autotrophic production of these biopolyesters.

Reported volumetric PHA productivity obtained by cyanobacteria are typically expressed as gram PHA per liter and day (g/(L d)), while PHA production with (semi)industrially used heterotrophic bacteria is generally expressed as gram per liter and hour (g/(L h)). In fact, it is remarkable that most reports of PHA production by cyanobacteria are restricted to the presentation of the final intracellular PHA fraction in biomass; detailed kinetic data for PHA accumulation and degradation, as usual elaborated for well-described heterotrophic PHA production process, are scarcely found in the present literature. Moreover, different to the exhaustively scrutinized heterotrophic PHA production strains, genetic engineering to improve PHA productivity in cyanobacteria played a minor role for a long time, and intensified efforts in this direction only started a few years ago (Singh and Mallick, 2017). At present, *Synechocystis* sp. PCC6803 or *Nostoc cycadae* are examples of the scarce number of cyanobacteria for which the entire genome has been completely deciphered. For *Synechocystis* sp. PCC6803, this encompasses the systematic revelation of the complete PHA biosynthesis pathways in cyanobacteria, which involves the key enzymes 3-ketothiolase, acetoacetyl-CoA reductase, and different PHA synthases encoded by the PHA biosynthesis genes. The subsequent expression of these enzymes has so far been reported for, e.g., *Synechocystis* sp. PCC6803 or *Chlorogloeopsis fritschii* PCC 6912 (Hein et al., 1998; Taroncher-Oldenburg et al., 2000).

To date, the best results for PHA production by wild type cyanobacteria were reported by Samantaray and Mallick (2012) for heterotrophic cultivation of the cyanobacterium *Aulosira fertilissima*, a diazotroph, when cultivated under alternating dark/light cycles in optimized cultivation medium; by this process, an intracellular mass fraction of up to 85 wt.-% PHA biomass was achieved. In addition, Bhati and Mallick (2015) reported the biosynthesis of PHB homopolyester and PHBHV copolyesters by the diazotrophic cyanobacterium *Nostoc muscorum* Agardh; for this process, a maximum mass fraction of 69 wt.-% PHA in biomass was reported after 7 days of cultivation, while PHA productivity and intracellular PHA fraction increased to 0.098 g/(L d) and 0.71 g PHA per g biomass, respectively, under phosphate limited conditions, and to 0.101 g/(L d) and 0.78 g PHA per g biomass, respectively, when nitrogen acted as the growth-limiting factor. Importantly, for both *A. fertilissima* and *N. muscorum* Agardh, the cultivations were carried out in simple illuminated shaking flasks without control of the process conditions, as would be possible in PBR cultivation setups. Hence, a lot of process development is still needed to achieve higher PHA productivity by cyanobacteria; the physiological potential of these organisms needs to be tapped by means of enhanced process engineering.

22.3.2 Enzymatic background of PHA biosynthesis by cyanobacteria

The enzymatic background of bacterial PHA biosynthesis is already well understood. In principle, carbon sources are converted to oxoacyl-CoA thioesters, in most cases to acetoacetyl-CoA, by the condensation of the central metabolite acetyl-CoA, catalyzed by 3-ketothiolase. These oxoacyl-CoA thioesters get reduced to (*R*)-hydroxyacyl-CoA thioesters, catalyzed by NADPH-dependent oxoacyl-CoA reductases; similar to ethanol generation from the metabolic intermediate acetaldehyde by yeast strains, this reduction regenerates reducing equivalents, which is why PHA biosynthesis is sometimes referred to as a "pseudofermentation" process (reviewed by Braunegg et al., 1998).

To understand better the enzymatic machinery catalyzing PHA biosynthesis in cyanobacteria, it is necessary to summarize briefly the current knowledge about the four classes of PHA synthases (polymerases), as reviewed by Chek et al. (2019) and Zou et al. (2017).

Class I PHA synthases (present in the Class I prototype PHA production strain *Cupriavidus necator*) are encoded by *phaC* genes, and of relatively high molecular mass of about 64 kDa. Such Class I PHA synthases catalyze the polymerization of the activated form of hydroxyalkanoates, namely hydroxyacyl-CoAs, with three to five carbon atoms. Hence, these synthases generate *scl*-PHA.

Class II PHA synthases (present, e.g., in the Class II prototype PHA production strain *Pseudomonas putida*) are also encoded by *phaC* genes, and of relatively high molecular mass of

about 63 kDa. Class II PHA synthases catalyze the polymerization of hydroxyacyl-CoAs with six to 14 carbon atoms, and generate *mcl*-PHA.

Class III PHA synthases (present, e.g., in the Class III prototype PHA production strain *Allochromatium vinosum*) are heteromeric enzymes. They consist of two subunits, each about 40 kDa of molecular mass, and are encoded by *phaC* and *phaE* genes. Similar to Class I synthases, Class III PHA synthases catalyze the polymerization of *scl*-hydroxyacyl-CoAs.

Class IV PHA synthases are a rather new group of PHA polymerizing enzymes; previously, they were considered an exceptional group of Class III synthases, found in spore-forming strains like *Bacillus megaterium* or *Bacillus cereus*. Importantly, Class IV PHA synthases are able to catalyze polymerization as well of *scl*- as of *mcl*-hydroxyacyl-CoAs, with a preference for *scl*-PHA building blocks.

The first report on a PHA synthase in cyanobacteria described PHA synthase activity determined in membrane fractions of the cyanobacterium *Spirulina* sp. MA19, which was cultivated in a nitrogen-limited medium to provoke PHA biosynthesis (Asada et al., 1999; Miyake et al., 1997). Remarkably, this PHA synthase revealed an outstandingly high temperature optimum of 65°C, which by far exceeds the temperature optimum of enzymes in most heterotrophic organisms (Miyake et al., 1997). In follow-up studies, the cyanobacterium *Synechocystis* sp. PCC6803 was cultivated on acetate, and accumulated about 0.1 g PHB homopolyester per gram of biomass. Genome analysis of this strain was carried out, and resulted in discovery of synthase genes that corresponded to an open reading frame (ORF) *slr1830* (labeled *phaC*) and to a collinear ORF *slr1829* upstream of *phaC* (labeled *phaE*). These *phaE* and *phaC* genes revealed high homology to the corresponding Class III PHA synthase genes subunits in non-oxygenic purple sulfur PHA-producing bacteria like *Chromatium vinosum*, *Thiocapsa pfennigii*, and *Thiocystis violacea*. These *Synechocystis* genes were expressed, either separately or together, in the bacterial hosts *Escherichia coli* and *Alcaligenes eutrophus* (today: *C. necator*). It was clearly shown that co-expression of both genes (*phaC* and *phaE*) is needed for PHA synthase activity (Hein et al., 1998). Later, it was demonstrated that the cyanobacterium *Synechocystis* sp. strain PCC6803 has a PHA-specific 3-ketothiolase (formerly known as β-ketothiolase), which is encoded by *phaASyn*, and an acetoacetyl-CoA reductase encoded by the gene *phaBSyn*. By similarity search of the complete genome sequence of this organism, it was shown that a cluster of two putative ORFs for these genes, namely *slr1993* (*phaASyn*) and *slr1994* (*phaBSyn*), which are collinear and co-expressed, is present in the genome of this cyanobacterium. Together with the PHA synthase from *Spirulina* sp. PCC6803, *phaASyn* and *phaBSyn* were transferred into the host organism *E. coli*; in a glucose medium, this transformed strain accumulated 0.123 g PHA per gram of biomass (Taroncher-Oldenburg et al., 2000).

Later, it was substantiated that occurrence of Class III PHA synthases is a widespread and typical feature in cyanobacteria. This was shown by Hai et al. (2001), who carried out molecular

characterization of PHA synthases from a total of 11 cyanobacteria. By Southern blot analysis with *phaC*-specific probes, sequence analysis of PCR products with *phaC*-specific oligonucleotide primers, cloning techniques, Western blot analysis using specific polyclonal anti-*PhaE* antibodies, and, finally, sequence analysis of the PHA synthase structural genes, the PHA production potential and the type of PHA synthases of these cyanobacteria were studied. Class III PHA synthases were detected in *Anabaena cylindrica* SAG 1403-2, *Chlorogloeopsis fritschii* PCC 6912, *Cyanothece* spp. PCC 7424, PCC 8303 and PCC 8801, *Gloeocapsa* sp. strain PCC 7428, and *Synechococcus* sp. strains MA19 and PCC 6715. In contrast, in *Cyanothece* sp. strain PCC 8955, *Gloeothece* sp. strain PCC 6501, and *Stanieria* sp. strain PCC 7437, Class III PHA synthase was not detected. Crude protein extracts and DNA of *Synechocystis* sp. strain PCC 6803 served as positive control (Hai et al., 2001).

More recently, specific activity PHA synthase obtained from *Synechocystis* sp. PCC 6803 was described in detail by Numata et al. (2015). In this study, both *phaC* and *phaE* were expressed together in a cell-free cultivation system. It turned out that the specific *in vitro* activity of the *phaCE* complex was in the same range as previously reported for Class I PHA synthases isolated from well-described heterotrophic PHA producers like *C. necator*, which clearly refutes earlier theories that the typically modest PHA productivity in cyanobacteria might be due to insufficient PHA synthase activity (Numata et al., 2015). Studying also *Synechococcus* sp. MA19, Miyake et al. (1997) further elucidated the enzymatic background of PHA biosynthesis by cyanobacteria. In crude extracts from cultures of this strain grown under parallel nitrogen deprivation and illumination, activity of PHA synthase was rather low; surprisingly, PHA synthase activity was determined solely in the membrane fractions from such cultures (Miyake et al., 1997). Later, it was shown that *Synechococcus* sp. MA19 PHA carbonosomes are linked to the photosynthetic pigments, which evidenced that PHA biosynthesis in such cyanobacteria occurs attached to the thylakoid membrane. PHB synthase activity in membrane fractions was increased further by adding acetyl phosphate to *Synechococcus* sp. MA19 cultures supplied with combined nitrogen. Moreover, the activity of the enzyme phosphotransacetylase, which catalyzes acetyl-CoA conversion to acetyl phosphate, was studied; it turned out that phosphotransacetylase activity occurred only in extracts from cells grown in nitrogen-free media (Asada et al., 1999). In this context, the enzyme acetyl phosphate possibly responds to the carbon-to-nitrogen balance, and triggers PHA metabolism in *Spirulina* sp. MA19. Consequently, decreasing phosphotransacetylase activity generates a high acetyl-CoA pool, and increases PHA fraction in biomass (Miyake et al., 1997).

Mallick et al. (2007) studied the impact of different compounds acting as inhibitors on PHA biosynthesis by *Nostoc muscorum*. Among the investigated compounds, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited the photosystem II in cultures of this strain grown photoautotrophically under phosphate deprivation; this resulted in suppression of NADPH synthesis, and thus reduced PHA accumulation. When supplying carbonylcyanide,

m-chlorophenylhydrazone, or dicyclohexylcarbodiimide, i.e., such compounds that uncouple phosphorylation and NAD(P)$^+$ reduction, PHA fraction in biomass was increased by 21% (carbonylcyanide m-chlorophenylhydrazone) and 17% (dicyclohexylcarbodiimide) from initially 8.5 wt.-% PHA in biomass. Supplementation of 2,4-dinitrophenol, which also uncouples phosphorylation and NAD(P) reduction, only impacted PHA accumulation insignificantly. When supplying specific inhibitors of glutamine synthetase and glutamate synthetase, namely L-methionine-DL-sulfoximine and azaserine, PHA biosynthesis was increased in cultures fixating molecular nitrogen and additionally supplied with NH_4^+. However, this was not the case when supplying the cultures with NO_3^- instead of NH_4^+. The addition of monofluoroacetate, a compound inhibiting the essential TCA enzyme aconitase, had a positive effect on PHB biosynthesis; this effect was diminished by adding α-ketoglutarate and DCMU. The addition of 2,3-butanedione, which specifically inhibits the enzyme phosphotransacetylase needed to activate PHA synthase, resulted in lower PHB accumulation both in heterotrophic phosphate or nitrogen limited cultures, and in autotrophic control cultures (Mallick et al., 2007).

Until 2015, only three enzymes playing a central role in PHB metabolism of cyanobacteria, specifically of the model strain *Synechocystis* sp. PCC, were described: PhaA, PhaB, and the heterodimeric PHB synthase PhaEC. Hauf et al. (2015) described for the first time cyanobacterial phasins (PhaP), which are PHA surface-coating proteins well-known for chemoheterotrophic PHA producers, mainly regulating the surface-to-volume ratio of PHB granules; in *Synechocystis* sp. PCC, these phasins are encoded by the *ssl2501* gene. By translational fusion of Ssl2501 with eGFP, a green fluorescent protein, the authors demonstrated that PhaP are closely associated with PHB granules. Knockout of *ssl2501* significantly reduced the number of PHA granules ("carbonosomes") per cell, while the average size of PHB granules increased, which is also analogous to typical phasins described for chemoheterotrophic PHA production strains. Although *ssl2501* knockout had almost no effect on PHB productivity, PHB synthase activity in these knockout mutants was drastically decreased (Hauf et al., 2015).

22.4 Factors affecting cyanobacterial PHA composition, production, and other storage compounds

22.4.1 Composition of PHA produced by cyanobacteria

Experiments by Stal et al. (1990) using *O. limosa* were also the first to report the production of a PHA by cyanobacteria that is not a simple PHB homopolyester, but a PHBHV copolyester. This is different to later studies by Stal carried out with *Gloeothece* sp. PCC 6909, a diazotrophic cyanobacterium, which turned out to produce the PHB homopolymer under the same cultivation conditions. The maximum PHA fraction in biomass amounted to 6 wt.-% and 9 wt.-%, respectively, for the two species (Stal, 1992).

Taepucharoen et al. (2017) cultivated the diazotropic, not cyst-forming cyanobacterium *Oscillatoria okeni* TISTR 8549 under photoautotrophic (permanent illumination with white light) and nitrogen-limited conditions for production of high-quality PHBHV copolyesters. Importantly, no structurally related 3HV precursor like propionate or valerate was added. Under these conditions, an intracellular PHBHV fraction of 14 wt.-%, with 5.5 mol-% 3HV in the copolyester, and a maximum PHBHV concentration of 0.103 g/L was reached. This study constitutes the first report of photoautotrophic PHBHV copolyester production. Nitrogen-limited cells grown in photoheterotrophic mode (acetate supply) under illumination did not show increased PHBHV accumulation (maximum PHBHV concentration 0.134 g/L, maximum PHBHV fractions in biomass 0.12 g/g, maximal 3HV content in PHBHV 3.3 mol-%), while intracellular PHBHV fraction increased under dark condition to 0.42 ± 0.08 g/g with 6.5 mol-% 3HV in the copolyester. Compared to typical data for PHB homopolyesters, the PHBHV copolyester produced by *O. okeni* displayed a melting temperature decreased by 5–7°C, an elongation at break increased by 4–7 times, and a Young's modulus increased by 2.3–2.5 times. These experiments demonstrated that the C2-compound acetate could be used as substrate for PHBHV biosynthesis by *O. okeni* especially in cultivations carried out in the dark, which evidences the presence of multiple biochemical pathways for supply with 3HV precursors (propionyl-CoA) for PHBV biosynthesis by cyanobacteria (Taepucharoen et al., 2017). Similar multiple pathways for PHBHV production from structurally unrelated substrates are well described for the haloarchaeon *Hfx. mediterranei* (Han et al., 2013; illustration of multiple pathways for PHBHV biosynthesis available in Koller, 2019c).

When supplied with short odd-numbered fatty acids (propionic acid, valeric acid) as 3HV-precursors to *A. cylindrica* 10C cultures, it was possible to produce the first tailor-made PHBHV copolyester by a cyanobacterium with detailed information about the monomeric composition (Lama et al., 1996). Similar to other PHBV production processes described in literature (Koller et al., 2008), addition of these precursors, especially of propionic acid, resulted in a considerable inhibition of microbial growth. The 3HB/3HV ratio in the produced copolyesters amounted to 7/3 or 2/3, respectively, independent of using either propionate or valerate as precursor, which demonstrates that copolyesters with high 3HV fractions can be produced by the cultivation of *A. cylindrica* supplied with the respective precursors. Further, the authors supplied the cells with ^{13}C-labeled acetate for metabolic studies; it was shown that the labeled carbonyl-carbon is selectively incorporated into the biopolyester (Lama et al., 1996).

22.4.2 Production and intracellular degradation conditions for PHA in cyanobacteria

Increased PHA biosynthesis under nutrient-limited conditions (nitrogen, phosphate, oxygen, sulfur, etc.), combined with excess carbon source availability, is typical for heterotrophic PHA production strains (Braunegg et al., 1998). The effect of nitrogen and sulfur limitation on

growth and PHA biosynthesis by the cyanobacterium *Synechococcus* sp. 6301 was first studied by Wanner et al. (1986), who used constant illumination, but limited CO_2 supply for their cultivation experiments. It was shown that sulfate limitation increased intracellular PHA fraction in biomass to about 10 wt.-%, while in nitrogen (nitrate) limited cultures, glycogen was accumulated as the predominant carbon-rich reserve material. Costa Coelho et al. (2015) investigated the cultivation of *Spirulina* sp. LEB 18 under different nutritional conditions. Growth of the strain was studied with and without inoculum adaptation and at varied concentrations of the carbon, nitrogen, and phosphate sources. Maximum biomass concentration (0.6 g/L), but a rather modest PHA fraction in biomass (13.4 wt.-%) was achieved when adapting the inoculum for 45 days and using 0.25 g/L sodium nitrate as nitrogen source, 4.4 g/L sodium hydrogen carbonate and 0.5 g/L potassium phosphate, while setups containing 0.05 g/L sodium nitrate, 8.4 g/L sodium hydrogen carbonate, and 0.5 g/L potassium phosphate achieved high intracellular PHA fraction (30.7%) at low biomass concentration (0.5 g/L). Inoculum adaptation increased biomass and PHA concentration by 7.0% and 20.5%, respectively.

Analogous to well-described non-phototrophic PHA-producing bacteria, PHA production by the cyanobacterium *A. fertilissima* was favored by nitrogen depletion and ample availability of carbon source. Sharma and Mallick (2005) reported similar results when studying PHA accumulation by the cyanobacterium *Nostoc muscorum*. Also in this case, cells revealed the highest PHA accumulation (0.086 wt.-% PHB in biomass) during the stationary growth phase, while during the lag phase or the exponential phase, only 0.041 wt.-% or 0.061 wt.-% PHB in biomass were achieved, respectively. Acidic pH values, permanent illumination, and addition of inorganic nitrogen sources (NH_4^+ or NO_3^-) decreased PHA biosynthesis, while phosphate limitation increased the intracellular PHA fraction.

Panda et al. (2005) studied these effects in more detail by cultivating *N. muscorum* and *Spirulina platensis* under phosphate-limited conditions; in these experiments, special emphasis was placed on the role of the intracellular phosphate pool and activity of the alkaline phosphatase enzyme, which indicates the bacterial phosphate status on PHA production by these cyanobacteria. PHA fraction in *N. muscorum*, after 4 days of phosphate limitation, increased to 22.7 wt.-%; at the same time, the intracellular phosphate pool was reduced by 0.02 µM phosphate per mg biomass, and the alkaline phosphatase reached its highest activity. *S. platensis* showed the highest phosphatase activity after 30 days of cultivation; however, this activity was only half of the enzyme's maximum activity in *N. muscorum*. Even after prolonged phosphate starvation, the PHA content in *S. platensis* was rather modest, with only 3.5 wt.-% PHA in biomass after 60 days of cultivation under phosphate-limited conditions; this demonstrates the lower impact of phosphate limitation for *S. platensis* than for *N. muscorum*. Supplying NADPH to phosphate-limited *S. platensis* cultures increased PHA biosynthesis in 10-, 20-, and 30-day-old cultures, which, on the one hand, substantiates the role of PHA synthesis in cyanobacteria in regeneration of reducing equivalents, and, on the other hand,

evidences that high intracellular phosphate pools result in insufficient intracellular NADH accumulation. However, in cells cultivated for 60 days under phosphate limitation, NADH supplementation did not increase PHA production, showing that the PHA biosynthesis system might not function fully after long-term phosphate limitation. In conclusion, phosphate deprivation supports PHA biosynthesis by cyanobacteria, but low intracellular phosphate pools are needed well before the enzymatic PHA biosynthesis machinery is impaired by long-term phosphate starvation (Panda et al., 2005).

Remobilization of PHA produced under nitrogen-limited conditions after re-feeding of nitrogen source was studied by Jau et al. It was shown that remobilization was dependent on both the light regime and the pH value of the cultivation medium. Under continuous illumination in 4-day cultivation setups, the PHA content in biomass was decreased by 80%, while only 40% of the storage material was degraded under dark conditions. Alkaline conditions (pH value above 10) favored both PHA accumulation and degradation (decrease of PHA by up to 90%). By electron microscopic observation of the PHA-rich cells, it was shown that the quantity and morphology of PHA granules changed during the degradation: granules became irregular, with less sharply demarcated shapes. Intracellular PHA degradation in the cyanobacterium *S. platensis* is faster than PHA biosynthesis, which is different to PHA anabolism and catabolism kinetics in most chemoheterotrophic microbes. According to the authors, this effect might be due to PHA biosynthesis in cyanobacteria being delayed by chlorosis, which evidences that factors impelling pigment synthesis, hence, the illumination regime, might also affect PHA biosynthesis (Jau et al., 2005).

22.4.3 Other storage compounds produced by cyanobacteria in parallel with PHA biosynthesis

In addition to PHA, glycogen and other polysaccharides are storage compounds frequently found in cyanobacteria; similar to PHA production, fixation of the required carbon starts with the Calvin cycle. Stal et al. (1990) investigated the different functions of these two reserve materials (glycogen and PHA) in the metabolic network of the marine cyanobacterium *Oscillatoria limosa*. These authors detected that the intracellular PHA fraction was growing during the late exponential phase of growth, and reached its maximum value during the stationary phase of growth. If transferred into carbon-limited fresh medium, *O. limosa* cultures rich in PHA rapidly started to degrade these PHA reserves under illumination, while in dark reaction, PHA degradation was not observed, whether with or without aeration. In contrast, polysaccharide (glycogen) reserves were degraded during the dark reaction in order to maintain the cell's metabolism (Stal et al., 1990). Further studies by Stal (1992) with dark reaction cultures of PHA-rich *Gloeothece* sp. PCC 6909 substantiated this finding; this strain also did not degrade its PHA reserves after stopping the light supply.

These results evidenced that PHA primarily acts as carbon reserve in cyanobacteria, while, for energy generation, cyanobacteria preferably degrade their glycogen storage. Further, PHA's role in regulating the intracellular redox state in phototrophic organisms like cyanobacteria was evidenced by the studies of De Philippis et al. (1992), who described the competition between nitrogenase and PHA synthesis enzymes for reducing equivalents in a photoheterotrophic purple non-sulfur bacterium; in this context, regeneration of the oxidized state of reduction equivalents by PHA biosynthesis is referred to as "pseudofermentation" due to its biochemical similarities to ethanol production by yeast as a response to oxygen limitation. The concomitant production of lipids, glycogen, and PHA in *Synechocystis* sp. PCC 6803 was studied by Monshupanee and Incharoensakdi (2014). Under photoautotrophic cultivation conditions, the sum of accumulation products peaked in the mid-stationary growth phase with about 0.4 g accumulation products (0.227 g glycogen, 0.141 g lipids, 0.024 g PHA) per gram of biomass. The mass fraction of accumulated products was increased to 0.615 g (0.368 g glycogen, 0.112 g lipids, 0.135 g PHA) per gram of biomass under nitrogen-limited conditions, which outperformed storage compounds production under phosphate-, sulfur-, iron-, or calcium-limited conditions investigated in parallel.

More recently, parallel intracellular production of carbohydrates and PHA was also reported for mixed microbial cultures enriched by cyanobacteria under different photoperiods and nutrient availability (Arias et al., 2018a), and under feast-and-famine feeding conditions (Arias et al., 2018b). For both storage compounds, sufficient carbon supply and parallel limitation of nitrogen (favoring PHA biosynthesis) or phosphate (favoring carbohydrate biosynthesis) were reported. In addition, Troschl et al. (2018) reported the cyclic nature of PHA and glycogen accumulation and utilization by *Synechocytis* sp. CCALA192 in pilot-scale experiments; as a new insight into the cyclic nature of storage compound metabolism in cyanobacteria, these authors emphasized the intracellular conversion of glycogen to PHA by older cells of this cyanobacterium.

De Philippis et al. (1992) studied *Spirulina maxima* under different cultivation conditions in order to shed further light on the function of PHA in the metabolism of cyanobacteria. In photoautotrophic batch cultures, grown either under balanced conditions or under nitrogen or phosphate deprivation, PHA fractions did not exceed 0.00005 wt.-% (50 µg PHA per g biomass). After long nitrogen starvation, the PHA fraction increased to 0.007 wt.-%, and 0.012 wt.-% after depletion of intra- and extracellular phosphate reserves; this was the first study reporting enhanced PHA production by cyanobacteria under phosphate-limited conditions. When suddenly increasing the photosynthesis activity by increasing illumination, or by shifting the cultivation temperature to 18°C, PHA biosynthesis was stopped completely. Under all these photoautotrophic growth conditions, considerably more glycogen than PHA was produced (De Philippis et al., 1992).

Production of an extracellular polysaccharide (EPS) was determined for *A. cylindrica* in parallel to PHA biosynthesis (Lama et al., 1996), which is analogous to reports for the bacteria

Azotobacter beijerininckii (Pal et al., 1999) and *Azotobacter vinelandii* (Brivonese and Sutherland, 1989), or the haloarchaeon *Hfx. mediterranei* (Koller et al., 2015; Parolis et al., 1996). Moreover, the characterization of an EPS produced by *Spirulina* strain LEB 18 revealed the presence of six different pentoses and hexoses plus pending sulfate groups (Martins et al., 2014).

22.5 Comparison of autotrophic and mixotrophic cultivation of cyanobacteria

22.5.1 Spirulina *sp. under different carbon-supply regimes*

As a follow-up to the early works of Carr (1966), who first reported the positive impact of reduced carbon compounds for PHA production by cyanobacteria, Vincenzini et al. (1990) cultivated different strains of the genus *Spirulina*, namely *Spirulina laxissima*, *Spirulina maxima*, and *Spirulina platensis* in alkaline mineral media under permanent illumination and supply of pure CO_2. Acetate or pyruvate were added to assess the impact of these compounds on PHA biosynthesis. Very low, yet detectable amounts of PHA were found in all investigated strains grown photoautotrophically; these show that PHA biosynthesis might be a universal feature of *Spirulina*, but not too important for supplying the cells with carbon. This situation changed massively when analyzing the same strains after 10 days of cultivation under co-supplementation of CO_2 and acetate; under these mixotrophic conditions, about 3 wt.-% of PHA in biomass was obtained, which is about the 10-fold amount of PHA stored in phototrophic control cultures, or in cultures supplied with CO_2 plus pyruvate (Vincenzini et al., 1990). These results clearly contradicted the reports of Campbell et al. (1982), where the supply of acetate did not positively affect PHA biosynthesis by *Spirulina* sp. cultures supplied with CO_2. Vincenzini et al. (1990) explained these outcomes by the high acetyl-CoA pool and restricted availability of free CoA in cultures supplied with acetate; the authors supposed that also for cyanobacteria, increased PHA biosynthesis could indicate unfavorable, unbalanced cultivation conditions. These results are in accordance with data published by Stal (1992), who described a beneficial role of acetate on PHA biosynthesis by *Gloeothece* sp. PCC 6909, while, in the same study, the PHA fraction in *O. limosa* did not increase when adding acetate.

Three different *S. platensis* spp. strains were studied in further experiments carried out by Jau et al. (2005). Under nitrogen-limited mixotrophic cultivation conditions, all three strains showed PHA accumulation, with the highest intracellular PHA contents amounting to about 10 wt.-%.

It should be stressed that PHA biosynthesis after addition of organic carbon sources is not increased in all cyanobacteria, and not even in all representatives of the genus *Spirulina*. In this context, Martins et al. studied autotrophic PHA biosynthesis by *Spirulina* sp. LEB 18 in mineral media with varied $NaNO_3$ and $NaHCO_3$ concentration, and under mixotrophic growth

conditions by partially replacing $NaHCO_3$ by glucose or acetate. The highest PHA content (44 wt.-%) was achieved by autotrophic cultivation of *Spirulina* sp. LEB 18 in a medium containing 0.25 g/L $NaNO_3$ and 8.4 g/L $NaHCO_3$ (Martins et al., 2014).

22.5.2 Nostoc *sp. under different carbon-supply regimes*

Sharma and Mallick (2005) also reported increased PHA biosynthesis by *N. muscorum* when supplying cultures of this strain with diverse organic carbon sources like acetate, sugars, or ethanol. The highest intracellular PHA content (35 wt.-%) was obtained when supplying *N. muscorum* cells cultivated for 7 days in the dark with 2 g/L acetate. Sharma et al. (2007) performed a five-level-four-factorial experimental design to study the interrelations of acetate, glucose, and K_2HPO_4 concentrations, and the period of dark reaction in more detail. Acetate and glucose addition, together with prolonged dark reaction periods, had a positive impact on PHA biosynthesis. The highest PHA content in biomass of 45.6 wt.-% was reached by a 95 h dark incubation period with 1.7 g/L acetate, 1.6 g/L glucose, and 0.005 g/L K_2HPO_4. Further, Bhati and Mallick (2012) revealed that, under photoautotrophic growth conditions, *N. muscorum* Agardh accumulated PHB homopolyester; when supplied with propionic or valeric acid as 3HV-related precursor compounds, the strain synthesized PHBHV copolyesters. Adding organic carbon compounds, such as acetate, fructose, or glucose, together with the 3HV precursors, considerably enhanced PHBHV copolyester biosynthesis; under dual substrate limitation (phosphate and nitrogen source) with 4 g/L acetate and 4 g/L valerate, intracellular PHBHV fractions in biomass increased to 60 wt.-%, which, at that time, was the highest PHBHV fraction ever determined in a wild type cyanobacterium. Later, the same group of authors further optimized these results via a multifactorial experimental design. At reduced substrate concentrations, even 69 wt.-% PHA in biomass was obtained after 7 days of incubation; moreover, PHA productivity increased to 0.098 g/(L d) (71 wt.-% PHA in biomass) under phosphate limited conditions, and to 0.101 g/(L d) (78 wt.-% PHA in biomass), when nitrogen acted as a growth-limiting factor (Bhati and Mallick, 2015).

Later, *Nostoc muscorum* Agardh was studied by Bhati and Mallick (2016) for PHA production under photoautotrophic conditions (10% CO_2 supply in the aeration stream) and after adding poultry waste as organic substrate. After 8 days of photoautotrophic incubation, biomass concentration reached 1.12 g/L, and the CO_2 fixation rate amounted to 0.263 g/(L d). Supplementation of poultry waste had a positive impact on the biomass yield, and nutrients were successfully removed from the poultry waste; under these mixotrophic conditions, a maximum PHBHV copolyester concentration of 0.774 g/L, corresponding to a PHBHV mass fraction in biomass of 65%, was reached, which is about the 11-fold mass fraction of that achieved in the photoautotrophic setups. This study demonstrates that the strain *N. muscorum* is a potential candidate for parallel CO_2 mitigation and poultry waste remediation, combined with production of value-added biopolyesters.

22.5.3 Anabaena *sp. under different carbon-supply regimes*

PHB biosynthesis by the diazotrophic cyanobacterium *Anabaena cylindrica* 10C was studied by Lama et al. under different growth conditions. Regarding PHA biosynthesis, *A. cylindrica* constitutes an exception among the genus *Anabaena*; in the case of *Anabaena variabilis*, *Anabaena toruloas*, or other *Anaebena* spp., PHA accumulation was not detected, under either autotrophic or heterotrophic cultivation conditions (Lama et al., 1996), which matches previous outcomes reported by Stal (1992). Without supplying combined nitrogen sources in autotrophic cultivations, the PHB fraction in biomass increased from only 0.00005 to 0.2 wt.-% after 3 weeks of cultivation, which might indicate again the rather modest role of PHA as energy reserve in cyanobacteria. Under heterotrophic cultivation conditions (addition of acetate), *A. cylindrica* biomass concentration significantly increased, and the intracellular PHA fraction was enhanced to 2 wt.-%; as soon as the organic carbon source acetate was depleted, the accumulated PHA was quickly degraded. Other organic carbon sources (e.g., glucose) showed a less significant increase in biomass and PHA formation; in the case of citrate, even less PHA was produced compared to autotrophic cultivation setups (Lama et al., 1996).

22.5.4 Synechocystis *sp. under different carbon-supply regimes*

PHB biosynthesis by *Synechocystis* sp. PCC 6803 under both nitrogen and phosphate limitation was studied by Panda and Mallick (2007) using stationary phase cultures of this strain; in addition, the impact of restricted gas exchange and supply of organic carbon sources was studied. All these factors resulted in increased accumulation of PHB. In particular, the synergistic effect of phosphate limitation and limited gas exchange, together with addition of organic carbon sources (fructose or acetate), resulted in the highest PHB content of up to 38 wt.-% in biomass; this constitutes an eightfold increase to the control cultivations grown under photoautotrophic conditions. Moreover, Monshupanee and Incharoensakdi (2014) cultivated *Spirulina* sp. PCC 6803 under nitrogen limitation, heterotrophic growth conditions by adding 4 g/L glucose, and optimized illumination intensity of 200 μE/(m^2 s). The synergistic effect of these factors resulted in increased accumulation of polysaccharides (41.3 wt.-% glycogen in biomass), lipids (16.7 wt.-%), and PHB (13.1 wt.-%), hence to a total mass fraction of storage compounds exceeding 71 wt.-%.

Mendhulkar and Shetye (2017) tested different experimental conditions like nitrogen and phosphate limitation and addition of exogenous carbon sources to study photoautotrophic and chemoheterotrophic growth of the cyanobacterium *Synechococcus elongatus*. It turned out that nitrogen-limited, phototrophic conditions resulted in the highest intracellular PHA fractions (17.15 wt.-%) when using sucrose (1%) as an exogenous carbon source. Also under chemotrophic conditions, the highest intracellular PHA fractions (13 wt.-%) were achieved when adding 1% sucrose to the cultivation medium. In phosphate-limited medium, maximum PHA fraction in biomass amounted to 7 wt.-% under phototrophic conditions when using

fructose (1%) as an exogenous carbon source; this is in a similar range to a mass fraction of 5.4 wt.-% PHA in biomass, which was reached when using a medium containing 1% glucose under chemotrophic conditions. In this study, it was shown that nitrogen limitation boosts intracellular PHA fractions in *S. elongates* compared to phosphate limitation under photoautotrophic conditions when supplying sucrose as an exogenous carbon source.

Shrimp wastewater, a resource rich in nutrients causing eutrophication like carbohydrates, nitrogen, and especially phosphate, was collected from the discharge water of a shrimp pond in Thailand, and used as a cultivation medium for the recombinant cyanobacterium *Synechocystis* sp. PCC 6803 strain ΔSphU by Krasaesueb et al. (2019). This organism lacked the phosphate regulator SphU, which enhanced its phosphate uptake performance. Cultivations were carried out for 14 days in a flat panel photobioreactor (19 L working volume) made of acrylic plastic under constant illumination by fluorescent lamps; aeration was accomplished by bubbling filtrated compressed air from a bottom tube. Temperature was maintained at ambient conditions (27–30°C), while the pH value was in the range of 7–9. Excellent nutrient removal rates of 96.99% (phosphate), 80.10% (nitrate), 67.90% (nitrite), and 98.07% (ammonium) from shrimp wastewater were reported. Nitrogen depletion caused by uptake of nitrate, nitrite, and ammonium ions provoked synthesis of PHA, which resulted in intracellular fractions of 32.5 wt.-% and maximum productivity of 12.73 mg/(L d).

Dutt and Srivastava (2018) studied the intracellular carbon flux in *Synechocystis* PCC 6803 cells under conditions of nitrogen deprivation with and without addition of labeled acetate (acetate-2-^{13}C), and investigated how photomixotrophic cultivation affects whether the PHA precursor acetyl-CoA originates from intracellular carbon recycling, or from the exogenous carbon source acetate. In addition to acetate, the inorganic carbon source $NaHCO_3$ was labeled by the isotope ^{13}C. It was shown that the pre-cultivation mode (photoautotrophic or photomixotrophic) significantly impacts glycogen concentration, but does not significantly effect PHA levels. Moreover, in cells pre-cultivated photoautotrophically, 26% of the carbon fixed after nitrogen deprivation fluxes to PHA accumulation; less carbon is directed toward PHA biosynthesis after addition of acetate at the resuspension phase or when cells are pre-cultivated photomixotrophically. When adding acetate at the onset of nitrogen limitation, intracellular PHA fractions were doubled; 44%–48% of the carbon from acetate was shifted to PHA biosynthesis, independent of the mode of pre-cultivation of cells. The experiments also showed that the intracellular carbon recycling is the major source of precursors for PHA synthesis (acetyl-CoA); in the absence of acetate, recycling contributes about 74%–87% of the carbon undergoing PHA biosynthesis, while acetate addition considerably reduces its contribution: in cultures pre-cultivated photoautotrophically and supplied with acetate under nitrogen limitation, the contribution of intracellular carbon decreases to about 34%. In a nutshell, these experiments evidence a common pool of metabolites being used for PHA biosynthesis, with primary contribution from the recycling of intracellular carbon in the absence of acetate.

Carpine et al. (2018) developed a kinetic dynamic model of autotrophic PHA biosynthesis by *Synechocystis* PCC 6803 using the biochemical networks simulator COPASI. The model assumed two types of cells, namely growing cells and PHA accumulating cells, to be present in the cultivation medium, and their nitrogen and phosphate internal quota. Dynamics of biomass growth and PHA biosynthesis were described via the microbial growth rate, the cell lysis rate, utilization rates for nitrate and phosphate, and the PHA biosynthesis rate. Model calibration, hence, calculation of kinetic parameters and yields, was accomplished by regression of results from experimental cultivations, which were performed in 0.8 L inclined bubble column photobioreactors under alternating light/dark cycles (16 h illumination, 8 h dark reaction) and an initial nitrogen source (nitrate) concentration between 0 and 1.5 g/L. Model validation was done by carrying out independent experimental runs. The developed model was of high predictive power regarding cell concentration, nitrogen and phosphate concentration, and intracellular PHA fraction. The impact of the selected parameters on cell growth and PHA biosynthesis was assessed by parameter sensitivity analysis. While dynamics of cellular growth were only insignificantly affected by a minor variation of maximum specific growth rate, of rate of transition to PHA-accumulating cells, and of maximum nitrate uptake rate, dynamics of PHA biosynthesis were predominantly sensitive to the variation of values of studied parameters. According to the authors, the new model may assist in developing and optimizing autotrophic PHA production processes (Carpine et al., 2018).

22.5.5 Aulosira *sp.*

High intracellular PHB fractions of 66 wt.-% in the diazotroph cyanobacterium *Aulosira fertilissima* were determined more recently by Samantaray and Mallick (2012), who cultivated this organism with 3 g/L acetate and 3 g/L citrate. Cultivating the strain with 5 g/L citrate under phosphate-limited conditions, followed by dark reaction of 5 days, resulted in 51 wt.-% PHB in biomass. When replacing citrate by 5 g/L acetate, the PHB fraction increased again to 77 wt.-%. Via response surface methodology, the process was further optimized. As a result, PHB fractions of up to 85 wt.-% were obtained with 2.6 g/L citrate, 2.8 g/L acetate, and 0.00558 g/L K_2HPO_4 after 5 days of cultivation, which is, as of 2019, the highest reported PHA content in a wild-type cyanobacterium. By subjecting *A. fertilissima* cultures pre-cultivated in a fructose-containing BG 11 medium to these optimized cultivation conditions, a PHB concentration of 1.59 g/L was reached, which is about 50 times higher than in autotrophic control setups. The same authors further studied *A. fertilissima* for PHBHV copolyester biosynthesis after supply of structurally related 3HV precursors (propionic or valeric acid). In a medium containing 5 g/L fructose plus 4 g/L valeric acid, a PHBHV copolyester fraction in biomass of 77 wt.-% and a volumetric productivity of 0.038 g/(L d) were achieved, while under phosphate-limited conditions, an almost threefold productivity (0.095 g/(L d)) was obtained. Microscopic surface analysis of the copolyester granules showed a regular and smooth surface, while the surface of PHB homopolyester granules of the same organism appeared irregular and porous. By X-ray

diffraction analysis, the semi-crystallinity of the copolyester was determined, which correlates to the semi-crystallinity of PHBHV samples produced by well-described chemoheterotrophic PHA production strains (Samantaray and Mallick, 2012).

22.6 Extremophilic cyanobacterial PHA producers

22.6.1 PHA production by thermophilic cyanobacteria

Microbial strains thriving under extreme environmental conditions are steadily gaining in importance as robust workhorses for numerous products of white biotechnology (Yin et al., 2014), *inter alia* for diverse extra- and intracellular biopolymers (Poli et al., 2011). Such extreme environmental conditions encompass high or low temperature (thermophiles and cryophiles, respectively), excessive salinity (halophiles), low and high pH values (acidophiles and alkaliphiles, respectively), extreme substrate concentrations (saccharophiles), or high contamination with pollutants like heavy metals (metallophiles). Cultivating such strains under these extreme conditions minimizes the need for sterility precautions normally indispensable for axenic cultivation processes, because the risk of microbial contamination can in many cases be neglected, which constitutes a considerable economic advantage of bioprocesses resorting to "extremophiles." The best-known examples are various haloarchaea from diverse genera, which operate best as PHA biopolyesters factories under salinities exceeding 5 M NaCl (Koller, 2019c), thermophilic PHA producers like *Chelatococcus daeguensis*, which operates best at 50°C (Cui et al., 2015), or, more recently, cryophilic PHA producers thriving best under extremely low temperatures like *Pseudomonas* sp. MPC_6, which performs as well at 4°C as at 30°C (Pacheco et al., 2019). During the last years, some studies have been published describing PHA production under extreme environmental conditions also by a range of cyanobacteria.

In the context of thermophilic PHA production strains, the cyanobacterium *Synechococcus* sp. MA19, isolated from a volcanic rock in Japan, accumulates high fractions of PHB under photoautotrophic cultivation conditions. As shown by Miyake et al. (1997), this organism thrives best at a high temperature of 50°C; under these temperature conditions, 21 wt.-% PHB were produced under nitrogen-limited photoautotrophic growth conditions in simple bottles aerated with 2% CO_2. Compared with the rather low PHA fractions in mesophilic cyanobacteria described before in this chapter (exception: *Aulosira fertilissima*), this can be regarded a significant step forward in PHA production using cyanobacteria; most of all, this study described for the first time a cyanobacterium as a potential candidate for PHA production on a larger scale. To reach optimum PHA productivity with this organism, CO_2 supply and the illumination regime had to be fine-tuned. Nitrogen-limited setups cultivated in the dark showed a further increase of the intracellular PH fraction (27 wt.-%), which is due to the degradation of the second storage compound, glycogen, under these conditions (Miyake et al., 1997), which again substantiates previous reports of Stal et al. (1990).

In contrast to glycogen degradation, the intracellular PHA concentration decreased only under conditions of illumination and addition of a combined nitrogen source. In addition, phosphate-limited cultivation conditions were tested to increase PHA production by thermophilic cyanobacteria; as shown by Nishioka et al. (2001), *Spirulina* sp. MA19 accumulates PHA at 50°C under autotrophic, phosphate-limited (intracellular phosphate levels (P_i) between 0.043 and 0.076 mmol) conditions. Using $Ca_3(PO_4)_2$ as an insoluble phosphate source, 260 h old cultures of this strain accumulated 55 wt.-% PHB in biomass, which corresponds to 2.4 g/L PHB and 4.4 g/L biomass; this is about twice the output obtained in cultivations sufficiently supplied with phosphate. When planning fed-batch cultivation processes with this strain aiming at enhanced PHA production, the authors underlined the importance of supplying phosphate at concentrations not exceeding a defined P_i level (Nishioka et al., 2001). Later, Nishioka et al. (2002) provided deeper insights into the effect of different illumination intensities and parallel phosphate deprivation on biomass biosynthesis by *Spirulina* sp. MA19 when cultivated at 50°C. It was shown that, in phosphate-limited cultures, PHB accumulation started already at the early cultivation stage under all tested illumination regimes, while no PHB biosynthesis was noticed in cultures sufficiently supplied with phosphate. Generally, higher illumination resulted in increased intracellular PHA content. Based on metabolic flux analysis, it was shown that biomass yield based on ATP synthesis amounts to 3.5 g biomass per mol ATP.

Only recently, the thermophile *Thermosynechococcus elongatus* E542, isolated from a hot spring, was studied by Liang et al. (2019) as a thermophilic photosynthetic cyanobacterial cell factory for parallel CO_2 utilization and biomass growth from simulated effluent gas. In a 0.6 L bubble column-type photobioreactor, this organism worked best at 55°C, and revealed expedient acceptance of high NO_x and SO_x levels present in the effluent and CO_2 concentrations in the aeration gas mix up to 15%. The entire genome of this strain was deciphered, which opened the door for further genetic manipulation of the organism. Unfortunately, the study does not report about the presence or absence of PHA inclusions in this organism.

22.6.2 PHA production by halophilic cyanobacteria

The marine cyanobacterium *Spirulina subsalsa*, an organism isolated from Indian coast samples, was investigated by Shrivastav et al. (2010). This organism turned out to produce more PHA at higher NaCl concentration, which is similar to findings for the chemoheterotrophic haloarchaeon *Hfx. mediterranei* (Koller, 2015; Lillo and Rodriguez-Valera, 1990). Properties (composition, mechanical properties, thermo-properties) of the PHA accumulated by *S. subsalsa* were characterized in-depth using DSC, FTIR, NMR, and, TGA; as a major result, this product also revealed polymer characteristics as described for PHA for other well-known PHA production strains (Shrivastav et al., 2010). As described before for the chemoheterotrophic bacterium *Halomonas* TD01, halophilic strains can conveniently be farmed in continuous cultivation processes, and even in unsterile, open cultivation systems. In

this context, *Halomonas* TD01 was cultivated in a two-stage open, unsterile continuous process for 2 weeks; the culture remained stable and free of microbial contamination. In the second stage of this process, where boosted PHA biosynthesis was favored by nitrogen limitation, the mass fraction of PHA in biomass amounted to 70 wt.-% (Tan et al., 2011). Such continuous cultivation setups are also required in the near future to enhance PHA productivity in extremophile cyanobacterial strains.

22.6.3 Cyanobacteria cultivated at extreme pH conditions

According to the literature, some members of the genus *Spirulina* grow optimally under extreme pH conditions. As an example, the pH optimum for growth and PHA accumulation of the strain *Spirulina platensis* was determined in the remarkably alkaline range between pH 9 and 11 (Jau et al., 2005; Panda et al., 2005; Ogbonda et al., 2007). In addition, such extreme pH optima offer advantages against competing microorganisms during the cultivation process, in analogy to extreme temperature and salinity optima; for this reason, open cultivation processes with *Spirulina* sp. have already been suggested in the literature. However, such "conserved" cultivation systems do not entirely warrant a contamination-free process during the whole cultivation period, in particular when running the process for extended periods. Moreover, emphasis should also be placed on the fact that open pond systems, which are frequently used for large-scale, outdoor open cultivation of microalgae, require occupation of large land areas; for many microalgae and cyanobacteria, cultivation up to high cell densities is frankly not possible in such simple systems due to the particular cultivation requirements of selected species (Mata et al., 2010). Fig. 22.5 collects the extremophilic cyanobacterial PHA producers.

22.7 Mixed microbial culture cultivations

Arias et al. (2018a) studied the effect of different photoperiods and conditions of nutrients' availability when cultivating a mixed culture of cyanobacteria isolated from wastewater in order to increase the intracellular PHA and polysaccharide accumulation. While PHA was studied as biobased plastic substitutes in this study, the polysaccharide fraction was of interest for further fermentative conversion into bioethanol. The first experimental setup was carried out under permanent illumination, while alternating light/dark cycles (12 h per cycle) were used in the second experimental run. Both nitrogen and phosphate limitation were studied under the two different illumination regimes. The highest PHB concentration (0.10 g/L) was achieved under phosphate-limited conditions and permanent illumination, whereas the most polysaccharide (0.84 g/L) was produced under nitrogen limitation and alternating light/dark cycles, which indicates the importance of nutrient-limited regimes to optimize production of storage compounds from such mixed microbial cultures.

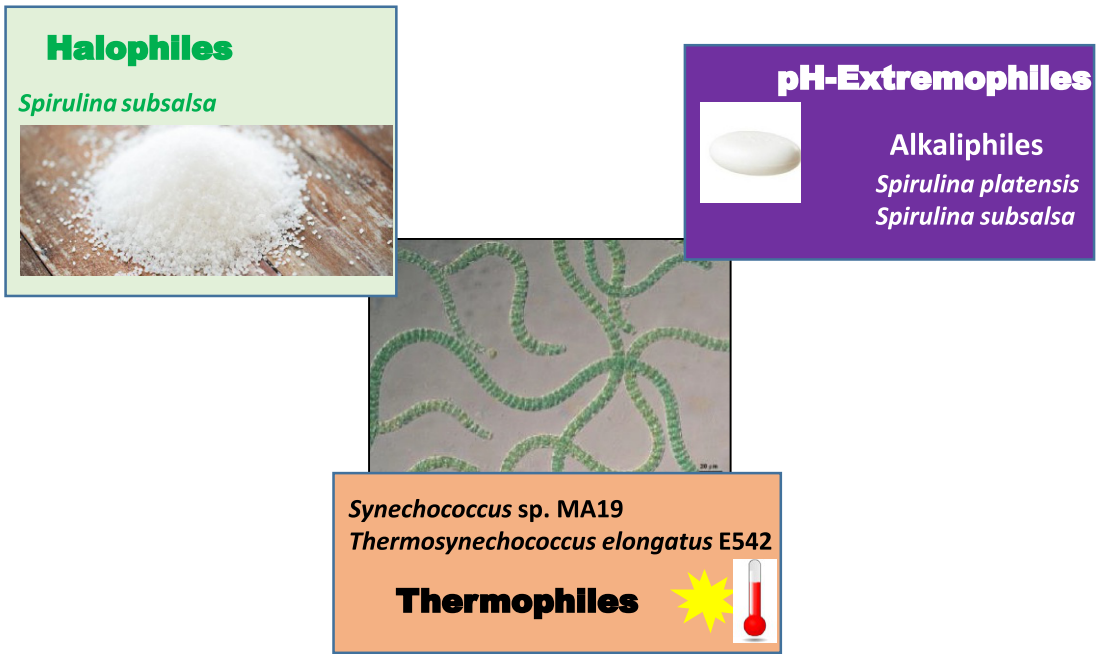


Fig. 22.5
Extremophilic cyanobacterial species as PHA producers.

The same group of authors (Arias et al., 2018b) used a sequencing batch reactor (SBR), operated in feast-and-famine mode, and different nutrients loads to enrich PHA-accumulating cyanobacteria from a mixed microbial culture taken from a wastewater body. The SBR was operated under aerobic conditions and alternating 12 h/12 h light/dark phases. The effect of carbon limitation, phosphate limitation, and carbon plus phosphate limitation was evaluated. After having reached steady-state conditions in each operational period, a portion of biomass was removed and used for separate batch tests (aerobic, 24 h/24 h dark/light cycles) to investigate the maximum accumulation levels of PHB and other storage compounds (polysaccharides). During the SBR operation, inorganic carbon source was predominantly used for cell growth and polysaccharide accumulation, while PHA fractions in biomass did not even reach 1 wt.-%. However, after complete nitrogen source depletion, almost 4 wt.-% PHA in biomass was produced. In addition, it turned out that phosphate limitation (with and without parallel limitation of carbon source) resulted in a culture more dominated by cyanobacteria and high carbohydrate content in biomass (43%–48%) than observed for carbon-limited cultures with high concentrations of nitrogen and phosphate sources (29%). The authors emphasized that optimized feeding and operating strategies can lead to enhanced nutrient removal from wastewater and concomitant biopolymer accumulation by mixed cultures enriched in cyanobacteria.

The filamentous nitrogen-fixing cyanobacterium *Nostoc muscorum* Agardh was studied by Bhati and Mallick (2016) for biomass and PHA production under photoautotrophic conditions. This cyanobacterium showed a profound rise in biomass yield, with up to 10% CO_2 supply in airstream with an aeration rate of 0.1 vvm. Maximum biomass yield of 1.12 g/L was recorded for an 8-day incubation period, corresponding to a CO_2-fixation rate of 0.263 g/(L d) at 10% (*v*/*v*) CO_2-enriched air. Poultry litter supplementation also had a positive impact on the biomass yield. The nutrient removal efficiency of *N. muscorum* was reflected in the significant reduction in nutrient load of poultry litter over the experimental period. A maximum PHBHV copolyester concentration of 0.774 g/L (65 wt.-% of biomass), a value almost 11-fold higher than the control, was recorded in poultry waste (10 g/L) supplemented cultures with 10% CO_2 supply under optimized conditions. This demonstrated that *N. muscorum* has expedient potential for CO_2 mitigation and poultry waste remediation while simultaneously producing ecologically benign biopolyesters.

22.8 Genetic engineering—A tool to boost PHA biosynthesis by cyanobacteria

It is a relatively easy task for molecular biologists and genetic engineers to subject unicellular phototrophic organisms like cyanobacteria or microalgae to genetic engineering if compared to microalgae, higher plants, or animals. This fact originates from the lack of cell differentiation in unicellular organisms. Indeed, genetic modifications of PHA-accumulating cyanobacteria were successfully performed and described in the case of members of the genera *Synechococcus*, *Synechocystis*, and *Thermosynechococcus*. Here, it should be emphasized that, in analogy to the reservations about higher genetically modified organisms, transgenic cyanobacteria pose potential risks if released into environmental systems. Therefore, such mutants should not be cultivated in open systems, in particular in those operated outdoors; their cultivation should exclusively be carried out in closed indoor systems under rigorous control, regulation, and absolute compliance with good laboratory practice principles (Pulz and Gross, 2004).

Synechocystis sp. PCC 6803 is one of the cyanobacteria most often genetically manipulated to increase its PHA production capability. This strain is exhaustively described regarding its growth requirements, metabolic pathways, and ways to transfer foreign genetic information into it. Moreover, it was the first photosynthetic organism with an entirely sequenced and deciphered genome, including the genes encoding the enzymes catalyzing PHA biosynthesis and degradation (Hein et al., 1998). To enhance PHA biosynthesis by this strain, Sudesh et al. (2002) transferred the *Ralstonia eutropha* PHA biosynthesis genes into *Synechocystis* sp. PCC 6803. The wild type and the transformed strain were cultivated for 15 days, 10 of which were in a BG11 medium, and 5 days were in a nitrogen-limited BG11 medium containing different organic carbon sources. Independent of the carbon source, it was found that *Synechocystis* sp. PCC 6803 (both the wild type and the transformant) can only produce PHB homopolyester,

even when supplied with 3HV, 3-hydroxypropionate (3HP), or 4-hydroxybutyrate (4HB) precursors. Moreover, higher PHA synthase activity was reported in the transformed strain than in the wild type. Surprisingly, this higher PHA synthase activity did not result in increased PHA biosynthesis; a similar PHA fraction in biomass was determined in both strains. Only when sodium acetate was applied as the carbon source did the recombinant *Synechocystis* sp. PCC 6803 strain show PHA biosynthesis superior to the wild type. Based on these findings, it was concluded that insufficient PHA synthase activity is not the rate-limiting step for PHA biosynthesis by cyanobacteria (Sudesh et al., 2002).

This conclusion was later substantiated by Lau et al. (2014), who transferred the genes encoding acetoacetyl-CoA synthase (*nphT7ss*) from *Streptomyces* sp. CL190, acetoacetyl-CoA reductase (*phaBcn*) from *C. necator*, and a highly active *Chromobacterium* sp. USM2 PHA synthase (*phaCcs*) into the cyanobacterium *Synechocystis* sp. PCC 6803. When cultivated for 7 days on modified BG-11 media supplied with 2–3 vol.-% CO_2, this recombinant organism, designated as C_{Cs}NphT7B_{Cn}, accumulated 14 wt.-% PHB in biomass, which is considerably more than obtained for the wild type strain (5 wt.-%), and among the highest values described for photosynthetic PHA production by *Synechocystis* sp. 6803. The highest PHA accumulation was obtained after 14 days of cultivation with supplementation of 0.4% (*w*/*v*) acetate under gas-exchange limiting conditions; here, the transformant C_{Cs}NphT7B_{Cn} accumulated 41 wt.-% PHA in biomass, while the wild type control strain reached a intracellular PHA fraction of only 21 wt.-%. This higher PHA biosynthesis potential of the transformant C_{Cs}NphT7B_{Cn} might be due to upregulation of the enzymes active in photosynthesis, e.g., ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), or enzymes needed for the electron transport chain. Better performance of these enzymes can generate more precursor molecules needed for PHA biosynthesis. Remarkably, lower expression levels of pivotal genes encoding for enzymes involved in PHA synthesis were also revealed by RNA-Seq analysis; hence, the regulation of these enzymes is not the determining factor to obtain high intracellular PHA fractions in cyanobacteria (Lau et al., 2014).

As shown by Osanai et al. (2013), influencing the sugar conversion constitutes a different strategy to increase the intracellular PHA content in *Synechocystis* sp. 6803. Since the catabolism of carbon sources is central for PHA biosynthesis, these authors overexpressed the sigma factor sigE, which is involved in activation of various genes expressing enzymes needed for sugar metabolism. To induce PHA accumulation, the sigE-overexpressing strain (GOX50) and the wild type control strain were cultivated under nitrogen-limited conditions, resembling those described before (Sudesh et al., 2002). In contrast to the study by Sudesh et al. (2002), who did not detect increased PHA fraction in their recombinant strain, GOX50 revealed a 2.3-fold higher intracellular PHA fraction (1.4 wt.-%) compared to the wild type. Hence, overexpression of the sigma factor sigE was considered an effective strategy to increase PHA biosynthesis, especially when compared to the cumbersome transfer of heterologous PHA biosynthesis genes, which resulted in PHB fractions in biomass not exceeding 1 wt.-%. In

analogy to the abovementioned study by Lau et al. (2014), PHA synthase levels in GOX50 were not increased, or were even decreased compared with the wild type strain (Sudesh et al., 2002).

In addition to overexpression and transfer of heterologous genes into *Synechocystis* sp. 6803, other approaches to enhance PHA biosynthesis in cyanobacteria were described. Among these approaches, the so-called "inverse metabolic engineering" was reported, which resorts to a global combinatorial strategy to identify those genetic loci responsible for a specific phenotype to be studied. Tyo et al. (2009) exploited this strategy by constructing a mutant library of *Synechocystis* PCC6803 via transposon insertion. This library screening identified a number of mutants able to produce high amounts of PHB; these mutants predominantly had disrupted *sll0461* and *sll0565* genes, which code for γ-glutamyl phosphate reductase (*proA*) and a hypothetical protein. After 2 weeks' cultivation in a BG11 medium, the Δ*sll0461* and Δ*sll0565* strains stored up to 8 wt.-% PHB in cell mass, which is significantly higher than the 3 wt.-% obtained with the wild type strain. Even higher intracellular PHA fractions (10 wt.-%) were obtained when supplementing the BG11 medium with acetate. These enhanced PHA fractions were predominantly detected at higher biomass concentration at the later stage of the cultivations, perhaps because of the nature of the disrupted genes. The gene *proA* plays a pivotal role in biosynthesis of proline, which, in turn, is described as an essential protecting osmolyte in higher plants. Disrupting the *proA* gene causes a stress response, which provokes higher PHA production, particularly at later stages of cultivation.

The strain *Synechocystis* PCC6803 also constitutes a promising candidate for the production of chiral (*S*)- and (*R*)-3HB monomers, which act as precursors for PHA and for various other fine chemicals with chiral centers. To design an effective (*S*)- and (*R*)-3HB production strain, Wang et al. (2013) removed *phaA2* and *phaB2* genes from this strain, and replaced them with *R. eutropha phaA* and *phaB* genes due to their higher efficiency in converting acetyl-CoA to the intermediate compounds (*R*)- or (*S*)-3-hydroxybutyryl-CoA. Further, the gene encoding thioesterase II (*tesB*) from *E. coli* was transferred into this organism, and the *phaE-phaC* operon was deleted from the *Synechocystis* genome. All three new genes were expressed assisted by the powerful promoter *Ptac*. This mutant strain produced considerably higher 3HB quantities using inorganic carbon sources (CO_2 or $NaHCO_3$) than all other strains investigated in this study; applying $NaHCO_3$ as the sole carbon source, 93.9 mg/L 3HB were reached after a cultivation period of 5 days, when using atmospheric CO_2 as the only carbon substrate, up to 533.4 mg/L 3HB were obtained within 21 days (Wang et al., 2013).

As a follow-up to the studies described above aiming at genetic engineering for PHA overproduction by *Synechocystis* sp. PCC6803, which were mainly based on increasing the expression of enzymes directly involved in its biosynthesis metabolism (encoded by *phaA*, *phaB*, *phaC*, and *phaE*), Carpine et al. (2017) engineered the central carbon metabolism of this cyanobacterium in a way to boost the PHA synthesis pathway on the one hand, and, on the other, to increase the intracellular level of acetyl-CoA, the central metabolite of PHB biosynthesis. Seven different genetic modifications of the metabolic network of this

cyanobacterium were tested, among them deletion of acetyl-CoA hydrolase (Ach) and phosphotransacetylase (Pta) (these enzymes cause loss of acetyl-CoA due to conversion to acetate and acetyl-P, respectively), and insertion of a gene encoding the expression of a *Bifidobacterium breve* phosphoketolase (XfpK), which should increase the acetyl-CoA levels. Biomass growth and PHA biosynthesis performance during photoautotrophic growth were compared between the wild type strain and the mutants in photobioreactor (inclined bubble column photobioreactor with 0.8 L working volume) experiments under alternating dark/light cycles; illumination was accomplished via fluorescence lamps. It turned out that the strain with all three genetic modifications, namely VfpK overexpression and deletion of both Pta- and Ach-encoding genes, displayed the highest PHA biosynthesis potential, with PHA concentrations of 0.232 g/L, intracellular PHA fractions in biomass of about 12 wt.-%, and a volumetric productivity of 7.3 mg/(L d), which are among the top values for CO_2-based PHA biosynthesis (Carpine et al., 2017).

Another approach to modify the central metabolic pathway of *Synechocystis* sp. PCC6803 was recently described by Monshupanee et al. (2019), who designed mutants with a deleted γ-aminobutyric acid shunt by deleting the gene *gdc*, which encodes the enzyme glutamate decarboxylase. This deletion left over an incomplete tricarboxylic acid cycle, as evidenced by the reduced activity of the central tricarboxylic acid metabolites succinate, malate, and citrate. Compared with the wild type strain, intracellular concentrations of lipids and glycogen remained in a similar range, while the concentrations of pyruvate and PHB increased by 1.23- and 2.50-fold, respectively. Under photoautotrophic conditions, PHB productivity was doubled in the *Δgdc* mutant if compared to the wild type.

A restricted number of cyanobacteria different to *Synechocystis* PCC6803 were successfully modified for enhanced PHA biosynthesis, one of them being *Synechococcus* sp. PCC7002. This organism is remarkably resistant to excessive irradiation, and exhibits fast growth kinetics (Ludwig and Bryant, 2012). Akiyama et al. (2011) developed an antibiotic-free plasmid expression system, which is considerably more stable than regular plasmids, and transferred it into *Synechococcus* sp. PCC7002. Plasmid stability was conserved by the gene *recA* located on the plasmid; this gene complements the lethal "*recA*-null mutation." In this context, the protein RecA is essential for bacterial DNA recombination and repair. In these setups, *recA* complementation acted as selective pressure to retain the plasmid inside the *recA* null mutant cells. Together with *R. eutropha pha* genes, the transformant stored up to 52 wt.-% PHA in biomass under nitrogen limitation; the produced polymer had similar average molar mass (about 0.5–1 MDa) to that reported for *R. eutropha*. A fraction of 52 wt.-% PHA in biomass is among the top PHA fractions ever reported for cyanobacteria, and considerably higher than first attempts to genetically modify this strain by insertion of *R. eutropha* PHA synthesis genes, where PHA content did not exceed 17 wt.-% (Miyasaka et al., 1998). Surprisingly, in the study by Akiyama et al., the authors noticed that the PHA consisted of 98 mol-% 3HB units, the rest being 3HV and lactic acid (Akiyama et al., 2011).

Further, Suzuki et al. studied the PHA biosynthesis potential of strain *Synechococcus* sp. PCC7942 (Suzuki et al., 1996). Since this strain is not an innate PHA producer, genes from *R. eutropha* encoding the entire PHB synthesis pathway (3-ketothiolase, acetoacetyl-CoA reductase, PHA synthase) were introduced into the cyanobacterium. Under photoautotrophic growth and nitrogen limitation, the first obtained transformant strain accumulated only 1 wt.-% PHB in biomass. When a stronger promoter was added, and acetate was supplied as an organic carbon source, more than 25 wt.-% PHA was obtained (Takahashi et al., 1998). This productivity was similar to results obtained using *Synechococcus* MA19, an innate PHA production strain, under photoautotrophic, nitrogen-limited growth. Although PHB homopolyester was produced by both strains, differences in distributions of PHB granules in the cytoplasm (size of granules, number of granules per cell) and regulative variations of PHB biosynthesis were reported (Asada et al., 1999).

By using the *psbA2* promoter, Khetkorn et al. (2016) constructed *Synechocystis* sp. PCC 6803 strains overexpressing *pha* genes (*phaA*, *phaB*, *phaC*, *phaE*) needed for PHA biosynthesis. These *pha*-overexpressing constructs displayed slightly lower growth rates than the wild type organism. Under nitrogen-limited conditions, those strains overexpressing *phaAB*, *phaEC*, and *phaABEC* showed considerably higher intracellular PHA fractions compared with the wild type. In *phaAB*-overexpressing cultures grown for 9 days under nitrogen-limited conditions, a maximum intracellular PHB fraction of 0.26 g/g was obtained, which constitutes an almost threefold increase compared with the wild type. When supplied with 0.4 wt.-% acetate under the same conditions, PHA accumulation in these genetically engineered cells increased further to 0.35 g/g. In this study, the wild type strain also displayed higher PHA fractions (13 vs. 10 mol-%) when grown mixotrophically with acetate. As a major outcome, the study showed enhanced PHA biosynthesis in *pha*-overexpressing *Synechocystis* sp. PCC 6803, especially *phaA* and *phaB*, when cultivated under nitrogen-limited conditions in a medium containing 0.4% wt.-% acetate (Khetkorn et al., 2016). Fig. 22.6 summarizes the genetic engineering efforts described in this text to improve PHA biosynthesis by cyanobacteria.

In addition to recombinant cyanobacteria, Hempel et al. (2011) modified a eukaryotic microalga, the diatom *Phaeodactylum tricornutum*, to make this innate non-PHA producer accumulate PHA. As a background, such microalgae often grow at high growth rates, convert CO_2 and sunlight into valued bioproducts, are rather easy to handle, and have minimal nutritional requirements (Koller et al., 2014). In Hempel et al.'s (2011) study, *R. eutropha* H16 PHA synthesis genes were transferred into *P. tricornutum*; expression of these genes was controlled by a nitrate-inducible promoter. After 7 days' cultivation in the presence of nitrate as a nitrogen source, the genetically modified *P. tricornutum* strain accumulated up to 10.6 wt.-% PHB in biomass; these results, while not too overwhelming at first glance, have to be assessed by considering that no enzyme engineering, strain adaptation, or large-scale screening was carried out prior to the experiments. This evidences the high potential of using eukaryotic microalgae as new sunlight-driven autotrophic factories for biopolyesters as a completely new

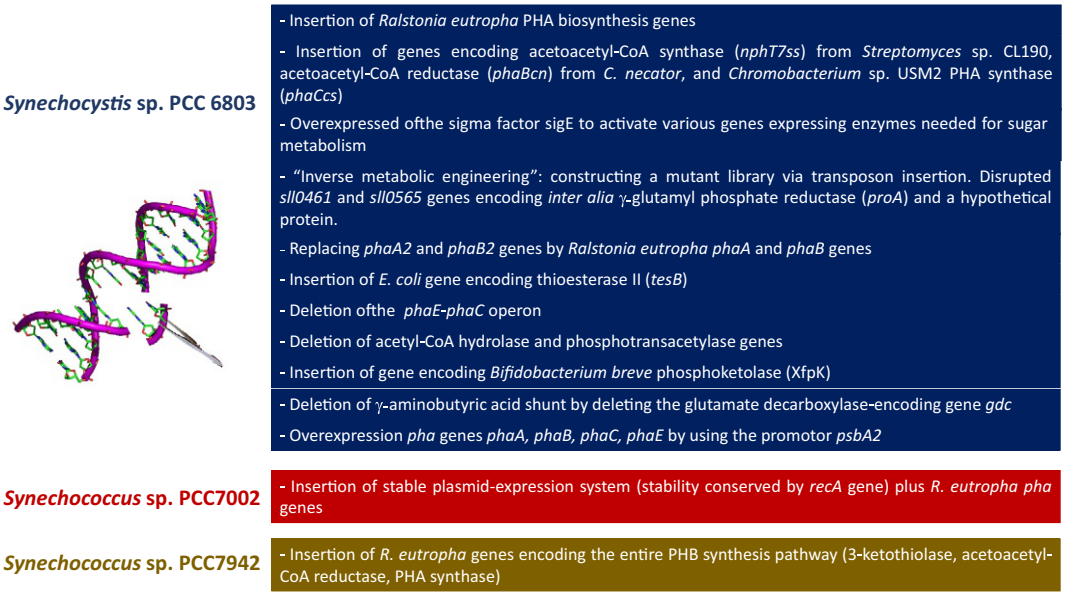


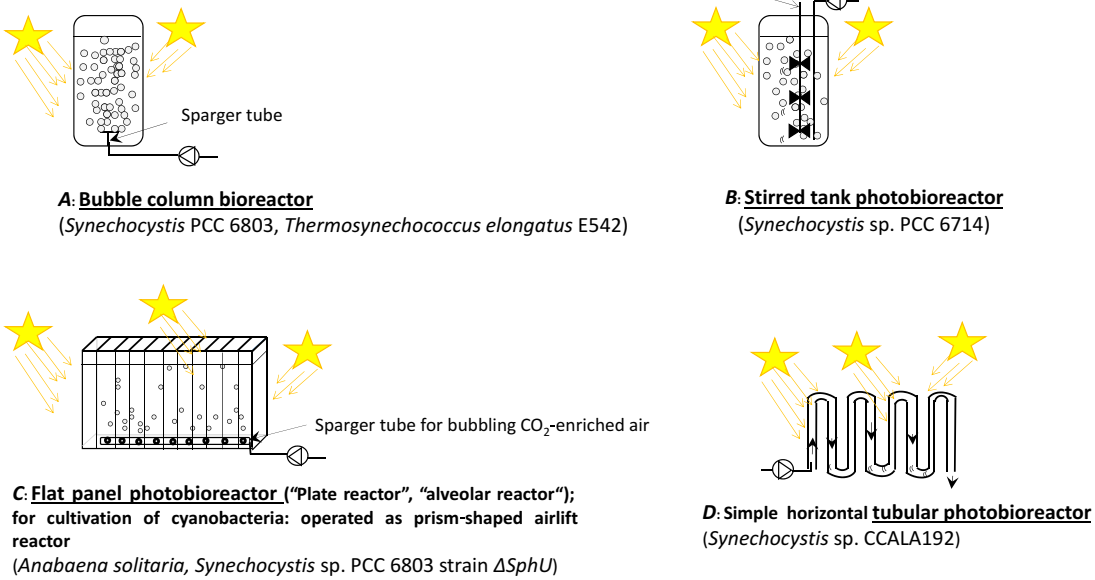
Fig. 22.6
Overview of genetic engineering approaches to improve PHA biosynthesis by cyanobacteria.

field of research. In the meantime, genetic modification of *P. tricornutum* was also tested to harness the strain for production of additional valued bioproducts, such as for increased accumulation of triacylglycerides as raw materials for production of third- and fourth-generation biodiesel (Daboussi et al., 2014).

22.9 Production scales and production techniques for cyanobacterial PHA production

In contrast to the large number of reports available for shaking-flask-scale PHA production using cyanobacteria (Drosg et al., 2015; Koller and Maršálek, 2015), only a small number of studies report on such processes under controlled cultivation conditions in closed photobioreactor (PBR) systems. In principle, the following basic types of photobioreactors were used to study PHA biosynthesis by cyanobacteria: bubble columns (Fig. 22.7A), stirred tank photobioreactors (Fig. 22.7B), flat panel bioreactors ("alveolar photobioreactors"; Fig. 22.7C), and tubular photobioreactors (Fig. 22.7D). Fig. 22.7 shows simplified schematic illustrations of these different types of photobioreactors.

Kamravamanesh et al. (2017) studied the previously unexplored cyanobacterium *Synechocystis* sp. PCC 6714 in different cultivation and nutritional limitation conditions according to defined and controlled parameters in a LED-illuminated 1.5 L (total volume) jacketed glass



A: **Bubble column bioreactor**
(*Synechocystis* PCC 6803, *Thermosynechococcus elongatus* E542)

B: **Stirred tank photobioreactor**
(*Synechocystis* sp. PCC 6714)

C: **Flat panel photobioreactor ("Plate reactor", "alveolar reactor"); for cultivation of cyanobacteria: operated as prism-shaped airlift reactor**
(*Anabaena solitaria, Synechocystis* sp. PCC 6803 strain *ΔSphU*)

D: **Simple horizontal tubular photobioreactor**
(*Synechocystis* sp. CCALA192)

Fig. 22.7
Illustration of different types of photobioreactors for cultivation of cyanobacteria for PHA biosynthesis under controlled conditions.

photobioreactor, bubbled with a mixture of sterile filtered air enriched with 2 vol-% CO_2. Under these photoautotrophic conditions, combined limitation of nitrogen and phosphate source resulted in the highest PHA accumulation. It turned out that specific growth rates were four times higher in controlled photobioreactor experiments than in pre-experiments carried out in illuminated shaking flasks. Intracellular PHA fraction and volumetric PHA productivity totaled 16.4 wt.-% and 59 ± 6 mg PHA /(L d), respectively, after 14 days of cultivation under nitrogen and phosphate limitation.

The first among these studies was published in Roberts' (2009) open accessible Master's thesis performed at the Australian University of Adelaide. Prior to PBR cultivations, different indigenous (Australian) PHB-producing cyanobacteria were screened in transparent flasks. Biomass produced in these flask cultures was supplied with air enriched with CO_2 and cultivated in a light-irradiated incubation cabinet. In this way, it was studied which of the tested cyanobacteria were robust against shear stress generated by bubbling, and which do not show unwanted formation of extracellular polysaccharides (biofilms) in order to prevent the adhesion of cells to the inside of the reactor. Compared with the other investigated species (*Anabaena flos-aquae*, *Microcystis flos-aquae*, *Microcystis aeruginosa*, *Nodularia spumigenia*, *Pseudanabaena* sp., and *Synechocystis* PCC 6803), *Anabaena solitaria* showed superior performance in terms of biomass growth and PHA biosynthesis. Consequently, *A. solitaria* was

chosen for controlled cultivation experiments in a flat panel PBR system (a.k.a. "alveolar bioreactor") designed by the Commonwealth Scientific and Industrial Research Organization (CSIRO). In particular, the dependence of PHA production on the illumination and temperature regime (500 µE/(m^2 s), 28°C; 500 µE/(m^2 s), 40°C, 500 µE/(m^2 s), 38°C, and 900 µE/(m^2 s), 38°C) was investigated. After 12 or 3 days of cultivation at illumination of 500 µE/(m^2 s) and temperature of 28°C and 40°C, PHA concentrations totaled 7 mg/L. Hence, *A. solitaria* displayed some PHB production potential under the investigated cultivation conditions, but the productivities were rather disappointing. By bubbling compressed air enriched with CO_2 at an aeration rate of 2.5 L per h from the PBR's bottom, the cells were airlifted and supplied with the inorganic carbon source (CO_2). Hence, this PBR system constitutes kind of a "flat plate bubble column." A 500 W halogen lamp served for external illumination of the PBR, and also gave off heat energy required for cell growth. The effect of different illumination strategies was investigated by adjusting the irradiation intensity by changing the distance between the PBR and the illumination source. At the onset of cultivation, the working volume amounted to 9.5 L; 8.5 L of sterilized BG-11 medium were inoculated with 1 L of pre-culture produced in shaking flasks (Roberts, 2009).

Spirulina sp. LEB 18 was used for cultivation in a 1.8 L vertical tubular reactor on waste from PHB extraction from biomass obtained by previous cultivations. A light intensity of 12 kW/m^2 and 12 h dark/light cycles were used for the experiments. PHA extraction from biomass was carried out by the combined utilization of sodium hypochlorite and acetone, and the generated waste remaining after removal of the crude PHA was collected and used for follow-up cultivations with 10, 15, 20, 25, and 30 vol.-% of waste; per kg of extracted biomass, 200 L of waste was obtained. The highest PHA fractions (10.6 wt.-%) were obtained when using 25% of waste, which was in the same range as for setups carried out in pure medium (da Silva et al., 2018). These experiments are analogous to previous reports for reutilization of saline cell debris after PHA recovery in follow-up cultivations using the haloarchaeon *Hfx. mediterranei* (Koller, 2015).

Samantaray et al. (2011) tested the use of nutrient-rich wastewater from intensive aquaculture for PHB biosynthesis by the diazotrophic cyanobacterium *Aulosira fertilissima* in a recirculatory bioreactor system. Fiber-reinforced plastic tanks were used for the experiments, and several parameters like depth, inoculum size, sedimentation, turbulence, and illumination were tested to evaluate the conditions for optimum productivity. The system displayed high bioremediation capability, as evidenced by the depletion of wastewater nutrients, which normally result in eutrophication, and, in parallel, produced valued *A. fertilissima* biomass for PHB production. The highest PHB productivity was achieved in sedimented fishpond discharge operated with permanent stirring at 20 cm culture depth and an initial inoculum size of 0.08 g per liter. Under optimized conditions for the summer, rainy, and winter seasons, PHB production amounted to 92, 89, and 80 g m^{-2}, respectively, corresponding to a theoretical annual harvest of about 17 t *A. fertilissima* biomass containing 14 t PHB per hectare cultivation

area in fishpond discharge, which goes in parallel with simultaneous annual treatment of about 33,000 m^3 wastewater.

A pilot-scale 200-L tubular photobioreactor cultivation setup was presented by Troschl et al. (2018) for cultivation of the cyanobacterium *Synechocystis* sp. CCALA192; CO_2 was used as the sole carbon source for the cultivations, which lasted 75 days, and were carried out as a two-stage process in a semi-continuous mode. After the onset of nitrogen depletion (5–7 days), PHA accumulation started, and the culture progressively switched from blue-green to yellowish. After 16–20 days, 90% of the cultivation broth was harvested (1.0 g/L biomass, average PHB fraction in biomass 12.5 wt.-%), while the residual 10% acted as inoculum for the subsequent cultivation cycle. When starting the new cultivation cycle with fresh nutrients, the yellowish culture switched to blue-green again, and PHA was degraded within 24–48 h. PHA biosynthesis started again after depletion of the nitrogen source, and the cycle started *de novo*. For the final phase of each cultivation cycle, a kind of maturation process was described; here, no CO_2 was consumed by the cells, but PHA concentration was still increasing by conversion of the second intracellular storage compound glycogen.

The strain *A. platensis* RRGK, a cyanobacterium isolated from an Indian freshwater lake, was cultivated by Kavitha et al. (2016) in an open, non-axenic raceway pond as the first report on outdoor autotrophic PHA production on a larger scale (volume: 2 m^3). Prior to cultivation, random mutagenesis of the cyanobacterium to increase PHA productivity was performed via UV-B radiation. After optimization of temperature, pH value, and concentration of the inorganic carbon source Na_2CO_3, about 2.2 g/L of biomass and 100 mg/L PHA were obtained in the open raceway pond cultivation.

After cultivation of high quantities of cyanobacteria in photobioreactors, downstream processing is needed to remove biomass from the liquid phase, and finally to recover PHA from biomass. In this context, biomass of the cyanobacterium *Chlorogloea fritschii* is characterized by the fact that it immediately sediments to the bottom of liquid cultures; hence, it is accessible to convenient downstream processing. The strain *C.* TISTR 8527 was subjected to a two-stage cultivation process for PHA production by Monshupanee et al. (2016). In the first stage, a high biomass concentration was generated under photoautotrophic cultivation conditions, while a second, heterotrophic, stage was carried out to boost PHA biosynthesis using acetate as the single organic carbon source. By optimizing this two-stage cultivation process, the yield of acetate-to-PHA conversion amounted to 0.51 ± 0.07 g/g, which is in a similar range to the theoretical maximum value. The authors stated that this two-stage process performs efficiently in converting simple organic substrates to PHA, reduces cultivation time, and results in enhanced overall volumetric productivity; it was suggested that this process might also work for other cyanobacterial cultivation processes (Monshupanee et al., 2016). Unfortunately, this experiment was carried out on a shaking flask scale, not in photobioreactors.

22.10 Conclusion

As described in detail in this chapter, plenty of research studies are available that demonstrate the potential of diverse meso- and extremophilic cyanobacteria as cell factories to generate PHA biopolyesters, both simple PHB homopolyester and copolyesters with enhanced material properties. In particular, the combination of phototrophic conversion of CO_2, in best case stemming from industrial effluent gases, and supply with organic, heterotrophic waste streams might pave the way to cost-efficient PHA production by cyanobacteria. This mixotrophic approach appears especially promising to overcome the rather modest productivities reported for pure phototrophic PHA biosynthesis. Moreover, mitigation of waste materials combined with PHA accumulation might also become more feasible by resorting to mixed culture approaches, a strategy which only now is sufficiently understood for mixed cultures enriched by cyanobacteria. Regarding the metabolic bottlenecks identified for individual cyanobacteria tested as PHA producers, it was shown that smart genetic engineering might help solve key issues such as low volumetric productivity, and could enhance substrate-to-PHA yields in such processes. However, such transformant strains were never tested on a reasonable scale, and using them requires strict compliance with safety regulations to prevent release of these engineered organisms into the environment.

To make PHA production by cyanobacteria a competitive strategy for the future, further work needs to be invested into development of optimized photobioreactor systems, which can be operated at larger production scale in accordance with special requirements of selected cyanobacterial production strains. This encompasses the mixing systems, gas supply and outgassing, and especially the illumination regime (fine-tuned supply of light with selected wavelengths according to the strain's needs, optimization of dark/light cycles). Most of all, more data will be needed from large-scale cultivation setups running for extended periods before any serious steps toward an industrial process will be possible.

However, provided the optimization of all these parameters will take place soon, it is likely that cyanobacteria will become the candidates of choice for solar-driven production of sustainable biopolyesters in the future.

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CHAPTER 23

UV-screening from microalgae

Ana Lucía Morocho-Jácome[a], Eleane de Almeida Cezare-Gomes[b], João Carlos Monteiro de Carvalho[b], Rafael Sauce[a], Catarina Rosado[c], Maria Valéria Robles Velasco[a], André Rolim Baby[a]

[a]Pharmacy Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil [b]Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, São Paulo, Brazil [c]CBIOS—Universidade Lusófona's Research Center for Biosciences and Health Technologies, Lisbon, Portugal

Chapter outline

23.1 Introduction 647
23.2 UV-induced skin problems 648
23.3 UV-absorbing compounds from microalgae 649
23.3.1 Carotenoids 649
23.3.2 Mycosporine-like amino acids 651
23.3.3 Scytonemin 653
23.4 Industrial applications of UV screening from microalgae 653
23.5 Conclusions 653
References 654

23.1 Introduction

Microalgae and cyanobacteria are photosynthetic microorganisms commonly found on Earth, mainly distributed in aquatic ecosystems (seawater and freshwater). They cann grow in extreme conditions and even in contaminated habitats. Such photosynthetic microorganisms are rich in active compounds such as polysaccharides, proteins, vitamins, essential amino acids, pigments, fibers, enzymes, and fatty acids (Safafar et al., 2015; Wang et al., 2015; Borowitzka, 2013). Nowadays, they are cultivated by batch, fed-batch, semicontinuous, and continuous processes under autotrophic, heterotrophic, or mixotrophic conditions to improve biomass production (Safafar et al., 2015; Borowitzka, 2013; Carvalho et al., 2014). Furthermore, cellular growth can be developed in open systems (Rodrigues et al., 2011; Danesi et al., 2004) or in engineered closed systems called photobioreactors (Carvalho et al., 2014; Morocho Jácome et al., 2012; Bresaola et al., 2019). As an interesting advantage, microalgae can profit from residual nutrients from different sources for growth with both high productivity levels and

Handbook of Microalgae-Based Processes and Products. https://doi.org/10.1016/B978-0-12-818536-0.00023-3

enhancement in lipid content (60%–65% dry biomass weight), total fibers (33%–50% higher than plants), proteins, and carbohydrates (Safafar et al., 2015; Caporgno et al., 2015; Wang et al., 2012; Coppens et al., 2014).

The growing demand for natural functional ingredients in food has increased microalgae production and their incorporation in foods (Bolanho et al., 2014). In this context, microalgae consumption has been promoted recently due to its health benefits, since it is being commercialized in nutraceutical products claiming antiinflammatory, antimutagenic, antioxidant, and antimicrobial properties (Sathasivam and Ki, 2018).

Furthermore, ultraviolet (UV) radiation induces many dangerous biological effects on organisms. Microalgae and cyanobacteria produce biological compounds with photoprotective properties under radiation stress, for instance, mycosporine-like amino acids (MMAs) and carotenoids, and other compounds such as scytonemin have also been isolated (Singh et al., 2017). Both carotenoids and flavonoids could prevent sunburn by UV radiation in humans with sensitive skin under light with low melanin levels (Stahl and Sies, 2012).

23.2 UV-induced skin problems

Skin, the most extensive organ of the human body, is an anatomical barrier that provides protection from pathogens and external threats. Damage induced by UV exposition can be minimized with a group of mechanisms developed by nature, named photoprotection. However, the protective capacity of melanin and other endogenous molecules can be exceeded by overexposure to UV radiation, provoking sunburn and skin damage (Pallela et al., 2010).

Aerobic metabolism processes in cells (e.g., signal transduction, gene expression) produce reactive oxygen species (ROS). These ROS can damage lipids, DNA, and proteins, consequently affecting enzyme functions, cellular membrane structures, and even gene expression. Moreover, ROS are involved in degenerative diseases. Antioxidant agents in skin could prevent the effects associated with ROS and avoid cell disruption and consequent damage. In addition, high UV exposure induces high levels of ROS and the accumulation in cells induces cell death, as apoptosis and necrosis. ROS also act in photoaging situations, such as melanoma, cutaneous inflammation, and even skin cancer. Finally, wrinkling and dryness of skin are produced by an excess in skin cell death (Pallela et al., 2010).

Microalgae compounds could be beneficial in tackling this problem, but their industrial production is under study since it is considered an expensive process when compared to chemical production (Panis and Carreon, 2016). Fortunately, there are many advanced studies making viable the production of microalgae on an industrial scale (Panis and Carreon, 2016; Cezare-Gomes et al., 2019).

23.3 UV-absorbing compounds from microalgae

High value-added bioproducts from microalgae and cyanobacteria biomass are being widely studied for food and even medical applications. Many reviews focusing on health benefits of carotenoids are available for discussion (Stahl and Sies, 2012; De Jesus Raposo et al., 2013a; Aburai et al., 2013; Grundman et al., 2018). However, information describing the cosmetic properties of such compounds is still scarce (Wang et al., 2015; Ariede et al., 2017).

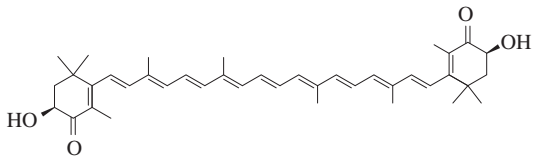
The main aim of this chapter is to summarize the UV-screening compounds from biomass of photosynthetic microorganisms as well as to improve the knowledge regarding microalgae compounds. For this reason, in this section, the information is organized not only to describe the chemical information of compounds but also to provide an explanation about their UV screening properties.

23.3.1 Carotenoids

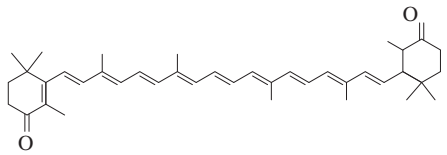
Carotenoids, natural pigments, comprise carotenes and xanthophylls. Hydrocarbon carotenoids have no substituent in their structures (β-carotene, lycopene), while xanthophylls are oxycarotenoids, which means that they are oxygen-containing molecules (e.g., astaxanthin, fucoxanthin, and lutein). There are many reviews regarding the different carotenoids produced by microalgae in nature (Sathasivam and Ki, 2018; Cezare-Gomes et al., 2019; Singh and Das, 2011; Saini et al., 2018). Fig. 23.1 shows common carotenoid structures from photosynthetic microorganisms.

These compounds exhibit UV protective effects by different mechanisms. Carotenoids contain eight units of isoprene, which form a polyene chain of conjugated double bonds establishing an extended π-electron system that absorbs both UV radiation and visible light, mainly blue and green (Singh et al., 2017). Photoprotection mediated by carotenoids could be achieved by both energy dissipation in the thylakoids and UV filter layer rich in nonoxygenated or oxygenated carotenoids at the chloroplast periphery or cell body. Moreover, carotenoids could act as provitamin A in skin and could protect against UV radiation and physical quenching, as well. Therefore, UV light could be directly absorbed by carotenoids to prevent sun damage in the skin (Stahl and Sies, 2012).

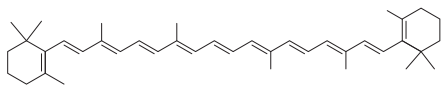
Carotenoids have the property of scavenging ROS, as peroxyl radicals and single molecular oxygen with concomitant formation of carotenoid radicals (Chisté et al., 2014). As is widely reported, β-carotene protects cells from free radicals. It is a potent antioxidant with UV light-protecting properties (additive in sunscreen products). It must be emphasized that the green microalga *Dunaliella salina* can produce more than 10% of dry biomass weight of β-carotene, and it is the largest natural source of this compound (Lamers et al., 2008).



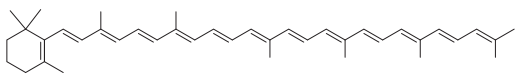
Astaxanthin



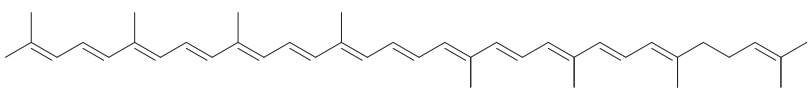
Cantaxanthin



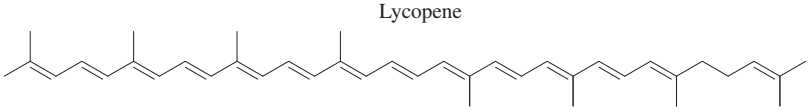
β-Carotene



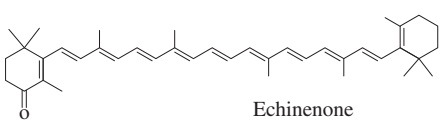
γ-Carotene



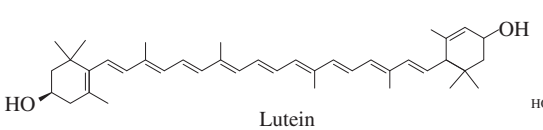
Lycopene



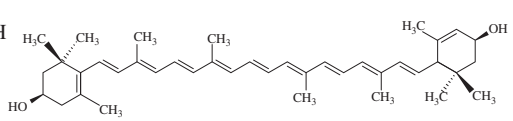
Phytoene



Echinenone



Lutein



Zeaxanthin

Fig. 23.1
Structures of some common microalgae carotenoids (Cezare-Gomes et al., 2019).

Fucoxanthin can also be produced by microalgae, including diatoms, and it has several applications in healthcare (Peng et al., 2011). It is an antenna pigment, which collects and transfers light energy to chlorophyll-protein complexes. Its unique molecular structure confers high energy transfer efficiency (80%). It is also involved in photoprotection and exhibits greater antioxidant activity than β-carotene (De Jesus Raposo et al., 2013a; Sathasivam et al., 2017; Ayalon, 2017). Furthermore, it could protect skin from photoaging (Peng et al., 2011).

Another green microalgae, *Haematococcus pluvialis*, has been studied to produce astaxanthin in different environmental conditions for decades (Wang et al., 2003; Lemoine and Schoefs, 2010; Varela et al., 2015). It is the major source of astaxanthin, containing 1.5%–3.0% of this natural pigment in its dry biomass. Such compounds have great commercial value; for instance, the Japanese company Fuji Chemical Industry markets the product AstaTROL (application in cosmetic product and personal care) containing astaxanthin from *H. pluvialis* (AstaTROL, 2019).

Furthermore, *H. pluvialis* was cultivated under high irradiance and not only was the light screening by astaxanthin (that absorbs light in the blue region) demonstrated, but also the protective effect of the photosystem, responsible for the photoprotective process, was shown (Wang et al., 2003). Moreover, some reports about the effects of these pigments in human health have been made (Wang et al., 2015; Tominaga et al., 2012), as well as the effective inhibition of skin cancer by daily astaxanthin supplementation (Rao et al., 2013).

Astaxanthin has also presented stronger antioxidant activities than β-carotene, zeaxanthin, cantaxanthin, and vitamin E. The natural pigment zeaxanthin can be used as a colorant in cosmetics (Bhosale and Bernstein, 2005), and cantaxanthin can create a tan color in the skin (Gong and Bassi, 2016). Lutein may also be used as coloring in the cosmetic industry (Kläui, 1982).

23.3.2 Mycosporine-like amino acids

MAAs, secondary metabolites, can be produced by many different organisms, mainly the ones that constantly receive high dosages of sunlight (marine and freshwater ecosystems), *e*.g., algae, protozoa, seaweed, corals, and fish (Řezanka et al., 2004; Bandaranayake, 1998; Gröniger et al., 2000; Shick and Dunlap, 2002; Sinha et al., 2007; Dunlap and Shick, 1998).

MAAs are molecules with water-soluble, colorless, and uncharged properties, having low molecular weight (<400 Da) (Chrapusta et al., 2017; Lawrence et al., 2019). They share their general structure with different substituents (amino acids), including a cyclohexenone or cyclohexenimine chromophore with the nitrogen substituent (Fig. 23.2) (Nakamura et al., 1982; Singh et al., 2008; Favre-Bonvin et al., 1976). In addition, with further carboxylation or demethylation, the UV absorption properties can be modified (Singh et al., 2008).

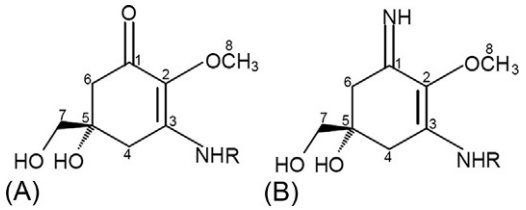


Fig. 23.2

(A) Aminocyclohexenone ring and (B) aminocyclohexeniminone ring (Bandaranayake, 1998).

MAAs can be synthesized through dietary intake. Their structures and type can vary with different species, and the variables of geographical location and environment characteristics are important for their specific production (Torres et al., 2004; Conde et al., 2004). Their production could also be increased by different levels and types of irradiation, such as visible light, UVA, or UVB (Conde et al., 2004).

Aminocyclohexenone derivatives have a cyclohexenone AC coupled to an amino acid, forming molecules like mycosporine-glycine, mycosporine-taurine, mycosporine-glutamine, and mycosporine-glutaminol (Torres et al., 2004; Carreto and Carignan, 2011). Otherwise, aminocyclohexenimine derivates have a carbon-nitrogen double bond, and can form molecules like shinorine, palythine, and palythinol (Wada et al., 2015; Klisch et al., 2007; Ishihara et al., 2017). Their structural properties are entirely related to their specific absorption spectra and high molar extinction coefficients (Conde et al., 2004). MAAs have the ability to absorb UV radiation between absorption wavelength peaks of 309 and 362 nm (covering the UVB and UVA spectrum), with high molar absorptivity between 2.81×10^4 and $5.00 \times 10^4\ M^{-1}\ cm^{-1}$ (Bandaranayake, 1998; Shick and Dunlap, 2002; Dunlap and Shick, 1998; Singh et al., 2008).

The most studied MMAs are mycosporine-glycine and shinorine, whose photostability is explained by their photo-excited states. They can dissipate about 98% of the energy absorbed as heat, without generating ROS (Conde et al., 2004). These characteristics provide strong evidence that MAAs could be an effective natural UV filter.

The majority of studies regarding MAAs were made in vitro, mainly with shinorine, porphyra-334, and mycosporine-glycine, as these MAAs are most abundant in nature (Lawrence et al., 2019). The best MAA examples acting as a filter to date are Helioguard 365 and Helionori, both containing *Porphyra umbilicalis* extract. Helioguard 365 is composed of porphyra-334 and shinorine, claiming protection against DNA damage and UVA-induced loss of cell viability, as well as being considered antiaging and skin protectant (Chrapusta et al., 2017). Helionori is composed of porphyra-334, palythine, and shinorine, and is claimed to be UVA photoprotective by preventing formation of sunburnt cells, protecting the metabolism of fibroblasts and keratinocytes, and preserving its membrane lipids, as well as protecting its DNA (Andre et al., 1999).

To date, there is little information about MAAs and their photoprotective potential. Moreover, their biosynthesis pathway is poorly understood, limiting production at an industrial scale with economic benefits (Lawrence et al., 2019). Therefore, making sunscreens with natural UV filters seems to be an appealing goal, linked to the use of more eco-friendly and safe compounds, enabling lower amounts of chemical filters.

23.3.3 Scytonemin

Scytonemin is a dimer of phenolic and indolic subunits. It is found in some terrestrial cyanobacterial species as a yellowish-brown lipid soluble pigment from the exopolysaccharide sheath (Singh et al., 2017; Rastogi et al., 2016). Studies show that the risk of damage provoked by the most lethal UVC radiation could be reduced by the sole use of scytonemin. It is also a stable compound under different stress conditions, such as high UV radiation and temperature, among other conditions (Rastogi et al., 2016; Rastogi and Incharoensakdi, 2014). For these reasons, it could be considered as a promising UV filter in cosmetic formulations.

23.4 Industrial applications of UV screening from microalgae

As discussed in the literature, microalgae pigment production is currently an expensive process that could increase the final raw material costs of sunscreen products in the market (De Jesus Raposo et al., 2013b). The potential domination of applications with astaxanthin derived from microalgae biomass over the synthetic alternatives may bring high-quality fisheries and expand its claim to use in the pharmaceutical/cosmetic industries, as well. However, astaxanthin extraction from *H. pluvialis* cultivation was evaluated at large scale (Panis and Carreon, 2016), and the cost of natural production was considerably higher than the synthetic one (€1536–€6403 instead of €880 kg^{-1}). Clearly, more efforts need to be made to produce natural compounds from microalgae for cosmetic applications. Table 23.1 shows some recent patents that demonstrate the industrial application of compounds from microalgae for cosmetic uses.

23.5 Conclusions

The use of sunscreens is an extremely convenient and effective way to protect the skin against UV damage and such products are composed of several ingredients, mainly UV filters, that could have their efficacy highly improved by adding natural compounds, like the ones obtained from microalgae. Recently, many consumers have become suspicious of chemical ingredients in cosmetics, creating a trend to go back to basics in cosmetic product formulation, which has led to the study of the potential use of new natural compounds from microalgae. Furthermore, the demand for natural and environmentally sustainable products, such as those containing compounds from microalgal biomass, has produced a significant increment in market value. Therefore, industrial production is growing not only for food (nutraceuticals and functional foods),

Table 23.1: Microalgae biocompounds in recent patents applications.

Microalgae	Biocompound	Claims for use in cosmetics	Reference
Haematococcus sp.	Astaxanthin	Sunscreen and antiaging products, skin hydration	Rastogi et al. (2016)
Haematococcus pluvialis	Astaxanthin Zeaxanthin Lutein	Skin hydration, antiwrinkle, caratolytics, peeling and mask via creams, ointments, gels, lotions or oils, moisturizing creams, sunblock products	Rastogi and Incharoensakdi (2014)
–	Neoxanthin Fucoxanthin Isofuncoxanthinol Lutein	Antiaging products, skin-care creams, facial makeup	De Jesus Raposo et al. (2013b)
Isochrysis sp. *Amphora* sp. *Phaeodactylum* sp.	Fucoxanthin	Antiaging, skin-whitening, and skin protection products	Sathasivam et al. (2017)

feeds (colorants and additives), and other applications, but also for cosmetic formulations (e.g., natural colorants, hair dyes, nail polish, eyeshadow, soap, lipsticks, creams, lotions, and sunscreen). Finally, researchers worldwide have found that microalgae-derived compounds can be utilized as cosmetic ingredients, as reported in this chapter. Thus we suggest more efforts to understand better not only the mechanisms but also the potential benefits of incorporation of compounds from microalgae in cosmetics.

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CHAPTER 24

Volatile organic compounds from microalgae

Karem Rodrigues Vieira, Pricila Nass Pinheiro, Leila Queiroz Zepka

Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Chapter outline

24.1 Introduction 659
24.2 Biosynthesis mechanism of volatile organic compounds in microalgae 660
24.3 Environmental factors affecting VOCs production from microalgae 666
24.4 Application of VOCs from microalgae 668
24.5 Techniques for VOCs recovery 678
24.6 Conclusions and future perspectives 681
References 682

24.1 Introduction

Microalgae comprise a diverse group of photosynthetic microorganisms; however, the term "microalgae" is not of a taxonomic term, but it is a common collective term a commercial terminology (Borowitzka et al., 2016). Regarding biotechnological exploitation, the most widely used species of microalgae belong to these classes: *Cyanophyceae*, *Chlorophyceae*, *Bacillariophyceae*, and *Chrysophyceae* (Borowitzka, 2018).

There is global interest in the exploitation of microalgae-based processes and products, fundamentally supported in the diversity chemical composition of the biomass, in addition to the broad spectrum of its secondary metabolites. Volatile organic compounds are secondary metabolites naturally emitted by microalgae (Santos et al., 2016a; Amavizca et al., 2017; Jacob-Lopes et al., 2019).

The profile of volatile compounds released by microalgae and cyanobacteria has been reported as a thriving source for the production of mixtures of VOCs from different chemical classes such as alcohol, aldehydes, ketones, hydrocarbons, esters, terpenes, and sulfur compounds (Santos et al., 2016b; Hosoglu, 2018).

Handbook of Microalgae-Based Processes and Products. https://doi.org/10.1016/B978-0-12-818536-0.00024-5

The emission of VOCs in the microalgae-based system depends on abiotic and biotic factors. Thus, the biosynthesis knowledge and environmental factors affecting the production can help identify them and target the most appropriate industrial application sector (Santos et al., 2016a; Achyuthan et al., 2017).

The use of the volatile compounds of microalgal culture may represent an improvement in the supply of inputs to a distinct sector of the industry, and once there is a growing interest in natural products guiding the development of the technologies that employ microorganisms, including microalgae, which can synthesize specific volatile organic compounds (Lukin et al., 2018).

Exploring the volatile compounds of microalgae is a possibility; however, it is scientifically challenging to apply these metabolites. Recent research shows that the VOCs produced by microalgae have a high energy potential. Substantial concentrations of VOCs are released and simultaneously reused as fuels in biocombustion processes (Santos et al., 2016a; Deprá et al., 2018; Severo et al., 2018).

In addition, microalgae have emerged as a promising technology for environmental applications because they balance sustainable vectors by reuse of pollutants, which are present in wastewater generated by industries (Santos et al., 2019). Wastewater, in addition to water pollution, also contributes to air pollution. These facts have affected industries, such as complaints from residents living near industrial facilities (Filipy et al., 2006; Lebrero et al., 2014). However, current research demonstrates that microalgae-based processes are an innovative technology for wastewater deodorization (Vieira et al., 2019).

Therefore, the objective of this chapter is to provide a comprehensive view of the volatile organic compounds formed in microalgae-based systems, focusing on the biosynthesis, culture conditions, and environmental factors that affect VOCs production, VOCs application, and the recovery techniques.

24.2 Biosynthesis mechanism of volatile organic compounds in microalgae

Microalgae-based systems release a wide spectrum of volatile organic compounds (VOCs). Metabolically, these VOCs are secondary metabolites, and biosynthesis depends mainly on the availability of carbon and nutrients as well as energy provided by primary metabolism (Dudareva et al., 2013; Santos et al., 2016a; Zuo, 2019).

Fundamentally, VOCs are produced from simple molecules through enzymatic pathways or degradation. Among the pathways for the production of these compounds are ketoacids, fatty acid derivatives, and the isoprenoid pathway. The VOCs belong to innumerable organic classes such as terpenes, alcohol, ketones, aldehydes, esters, hydrocarbons, carboxylic acids, and sulfurized compounds (Liao et al., 2016; Santos et al., 2016a,b).

From the 2-keto acid pathway, a diversity of volatile compounds is obtained, such as aldehydes, alcohols, esters and carboxylic acids, which can be synthesized through the 2-keto acid pathway (Fig. 24.1). The 2-ketoacid pathway covers sequential biochemical reactions such as extension, decarboxylation, isomerization, reduction, dehydration, and esterification of some branched-chain amino acids (e.g., leucine and valine). For example, for 1-butanol, 3-methyl-butanal, and 2-methyl-butanal subsequently reduced to 3-methyl-butanol and 2-methyl-butanol, the reaction can be extended to form 1-hexanol and other alcohols (Hasegawa et al., 2012; Lan and Liao, 2012; Liao et al., 2016).

Most microalgae groups possess two isoprenoid biosynthesis pathways: the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Table 24.1), responsible for the synthesis of isopentenyl diphosphate (IPP) and its molecular isomer dimethylallyl diphosphate (DMAPP) (Chappell, 2003; Lichtenthaler et al., 1997).

For both MEP and MVA routes (Fig. 24.2), DMAPP serves as the primer for the sequential and linear chain elongation, catalyzed by the respective enzymes. Consecutive additions of IPP in a head-to-tail fashion yield in sequence C10 geranyl diphosphate (GPP), C15 farnesyl diphosphate (FPP), and C20 GGPP. The series of reactions are catalyzed by enzymes geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS), respectively (Liao et al., 2016).

These carbon precursors are transformed rapidly into different terpenoids, as carotenoids and their oxidative and enzymatic cleavage products—for example, VOCs as α-ionone, β-ionone, and β-cyclocitral (Hosoglu, 2018; Lee et al., 2017; Van Durme et al., 2013).

Among numerous cyanobacteria volatile compounds, geosmin and 2-methylisoborneol (2-MIB) have been extensively studied due to undesirable outbreaks of taste and odor. Synthesis of 2-methylisoborneol (2-MIB) (Fig. 24.3) starts with the methylation of the precursor geranyl diphosphate (GPP) in 2-methylgeranyl diphosphate, which is cyclized in

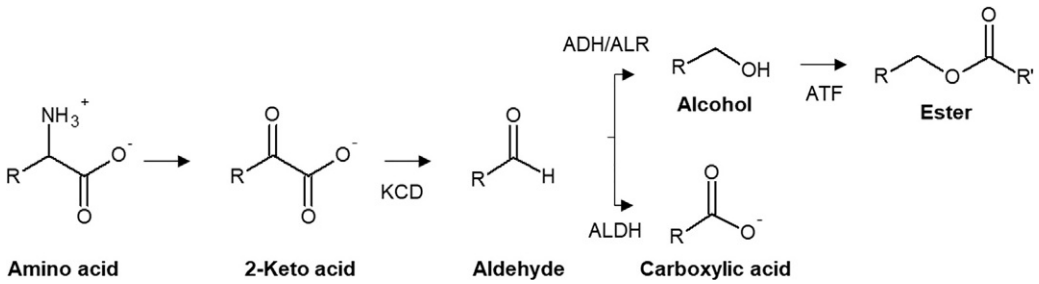

Fig. 24.1

Scheme of metabolic 2-ketoacid pathway for production of VOCs of different organic classes. *KDC*, 2-keto acid decarboxylase; *ADH*, alcohol dehydrogenase; *ALR*, aldehyde reductase; *ALDH*, aldehyde dehydrogenase; *ATF*, alcohol O-acyltransferase.

Table 24.1: Distribution MVA and the MEP pathways in different species.

Phylum	Class	Representative species	Pathway		Reference
			MVA	MEP	
Cyanophyta	*Cyanophyceae*	*Synechocystis* sp.	–	+	Disch et al. (1998)
Glaucophyta	*Glaucophyceae*	*Cyanophora paradoxa*	–	+	Grauvogel and Petersen (2007)
Rhodophyta	*Cyanidiophyceae*	*Galdieria sulphuraria*	+	+	Schwender and Seemann (1996)
		Cyanidium caldarium	+	+	Disch et al. (1998)
Chlorophyta	*Chlorophyceae*	*Scenedesmus obliquus*	–	+	Disch et al. (1998), Schwender and Seemann (1996)
	Trebouxiophyceae	*Chlorella fusca*	–	+	Disch et al. (1998)
	Prasinophyceae	*Tetraselmis striata*	–	+	Schwender and Gemu (2001)
Euglenophyta	*Euglenophyceae*	*Euglena gracilis*	+	+	Disch et al. (1998), Kim et al. (2004)
Heterokontophyta	*Chrysophyceae*	*Ochromonas danica*	+	+	Disch et al. (1998)
	Bacillariophyceae	*Phaeodactylum tricornutum*	+	+	Cvejic and Rohmer (2000)
		Nitzschia ovalis	+	+	Cvejic and Rohmer (2000)
Cryptophyta	*Cryptophyceae*	*Guillardia theta*	?	+	Frommolt et al. (2008)

This table is not comprehensive. "+" indicates proof of existence of pathway, "–" indicates absence of the pathway, and "?" indicates unknown if present.

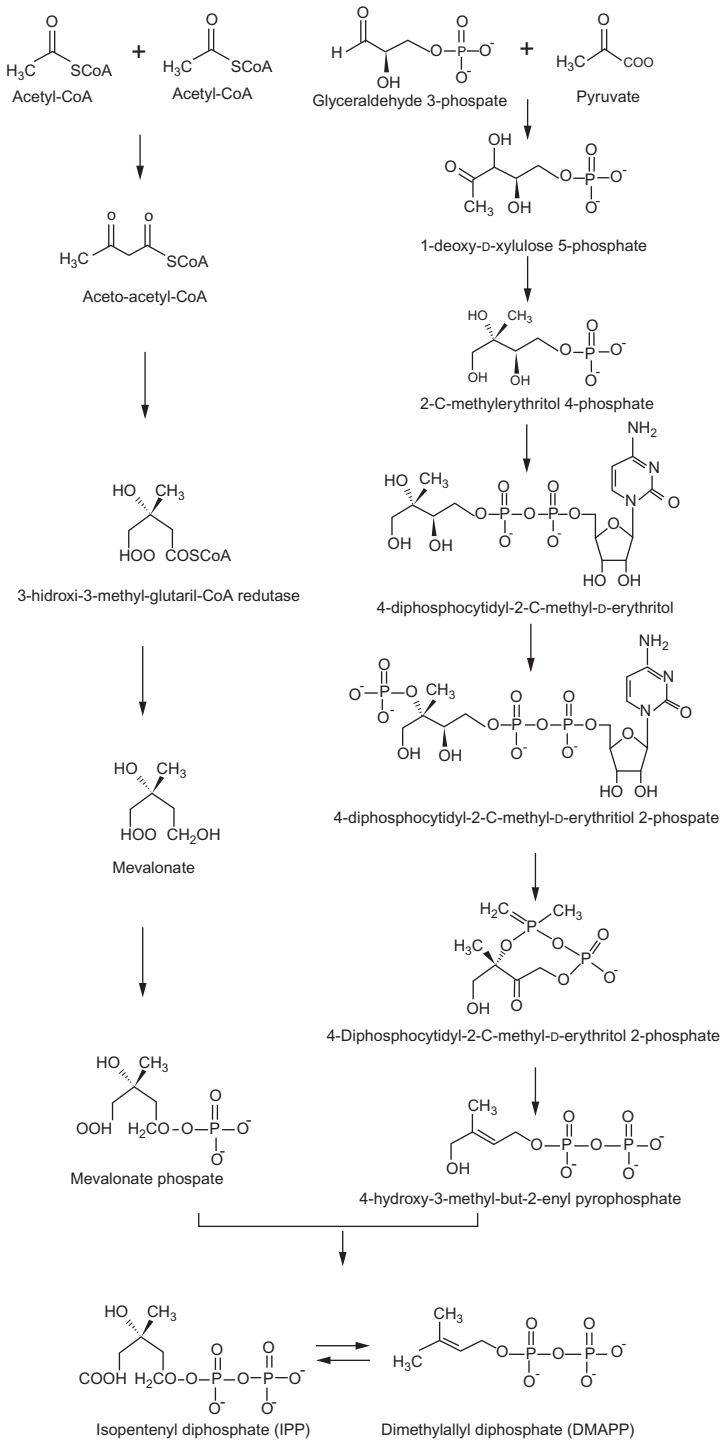

Fig. 24.2

Two pathways for the formation of isoprenoid. (A) Mevalonic acid (MVA) pathway. (B) Methylerythritol phosphate (MEP) pathway.

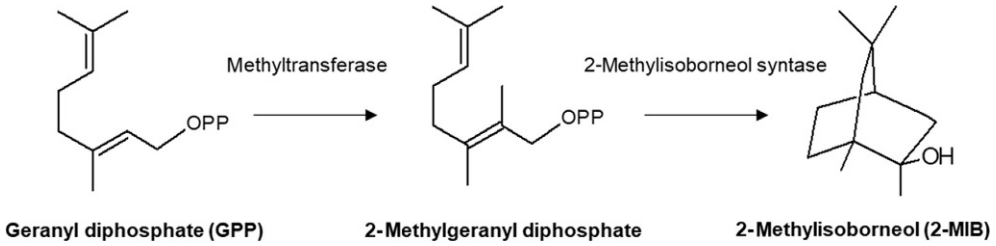

Fig. 24.3
Scheme of 2-methylisoborneol (2-MIB) biosynthetic pathway.

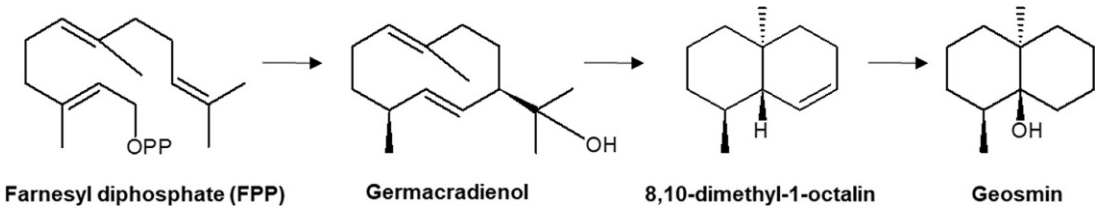

Fig. 24.4
Overview of geosmin synthesis route.

2-MIB (Lee et al., 2017). The cyclization of farnesyl diphosphate (FPP) to geosmin occurs in four stages (Fig. 24.4); farnesyl diphosphate to germacradienol is converted to 8,10-dimethyl-1-octalin, forming to geosmin, and is catalyzed by geosmin synthase (Liato and Aïder, 2017; Meena et al., 2017; Van Durme et al., 2013; Watson et al., 2016).

A range of VOCs, including classes such as ketones, aldehydes, hydrocarbons, and alcohols, can be produced from fatty acid degradation (Santos et al., 2016a). The fatty acid pathway starts with acetyl-CoA using malonyl-CoA as a building block, based on a series of cyclic reactions catalyzed by the multienzymatic system, denominated fatty-acid synthase (Fig. 24.5) (Peralta-Yahya et al., 2012; Zhou et al., 2018).

Aliphatic ketones can be formed from lipid degradation (Santos et al., 2016a). The aldehydes2,4-decadienal and 2,4,7-decatrienal are derivative products of arachidonic or eicosapentaenoic acid, catalyzed by lipoxygenase/hydroperoxid lyase. The fatty acids linoleic or linolenic acid are the precursors of aldehydes compounds such as nonanal, hexanal, and2-pentanal, which can subsequently be reduced to alcohols by dehydrogenases (Adolph et al., 2003; Jerković et al., 2018; Santos et al., 2016a,b; Yu et al., 2014).

Unbranched hydrocarbon production is achieved mainly by two families of enzymes: acyl-acyl carrier protein reductase (AAR) and an aldehyde decarbonylase (AAD), which catalyzes a

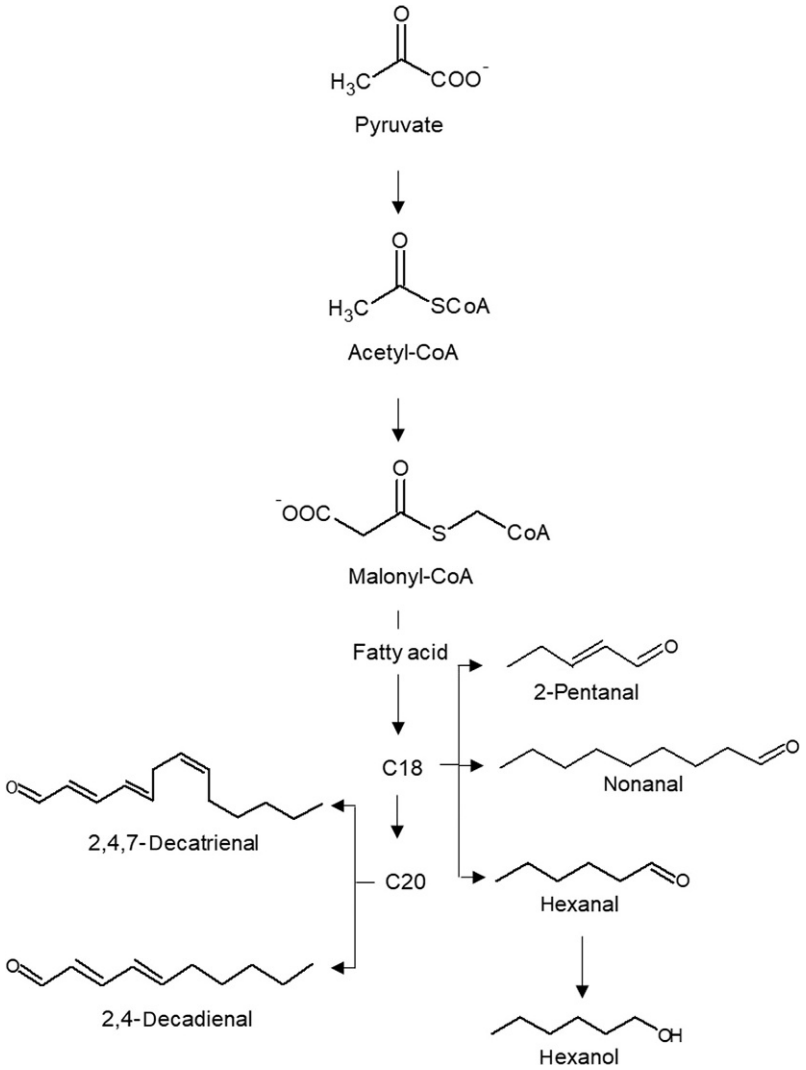

Fig. 24.5
Schematic representation of biosynthetic pathways of the fatty acids and its volatile derivatives.

number of mechanisms converting fatty acid intermediates into alkanes and alkenes (Milovanović et al., 2015; Santos et al., 2016a).

Sulfur compounds, such as dimethylsulfide (DMS), dimethyldisulfide (DMDS), and dimethyltrisulfide (DMTS), are potent volatile compounds due to their low odor threshold values, liberated by many microalgae (Achyuthan et al., 2017; Watson and Jüttner, 2017). The most important volatile sulfide produced is dimethylsulfide (DMS) (Watson and Jüttner, 2017).

The DMSP arises from the amino acid methionine, which is the forerunner of the 2-keto acid 4-methylthio-2-oxobutyrate, through transamination (see Giordano et al. (2005) and their references), followed by a reduction reaction catalyzed by 4-methylthio-2-oxobutyrate reductase, transforming in 4-methylthio-2-hydroxybutyrate, using a nicotinamide adenine dinucleotide phosphate molecule (Giordano and Prioretti, 2016).

The next stage in the mechanism is the *S*-methylation of 4-methylthio-2-hydroxybutyrate to 4-dimethylsufonio-2-hydroxybutyrate, which is finally transformed at the DMSP compound through oxidative decarboxylation (Giordano et al., 2005; Giordano and Prioretti, 2016). The demethiolation of dimethylsulfoniopropionate produces methanethiol, which can be converted into dimethylsulfide (DMS) by methylation (Fig. 24.6) (Achyuthan et al., 2017; Curson et al., 2017).

In order to exploit VOCs in microalgae-based systems successfully, a good understanding of physiology, biosynthesis, and mode of cultivation is essential. This will enable selection of controlled and appropriate growth conditions and optimization of yield biomass as the productivity of desirable volatile compounds (Santos et al., 2016a).

24.3 Environmental factors affecting VOCs production from microalgae

When we consider the biosynthesis of VOCs by microalgae, though dependent on the species, their production can be modified by various factors, such as culture system, nutritional conditions, light intensity, temperature, and growth phase (Milovanović et al., 2015; Van Durme et al., 2013; Achyuthan et al., 2017).

In general, the most widely used system for microalgae cultivation is the photoautotrophic system, where species are cultivated by inorganic carbon (CO_2) bioconversion and light energy absorption. In photosynthetic cultures, these microorganisms can biosynthesize CO_2 very efficiently and biotransform it into VOCs (Perez-Garcia et al., 2011; Perez-Garcia and Bashan, 2015; Claassens et al., 2016; Gong et al., 2018).

In addition, some microalgae species have the versatility to grow in the absence of light, where organic substrates are assimilated through aerobic respiration. In heterotrophic cultures, exogenous carbon sources such as glucose, fructose, and sucrose showed a variable profile of volatile compounds (Francisco et al., 2014; Perez-Garcia and Bashan, 2015; Santos et al., 2016b, 2018).

In mixotrophic cultivation, microalgae employ the phototrophy and heterotrophy systems simultaneously, using different energy sources, such as organic carbon and inorganic carbon in the presence of light. This cultivation system has an additive effect that increases biomass productivity and consequently the formation of volatile compounds (Bhatnagar et al., 2011; Perez-Garcia and Bashan, 2015; Santos et al., 2018).

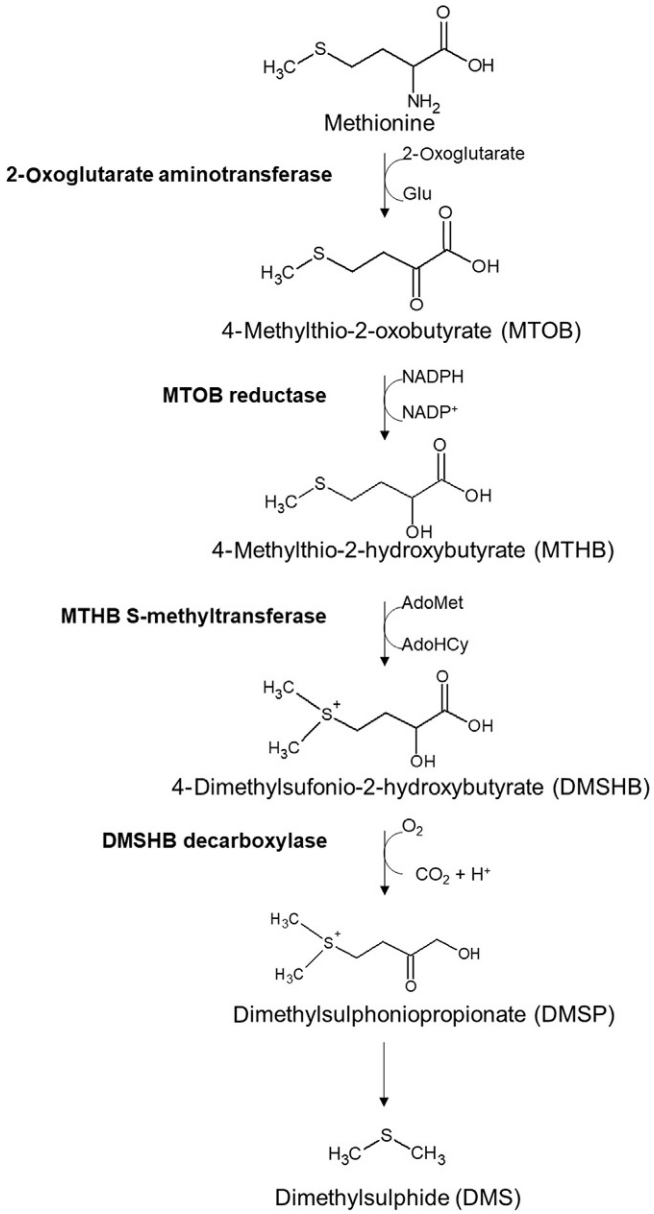

Fig. 24.6

Dimethylsulfide biosynthetic pathway in microalgae. *MTOB*, 4-methylthio-2-oxobutyrate; *MTHB*, 4-methylthio-2-hydroxybutyrate; *DMSHB*, 4-dimethylsufonio-2-hydroxybutyrate.

In microalgae-based systems, the nutrition conditions can influence the emission of the VOCs from algae. In addition to phosphorus and nitrogen sources, their concentrations may also affect the secondary metabolism of this microorganism (Zuo et al., 2018a; Zuo, 2019).

The cyanobacteria *Microcystis flos-aquae* released different VOCs, (sulfur compounds, terpenoids, hydrocarbons, aldehydes, and esters) when they were supplied with different nitrogen sources such as $NaNO_3$, $NaNO_2$, NH_4Cl, urea, serine, lysine, and arginine (Zuo et al., 2018a; Xu et al., 2017).

In previous studies, Hasegawa et al. (2012) demonstrated that *Microcystis aeruginosa* cyanobacteria cultures increased the emission of β-cyclocitral, 2-methyl-1-butanol, 2-phenylethanol, and 3-methyl-1-butanol under non-N condition (Hasegawa et al., 2012). Similar results have also been reported for cyanobacteria *Microcystis flos-aquae* and *Microcystis aeruginosa* under distinct sources and phosphorus concentration (Zuo et al., 2018b; Ye et al., 2018).

Light promotes terpenoid emission, which due to the availability of energetic cofactors and carbon intermediates increases the availability of DMAPP, the immediate precursor of the MEP pathway. Thus, isoprene and monoterpenes are synthesized via MEP and are released from microalgae after direct synthesis, due to no storage structures (Shaw et al., 2003; Niinemets and Sun, 2015; Liao et al., 2016; Englund et al., 2018).

Elevated temperatures promote the emission of alcohols, aldehydes, and hydrocarbons, which are formed via oxidative degradation of fatty acids and carotenoid derivatives as β-cyclocitral, α-ionone, β-ionone, and geranylacetone (Jüttner, 1984; García-Plazaola et al., 2017).

Another factor that affects the emission of volatile compounds in microalgae-based systems is the growth phases. Zhou et al. (2017) reported that the chemical classes of VOCs produced showed differences between the three growth phases. Aldehydes and alcohols of different microalgae species did not show the same tendency and concentration in the growth phases. Alkanes presented the highest concentration in the exponential phase, but decreased from the stationary phase, while ketones in the species studied showed similar increasing trends from the exponential to the stationary phase (Zhou et al., 2017).

The occurrence of VOCs in microalgae is a consequence of their versatile metabolism; thus, understanding the microalgae culture conditions can provide a better knowledge basis for the production of VOCs with industrial potential (Santos et al., 2016a, 2018).

24.4 Application of VOCs from microalgae

Chemicals obtained from microalgae-based systems are sold at prices 1000 times higher than those of synthetic chemicals (Santos et al., 2016b). The most important product of microalgae biotechnology in relation to the amount of production and economic value is its biomass.

However, an emerging trend toward knowledge production of low molecular weight compounds from renewable sources has been noted (Schirmer et al., 2010; Choi and Lee, 2013).

Volatile organic compounds generated by microalgae with commercial appeal include propanol, butanol, 3-methyl-butanol, hexanol, hexanal, β-cyclocitral, and β-ionone (Smith et al., 2010; Santos et al., 2016b). Berger (2009) reported that flavors from microorganisms can compete with traditional sources. The screening for overproducers, elucidation of metabolic pathways and precursors, and application of conventional bioengineering has resulted in a set of more than 100 commercial aroma chemicals derived via biotechnology. Table 24.2 shows a diversity of volatile compounds that were detected in different strains of microalgae from controlled cultures, as well as a comparison of chemically synthesized compounds and those found naturally in plants.

The global market of VOCs was worth US$3.85 billion in 2015, and has a predicted compound annual growth rate of 6.2% until 2024. Terpenes are the predominant class of compounds in this market (Sales et al., 2018). Other classes also of great interest are the alcohols and the aldehydes, which are important aroma components widely applied to the cosmetic, perfumery, and food industries (Longo and Sanromán, 2006).

The VOCs from microalgae have specific advantages regarding extraction from natural sources and chemical synthesis, mainly in terms of not having seasonal and environmental issues, due to the ability of microalgae to be cultivated on non-arable land, making their use commercially attractive for the source of fine chemicals and the food sector, despite the higher production costs (Borowitzka, 2018).

However, the full use of the volatile fraction of microalgal biomass may represent an improvement in the supply of a large volume of inputs to many different types of industry. Concerning the petrochemical industry, hydrocarbons and short-chain alcohols are interesting to generate bioenergy (Severo et al., 2018).

VOCs from microalgae have demonstrated an energy potential of 86.32 $MJ\,kg^{-1}$ (Table 24.3), representing nearly twice as much energy content when compared to traditional fuels, such as gasoline (47.30 $MJ\,kg^{-1}$) and diesel (44.80 $MJ\,kg^{-1}$). However, to meet the energy demands that a combustion system requires, a biotechnological process becomes necessary that produces these compounds in high volumes, which does not currently exist (Deprá et al., 2018).

Microalgae-based systems can also be used for wastewater deodorization (Vieira et al., 2019). The treatment techniques commonly employed in WWTPs for odor removal are chemical scrubbing and bio-filter (Lebrero et al., 2011; Alinezhad et al., 2019). However, these technologies present disadvantages such as produce secondary pollutants, a long period of adaptation required for the microbial population (weeks or even months), and high water consumption, which can make the long-term process onerous and costly (Lebrero et al., 2011).

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted.

Chemical name	Microalgae	Chemical	Natural	References
		Terpenes		
α-ionone	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochlopsis*	+	+	Van Durme et al. (2013)
β-cyclocitral	*Botryococcus braunii*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Nostoc* sp., *Phormidium autumnale*, *Rhodomonas* sp., *Spirulina platensis*, *Tetraselmis chuii*	+	–	Van Durme et al. (2013), Milovanović et al. (2015), Santos et al. (2016b), Lee et al. (2017)
β-ionone	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochlopsis*, *Spirulina platensis*, *Nostoc* sp.,	+	+	Van Durme et al. (2013), Milovanović et al. (2015)
Geosmin	*Anabaena lemmermannii*, *Anabaena circinalis*, *Anabaena solitaria*, *Anabaena viguieri*, *Aphanizomenon gracile*, *Geitlerinema splendidum*, *Leibleinia subtilis*, *Microcoleus* sp., *Phormidium allorgei*, *Phormidium amoenum*, *Phormidium breve*, *Phormidium cortianum*, *Phormidium formosum*, *Phormidium simplicissimum* *Phormidium* sp.,	+	–	Watson (2003), Liato and Aïder (2017), Lee et al. (2017)
2-methylisoborneol	*Oscillatoria curviceps*, *Oscillatoria limosa*, *Oscillatoria tenuis*, *Oscillatoria variabilis*, *Phormidium autumnale*, *Phormidium breve*, *Phormidium calcícola*, *Phormidium favosum*, *Phormidium tenue*, *Phormidium* sp.	+	–	Watson et al. (2016), Lee et al. (2017)
Geraniol	*Synechococcus*			Jüttner and Hans (1986)
Menthol	*Phormidium autumnale*	+	+	Vieira et al. (2019)

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
Citronellol	*Oocystis pusilla*	+	+	Ghasemi et al. (2009)
Linalool	*Chlorella* sp., *Chlamydomonas* sp., *Oocystis pusilla*	+	+	Ghasemi et al. (2009), Rasoul-Amini et al. (2010)
		Aldehyde		
Benzaldehyde	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
Heptanal	*Botryococcus braunii*, *Rhodomonas* sp., *Thalassiosira weissflogii*, *Dicrateria inornata*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
Hexanal	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Phormidium autumnale*, *Schizochytrium limacinum*	+	+	Van Durme et al. (2013), Santos et al. (2016b), Hosoglu (2018)
2-methylpropanal	*Phormidium autumnale*, *Nannochloropsis oculata*, *Chaetoceros calcitrans*, *Thassiosira weissflogii*, *Platymonas helgolandica*, *Nitzschia closterium*	+	+	Santos et al. (2016b), Zhou et al. (2017)
3-methylbutanal	*Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Phormidium autumnale*	+	+	Van Durme et al. (2013), Santos et al. (2016b)
Nonanal	*Botryococcus braunii*, *Rhodomonas* sp., *Nannochloropsis oculata*, *Chlorella vulgaris*, *Thalassiosira weissflogii*, *Nitzschia closterium*, *Chaetoceros calcitrans*, *Platymonas helgolandica*, *Crypthecodinium cohnii*, *Schizochytrium limacinum*, *Chlorella prothecoides*	+	+	Van Durme et al. (2013), Zhou et al. (2017), Xu et al. (2017), Hosoglu (2018)

Continued

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
2,6-nonadienal	*Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*, *Chlorella vulgaris*	+	–	Zhou et al. (2017), Hosoglu (2018)
2-octenal	*Botryococcus braunii*, *Nannochloropsis oculata*, *Thalassiosira weissflogii*, *Nitzschia closterium*, *Chaetoceros calcitrans*, *Dicrateria inornata*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
2-pentenal	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	–	Durme et al. (2013), Zhou et al. (2017)
Acetaldehyde	*Phormidium autumnale*	+	+	Vieira et al. (2019)
	Sulfurs			
Benzothiazole	*Phormidium autumnale*, *Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*	+	–	Santos et al. (2016b), Zhou et al. (2017), Vieira et al. (2019)
Dimethyl disulfide	*Rhodomonas* sp., *Tetraselmis chuii*	+	–	Van Durme et al. (2013), Lee et al. (2017)
Dimethyl sulfide	*Chaetoceros calcitrans*, *Chlorella protothecoides*, *Chlorella vulgaris*, *Crypthecodinium cohnii*, *Nannochloropsis* sp., *Oscillatoria chalybea*, *Oscillatoria tenuis*, *Phormidium autumnale*, *Plectonema boryanum*, *Synechococcus cedrorum*, *Tetraselmis chuii*, *Thalassiosira weissflogii*	+	+	Watson (2003), Van Durme et al. (2013), Zhou et al. (2017), Hosoglu (2018), Lee et al. (2017)
Dimethyl trisulfide	*Rhodomonas* sp., *Tetraselmis chuii*	+	–	Van Durme et al. (2013), Lee et al. (2017)

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
		Alcohol		
Benzyl alcohol	*Phormidium autumnale*, *Crypthecodinium cohnii*, *Schizochytrium limacinum*, *Chlorella prothecoides*, *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Santos et al. (2016b), Zhou et al. (2017), Hosoglu (2018)
cis-2-penten-1-ol	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	–	Van Durme et al. (2013), Zhou et al. (2017)
Ethanol	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
1-hexanol	*Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Phormidium autumnale*	+	+	Van Durme et al. (2013), Santos et al. (2016b)
3-hexen-1-ol	*Chlorella vulgaris*	+	–	Van Durme et al. (2013)
2-ethyl-1-hexanol	*Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*, *Spirulina platensis*, *Nostoc* sp.	+	–	Milovanović et al. (2015), Zhou et al. (2017)
Cyclohexanol	*Phormidium autumnale*	+	–	Vieira et al. (2019)
Isobutanol	*Phormidium autumnale*	+	+	Santos et al. (2016b)
2-methylbutanol	*Tetraselmis* sp., *Nannochloropsis*, *Chlorella vulgaris*	+	+	Van Durme et al. (2013)
3-methylbutanol	*Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Phormidium autumnale*	+	+	Hasegawa et al. (2012), Van Durme et al. (2013), Santos et al. (2016b), Vieira et al. (2019)

Continued

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
1-octen-3-ol	*Rhodomonas* sp., *Nannochloropsis oculata*, *Chlorella vulgaris*, *Crypthecodinium cohnii*, *Chlorella prothecoides*, *Tetraselmis chuii*, *Schizochytrium limacinum*	+	+	Van Durme et al. (2013), Hosoglu (2018)
2-phenylethyl alcohol	*Crypthecodinium cohnii*	+	+	Hosoglu (2018)
1-pentanol	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
1-penten-3-ol	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia Closterium*, *Phormidium autumnale*	+	+	Van Durme et al. (2013), Zhou et al. (2017), Vieira et al. (2019)
2-methyl-1-pentanol	*Phormidium autumnale*	+	–	Vieira et al. (2019)
	Hydrocarbons			
2,4-dimethylheptane	*Scenedesmus obliquus*	+	+	Severo et al. (2018)
Dodecane	*Microcystis flos-aquae*, *Microcystis aeruginosa*	+	–	Xu et al. (2017), Zuo et al. (2018a,b)
Heptadecane	*Spirulina platensis*, *Nostoc* sp., *Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*, *Microcystis flos-aquae*, *Microcystis aeruginosa*	+	–	Milovanović et al. (2015), Zhou et al. (2017), Xu et al. (2017), Zuo et al. (2018a,b)
Hexadecane	*Spirulina platensis*, *Nostoc* sp., *Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*, *Microcystis flos-aquae*, *Microcystis aeruginosa*	+	–	Milovanović et al. (2015), Zhou et al. (2017), Xu et al. (2017), Zuo et al. (2018a,b)

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
Pentadecane	*Spirulina platensis*, *Nostoc* sp., *Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*	+	–	Milovanović et al. (2015), Zhou et al. (2017)
Tetradecane	*Spirulina platensis*, *Nostoc* sp., *Nitzschia closterium*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, *Platymonas helgolandica*, *Nannochloropsis* sp., *Dicrateria inornata*, *Microcystis flos-aquae*, *Microcystis aeruginosa*	+	–	Milovanović et al. (2015), Zhou et al. (2017), Xu et al. (2017), Zuo et al. (2018a,b)
Tridecane	*Microcystis flos-aquae*, *Microcystis aeruginosa*	+	–	Xu et al. (2017), Zuo et al. (2018a,b)
	Furan			
2-ethylfuran	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	–	Van Durme et al. (2013), Zhou et al. (2017)
2-penthylfuran	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
	Ketones			
3-hydroxy-2-butanone	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
Acetyl valeryl	*Phormidium autumnale*	+	+	Vieira et al. (2019)
2,3-butanedione	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017)

Continued

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
2-heptanone	*Phormidium autumnale*	+	+	Vieira et al. (2019)
6-methyl-5-hepten-2-one	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochlopsis*, *Phormidium autumnale*	+	+	Van Durme et al. (2013), Santos et al. (2016b), Vieira et al. (2019)
2-octanedione	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	–	Van Durme et al. (2013), Zhou et al. (2017)
2-nonanone	*Phormidium autumnale*	+	+	Vieira et al. (2019)
3,5-octadien-2-one	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*, *Chaetoceros calcitrans*, *Dicrateria inornata*, *Platymonas helgolandica*	+	–	Van Durme et al. (2013), Zhou et al. (2017)
2-propanone	*Scenedesmus obliquus*	+	+	Severo et al. (2018)
2,3-pentenedione	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Nitzschia closterium*	+	+	Van Durme et al. (2013), Zhou et al. (2017).
1-penten-3-one	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Chlorella vulgaris*, *Nitzschia closterium*, *Chaetoceros calcitrans*, *Dicrateria inornata*, *Platymonas helgolandica*	+	+	Van Durme et al. (2013), Zhou et al. (2017)
Ester				
Methyl octanoate	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis* sp., *Nannochlopsis*, *Crypthecodinium cohnii*, *Chlorella prothecoides*, *Tetraselmis chuii*, *Schizochytrium limacinum*	+	+	Van Durme et al. (2013), Hosoglu (2018)

Table 24.2: VOCs detected in different strains of microalgae, and a comparative with chemical production and naturally extracted—cont'd

Chemical name	Microalgae	Chemical	Natural	References
Methyl 3-methyl 2-hydroxybutanoate	*Phormidium autumnale*	+	–	Vieira et al. (2019)
Methyl phenylacetate	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochlopsis*, *Crypthecodinium cohnii*, *Chlorella prothecoides*, *Schizochytrium limacinum*	+	–	Van Durme et al. (2013), Hosoglu (2018)
2-methoxy-2-methylpropane	*Scenedesmus obliquus*	+	–	Severo et al. (2018)

(+) indicates production or (−) no VOCs production chemically produced and naturally extracted.

Table 24.3: Volatile organic compounds generated by microalgae and their energy potential.

Volatile organic compounds	Energy potential ($MJ\,kg^{-1}$)
2-ethyl-1-hexanol	5.42
2-propil-1-heptanol	6.72
2-methylbutanal	3.24
Hexanal	3.88
2,4-heptadienal	2.97
2,4-decadienal	6.0
2-methoxy-2-methyl-propane	3.48
3,3-dimethyl-hexane	5.42
2,4-dimethyl-heptane	6.07
4,7-dimethyl-undecane	8.39
2-propanone	1.94
2,4-dimethyl-3-pentanone	4.53
4-octen-3-one	5.18
6-methyl-5-hepten-2-one	4.94
Acetophenone	4.22
β-ionone	7.70
2-phenylpropene	4.87
Total	86.32

Adapted from Deprá et al. (2018).

Limited research is available on the economic implications of investment and operational costs of microalgae-based systems (Banerjee and Ramaswamy, 2019). However, microalgae have the advantage of performing wastewater treatment, removing inorganic nutrients and fetid compounds, and in parallel, providing high-value biomass production with the potential to exploit multiple products (Leite et al., 2019; Jacob-Lopes et al., 2019).

These facts make it clear that microalgae-based systems are seen as an alternative that allows for marked improvements in wastewater treatment plants, and may result in the extinction of some traditional unit operations, like odor removal technologies. In addition, this process results in the production of VOCs with odor descriptors of interest to different industries (Vieira et al., 2019).

Finally, although hundreds of VOCs have been identified in cultures of microalgae, the induction of synthesis is in most cases unknown, and the separation and recovery of the compounds need to be optimized. Thus, their insertion into commercial products is subject to further research and development (Pinheiro et al., 2019).

24.5 Techniques for VOCs recovery

Commercial production of volatile organic compounds obtained biotechnologically requires economic profitability. The biosynthesis of microalgae-based products is generally limited by low productivity or low concentrations of main compounds in the bioreactor. In order to achieve high yields and productivity, it is important to choose a reactor design carefully and select a convenient system for the recovery of volatile compounds (Akachaa and Gargouri, 2015).

Currently, some techniques can be exploited for the separation and recovery of VOCs in bioreactors, such as adsorption, condensation, absorption, and membrane-based techniques, which may assist microalgae-based processes when a compound or a group thereof need to be obtained separately (Wylock et al., 2015; Try et al., 2018; Saffarionpour and Ottens, 2018).

In the condensation-based recovery system, the gas stream of the headspace of the bioreactor passes through the vertical trap column placed in a cryogenic bath containing liquid nitrogen, which allows VOC vapor to condense (Saffarionpour and Ottens, 2018). Condensation is used to separate VOCs from a plant matrix, or microalgae biomass, that consists mainly of carbohydrates and nonvolatile lipids (Lukin et al., 2018).

Another technique is adsorption, widely used in the recovery of VOCs from the bioreactors, being a process based on the ability of a solid (e.g., adsorbent) to connect a gaseous component (e.g., adsorbate) to its surface (Saffarionpour and Ottens, 2018). The adsorption of volatile compounds in solid materials, as in microalgae biomass, is more widely used for the quantitative analysis of these compounds on a laboratory scale (Lukin et al., 2018). This type of adsorbent is used to remove VOCs from industrial gases, as well as in wastewater treatment plants (Lebrero et al., 2011).

In the absorption technique, a gas stream is put into contact with a liquid in order to transfer one or several gaseous components into the liquid phase. The absorption devices can be used as a single operation with a reactant dissolved in the liquid phase, or can be used with a

non-reacting liquid. This device is coupled with an adsorbent, in order to regenerate the absorbing liquid (Wylock et al., 2015). This is the principle of bioscrubbers, which are applied in wastewater treatment plants as a biological treatment in the odor removal (Lebrero et al., 2011).

Overall, adsorption is in principle highly comparable with absorption and can be useful for both wastewater odor abatement and industrial recovery of VOCs, of the liquid and gaseous phase in microalgae-based systems (Lebrero et al., 2011; Lebrero et al., 2014; Lukin et al., 2018).

Membrane-based techniques, known as pervaporation, have as the principle of separating liquid mixtures through a dense membrane with the gas flow (Try et al., 2018). Pervaporation demonstrates significant advantages for the recovery of aroma compounds and hydrophobic molecules (Lukin et al., 2018). Pervaporation is an emerging technology with significant potential to recover alcohols and other biofuels efficiently from a microalgae bioreactor (Vane, 2005). Heymes et al. (2007) investigated the possibility of removing VOCs from industrial gases by a combination of absorption and pervaporation. Table 24.4 shows the technologies and VOC recovery efficiency.

The techniques proposed for the recovery of VOCs aim to minimize their losses and recover the major components, which are valuable in producing a high-quality final product for industrial application. These technologies can be applied in different industry for VOCs recovery such as the chemical, petrochemical, and pharmaceutical industries, and the food processing industry. In addition, they can be used in the treatment of gaseous wastewater released by these industries, contributing to reduction of olfactive and environmental pollution (Wylock et al., 2015; Saffarionpour and Ottens, 2018).

The recovery of volatile compounds from microalgae is challenging because the compounds are present at low concentrations; the biomass is present as solid content; within the solid phase the volatile may be intracellular or membrane-bound; and the compound may be located and distributed in different phases, such as solid, liquid, and gaseous (López-pérez et al., 2017; Achyuthan et al., 2017).

Thus, industrial recovery may be particularly limiting; as such, VOCs are expected to partition between different phases, sometimes requiring different recovery techniques for each phase, and in some cases additional steps. Fig. 24.7 shows the application possibilities of VOCs recovery techniques in microalgae-based systems.

Aeration and production of CO_2 can lead to loss of VOCs in the headspace of microalgae bioreactors as a result of the volatility of the molecules. According to Mackay and Yuen (1980), chemical substances can be found in the following volatility classes, based on Henry's law constant H: highly volatile, $H > 1 \times 10^{-3}$ atm m^3 mol^{-1}; volatile, $1 \times 10^{-5} < H < 1 \times 10^{-3}$ atm m^3 mol^{-1}; with slow volatilization for $3 \times 10^{-7} < H < 1 \times 10^{-5}$ atm m^3 mol^{-1}; and with negligible

Table 24.4: Comparison of the characteristics of the potential technologies for recovery of VOCs.

Technologies	Membranes separation	Condensation	Adsorption	Absorption
Industrial applications	Environmental depollution; solvent recovery	Environmental depollution; food volatile compounds recovery	Environmental depollution; capture of VOCs	Environmental depollution; odor reduction
Type of VOCs	Alcohols, alkanes, aromatic hydrocarbons	Hydrocarbons, ketones, aldehydes, alcohols, furan	Esters, aldehydes, alcohols, hydrocarbons	Hydrocarbons
Mediators	Polymer membranes	Liquid nitrogen	Activated carbon; porous resin	Water; high-boiling hydrocarbons
Recoveries efficiency	>90%	>95%	>99%	95%–99%
Advantages	Cyclic operating; easy recycling of membranes; no additive required; no further treatment of recovered VOCs; operates under mild conditions	Ideal for high concentrated gas stream	Good recovery efficiency	Easy to set up; reuse of absorbent liquid; used in a wide range of concentration
Disadvantages	Costly and rarely available membranes; susceptibility of membranes to fouling and bacterial growth (inducing clogging and possibly VOCs alteration)	High energy consumption; cooling fluid use; not suitable for compounds with boiling points above 37°C	Less selectivity; poor regeneration of adsorbent; use of solvent for desorbing; susceptible to clog; not suitable for cyclic operation; require humidity control	Use of a large amount of absorbing liquid; the need for posttreatment for the regeneration of absorbing liquid

Adapted from Wylock, C., Eloundou Mballa, P.P., Heilporn, C., Debaste, F., Fauconnier, M.-L., 2015. Review on the potential technologies for aromas recovery from food industry flue gas. Trends Food Sci. Technol. 46(1), 68–74. https://doi.org/10.1016/j.tifs.2015.08.002.

volatilization for $H < 1 \times 10^{-7}\,atm\,m^3\,mol^{-1}$. With vast diversity across VOCs from microalgae, the volatility of individual molecules varies greatly, requiring different recovery approaches (Lukin et al., 2018).

From the techniques presented, condensation seems less suitable for the recovery of hydrophobic VOCs from microalgae-based processes, because of the large volumes of water evaporated and the presence of compounds with low concentration. However, pervaporation shows higher potential for hydrophobic VOCs recovery from a bioreactor. The wide use of absorption for the removal of odorous volatile organic compounds from industrial gases, like

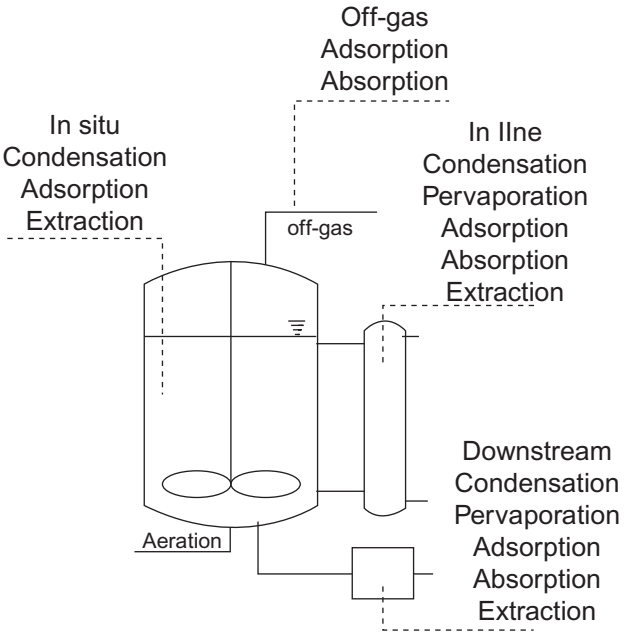

Fig. 24.7

Application possibilities of VOCs recovery techniques at different points in the microalgae-based system. Definitions: *in situ*, product recovery in the bioreactor during production; *off-gas*, product recovery from the reactor off-gas during production; *in line*, product recovery in the external loop during production; *downstream*, external product recovery after production.

wastewater odor abatement, makes its application for VOCs recovery from a microalgae-based system imaginable (Heymes et al., 2007; Lukin et al., 2018).

24.6 Conclusions and future perspectives

Microalgae can produce a variety of volatile compounds, and knowledge about the characterization and morphology of the microalgae, metabolic pathways, VOCs biosynthesis, and optimization of culture systems enables exploitation for many relevant commercial applications. However, some hurdles must be overcome for these bioprocesses to be included in the market, such as improving biochemical and genetic engineering strategies to boost VOCs production, because until now the yields of the products have been too low to make the biotechnological process competitive. Moreover, microalgae VOCs are a blend of compounds, and in order to select the most advantageous volatile compound recovery technique, it is necessary to investigate the location of the target compound within the biochemical system as well as the volatility of the target molecule and its partitioning between the phases under real production conditions.

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CHAPTER 25

Microalgae as enzymes biofactories

Michele Rigon Spier[a]*, Bianca Peron-Schlosser[a], Luana Cristina Paludo[a], Luis Alberto Gallo-García[a], Cristina Maria Zanette[b]

[a]*Post Graduate Program in Food Engineering, Federal University of Paraná, Curitiba, PR, Brazil*
[b]*Department of Food Engineering, Midwestern State University (UNICENTRO), Guarapuava, Brazil*
**Corresponding author.*

Chapter outline

25.1 Introduction 687
25.2 Microalgal enzymes 689
25.2.1 Amylases 689
25.2.2 Peroxidases 693
25.2.3 Proteases 694
25.2.4 Lipases 694
25.2.5 Phytases 695
25.2.6 Superoxide dismutases (SODs) 695
25.2.7 Carbonic anhydrase 696
25.2.8 Laccases 698
25.2.9 Galactosidases 698
25.2.10 L-asparaginase 699
25.3 Final considerations 700
References 701

25.1 Introduction

For more than 100 years, microalgae have been studied. Nowadays, open-pond systems have increased their cultivation on a large scale. The algal biomass world market is estimated at USD 3.8–5.4 billion, and its production is approximately 7000 tonnes per year (Brasil et al., 2017b; Enzing et al., 2014). The forecast report on the global microalgae market for the period 2017–2026 predicts that the market will grow moderately by 2026. World microalgae sales are predicted to exceed USD 75 million by 2026 (Persistence Market Research, 2018).

Microalgae are unicellular microorganisms found in marine and freshwaters, with the capacity to convert the solar energy, through photosynthesis, into chemical energy due to the presence of

Handbook of Microalgae-Based Processes and Products. https://doi.org/10.1016/B978-0-12-818536-0.00025-7

pigments (e.g., chlorophyll). The category includes a range of eukaryotic algae and prokaryotic (cyanobacteria). These microorganisms are considered an ideal feedstock for large-scale biomolecules production, since they are biofactories fed by solar energy with minimum nutritional requirements. They use sunlight with great efficiency, which increases their potential for energy use or for the production of compounds with high added value (e.g., biofuels, food supplements, animal feed, pharmaceuticals, nutraceuticals) (Andersen and Kawachi, 2005; Brasil et al., 2017b; Collins et al., 2014; Gangl et al., 2015; Masojídek and Torzillo, 2009; Pulz and Gross, 2004). In addition, microalgae can grow under heterotrophic, photoautotrophic, and/or mixotrophic conditions. Under photoautotrophic conditions, microalgae need light, CO_2, water, some salts, and a nitrogen source, which is an advantage for industry (Georgianna et al., 2013).

While there is no production of microalgae by enzymes on a large scale, it has been reported that microalgae have a great capacity for the synthesis of enzymes for industrial applications such as amylases, galactosidases, and phytases, among others (Brasil et al., 2017a). The application of these enzymes is in different industries (e.g., pharmaceutical, food, textile, fuel, detergent) and it is estimated that in 2024, production will be USD 9.63 billion (Grand View Research, 2016). The demand for these biomolecules has been growing over the years due to the wide range of applications in industry (Joshi and Satyanarayana, 2015).

In industrial terms, the production of extracellular enzymes is simpler than the production of intracellular enzymes. It could be produced on a large scale, making the purification process easier and cheaper (Hosamani and Kaliwal, 2011). Several biomolecules such as enzymes, are produced intracellulary by microalgae. So, for industrial production, it requires more unit operations steps for microalgal cell wall disruption aiming enzymes recovery, separation, and purification. It presents disadvantages comparing to microbial cells which synthesis and excrete it, because in this case, eliminates at least one unit operations of cell wall disruption; also they have a longer cultivation time compared to other microorganisms genus (yeast and bacteria). Nevertheless, major technological advances have improved lysis techniques by providing high yields in the extraction of these metabolites (Brennan and Owende, 2010; Gil-Chávez et al., 2013).

According to Brasil et al. (2017b) it is impossible to compare the production of industrial enzymes from microalgae with those from bacteria or fungi. Taking advantage of using all microalgal biomass in an integrated biorefinery approach (Gifuni et al., 2019) is a promising alternative to minimize the cost of producing microalgal enzymes and improving the economic sustainability of the process (Brasil et al., 2017a). Fig. 25.1 shows the applications of a microalgae biorefinery.

Therefore, this chapter focuses on the potential of microalgae for the synthesis of enzymes for industrial applications. Enzymes described include amylases, galactosidases, proteases, phytases, laccases, peroxidases, lipases, asparaginases, antioxidant enzymes, and carbonic anhydrase.

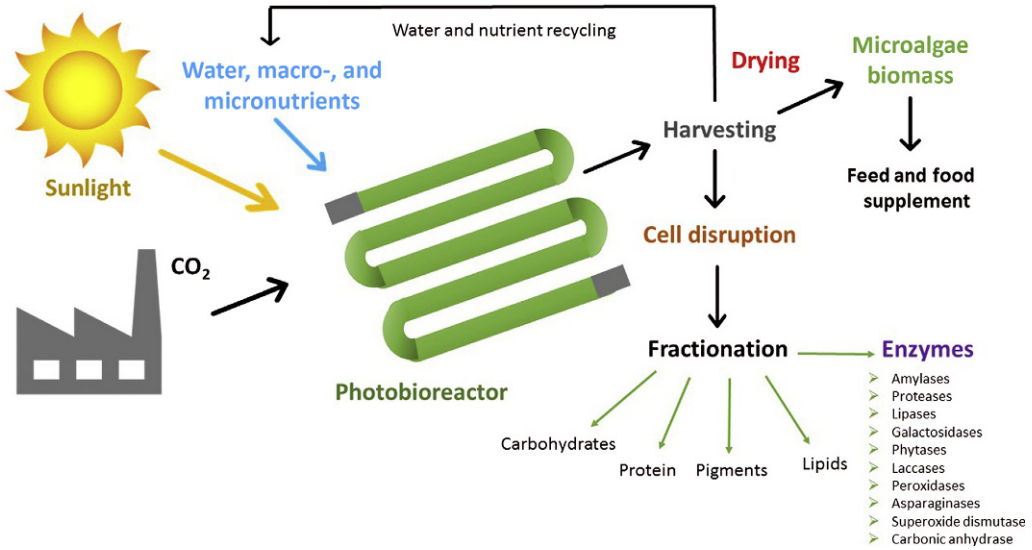

Fig. 25.1
Schematic illustration of microalgae biorefinery and the production of high-value-added products such as carbohydrates, proteins, pigments, lipids, and enzymes.

25.2 Microalgal enzymes

The increased application of biocatalysts in industrial processes has led to research on new sources of microbial enzymes. The current demand for enzymes is large and the production is still not adequate to meet industrial demand (Mogharabi and Faramarzi, 2016).

Enzymes obtained from microorganisms are more stable than those isolated from plants and animals; therefore, microalgae are a promising source for enzyme production. Microalgae can grow with low nutritional requirements with high biomass production and are susceptible to genetic manipulation (Gong and Jiang, 2011; Mogharabi and Faramarzi, 2016).

Although there is no industrial production of enzymes from microalgae, some studies report the ability of microalgae cells to synthesize enzymes (Table 25.1) (Brasil et al., 2017b). According to Radakovits et al. (2010), over the last decade significant advances have been achieved in algal genomics, which suggest that algae are a low-cost potential host for the production of enzymes, recombinant proteins, and new metabolites.

25.2.1 Amylases

Amylases are a group of enzymes that are part of the family of glycohydrolases (GH), which carry out the hydrolysis of starch, oligosaccharides, and polysaccharides (Mohanan and Satyanarayana, 2019). Several amylases belong to the GH group, such as α-amylases (EC

Table 25.1: Examples of enzymes produced by microalgae and their potential industrial applications.

Enzyme	Microalgae	Application	Author
Amylase	*Spirogyra* sp. *Chlorella sorokiniana* *Scenedesmus* sp. *Chlamydomonas reinhardtii* *Dunaliella marina* *Oedogonium* sp. *Rhizoclonium* sp. *Klebsormidium* sp. *Rhizoclonium hieroglyphicum*	Starch conversion, detergent industry, fuel alcohol production, food industry, textile industry, paper industry	Patil and Mahajan (2016) Kombrink and Wöber (1980) Levi and Gibbs (1984) Manoj et al. (2018)
Protease	*Cladophora vagabunda* *Chlorella vulgaris* *Arthrospira platensis* *Anabaena variabilis*	Detergent industry, food industry, pharmaceutical industry	Nanni et al. (2001) Yada et al. (2005) Maldener et al. (1991) Lockau et al. (1988) Silva et al. (2017)
Superoxide dismutase	*Phaeodactylum tricornutum* *Porphyridium* sp. *Anabaena* sp. *Synechococcus* sp. *A. platensis* *Pseudanabaena* sp. *Synechococcus nidulans* *Anabaena cylindrica* *Porphyridium cruentum*	Therapeutic potential (due to antioxidant and antiinflammatory actions, SOD exerts preventive and agrochemical effects to control oxidative stress induced by free radicals or reactive oxygen species in practically all tissues) and cosmetic industry	Guzmán-Murillo et al. (2007) Gunes et al. (2015) Canini et al. (1992) Misra and Fridovich (1977) Priya et al. (2007) Ismaiel et al. (2014) Sannasimuthu et al. (2018)
Carbonic anhydrase	*Desmodesmus* sp. *Kirchneriella* sp. *Acutodesmus* sp. *Chlamydomonas reinhardtii* *Chlorella* sp. *Pleurochrysis carterae* *Phaeodactylum tricornutum* *Dunaliella salina* *Dunaliella parva* *Chlorella vulgaris* *Dunaliella peircei* *Dunaliella primolecta* *Dunaliella tertiolecta*	Biomedical applications (artificial lungs, biosensors and CO_2 sequestration systems, drugs) and biofuel production	Swarnalatha et al. (2015) Badger and Price (1994) Satoh et al. (2001) Dionisio-Sese and Miyachi (1992)
Peroxidase	*Coelastrella* sp. *Porphyridium purpureum* *Phaeodactylum tricornutum* *Dunaliella tertiolecta* *Euglena gracilis Z* *E. gracilis* var. *bacillaris* *Astasia longa* *Galdieria sulphuraria*	Paper industry (analysis and diagnostic kits, discoloration of synthetic dyes), food industry (peroxidase has been widely used as an indicator of the suitability of vegetable bleaching due to its relatively high thermal stability and wide distribution)	Baldev et al. (2013) Oesterhelt et al. (2008) Murphy et al. (2000) Overbaugh and Fall (1985) Overbaugh and Fall (1982)

Table 25.1: Examples of enzymes produced by microalgae and their potential industrial applications—cont'd

Enzyme	Microalgae	Application	Author
Phytase	*Synechococcus lividus* *S. bigranulatus* *Chroococcidiopsis thermalis*	Food and animal feed industries (hydrolyze the ester bonds of phosphatidylinositol and release phosphorus from materials of plant origin, as well as destroying the existing chelates in the phytate structure and releasing nutrients such as minerals, proteins, and starch)	Klanbut et al. (2002)
Galactosidase	*Poterioochromonas malhamensis* *Tetradesmus obliquus* *Dunaliella tertiolecta* *Chlorella minutissima* *Nannochloropsis oculata* *Scenedesmus obliquus* *Acutodesmus obliquus* *Cosmarium* sp.	Food, sugar, pulp, paper, and animal feed industries; β-galactosidase is used to hydrolyze the lactose of dairy products	Dey and Kauss (1981) Suwal et al. (2019) Zanette et al. (2019) Bentahar et al. (2018) Girard et al. (2014) Davies et al. (1994)
Laccase	*Tetracystis aeria* *Chlamydomonas moewusii* *Scenedesmus vacuolatus* *A. platensis*	Used for bioremediation in the pulp, paper, and food industry for its ability to use ambient O_2 to oxidize various compounds including mono and polyphenols, amines, aromatic amines, and methoxyphenols with concomitant reduction of oxygen to water; as well as for biotechnological applications, such as tea and wine clarification, biodegradation of industrial effluents and xenobiotics, discoloration of dyes, ethanol production, and use in biosensors	Otto et al. (2010) Afreen et al. (2017) Otto et al. (2015)
Lipase	*Botryococcus sudeticus* *Nannochloropsis oceanica* *Isochrysis galbana* *A. platensis*	Detergent, food, and flavor industry, biocatalytic resolution of pharmaceuticals, esters, and amino acid derivatives, manufacture of fine chemicals, agrochemicals, use as biosensor, bioremediation, and in cosmetics and perfumery	Yong et al. (2016) Savvidou et al. (2016) Hubert et al. (2017) Godet et al. (2012) Demir and Tükel (2010)

Continued

Table 25.1: Examples of enzymes produced by microalgae and their potential industrial applications—cont'd

Enzyme	Microalgae	Application	Author
L-asparaginase	*Chlamydomonas* sp. *Chlorella vulgaris* *Arthrospira maxima* *A. platensis* *Synechococcus elongatus*	Food industries (to reduce the acrylamide formation during food processing) and pharmaceuticals industries (treatment of patients with acute lymphocytic leukemia)	Paul and Cooksey (1979) Ebrahiminezhad et al. (2014) Prihanto and Wakayama (2014) El Baky and El Baroty, 2016Kebeish et al. (2016)

3.2.1.1, 1,4-α-D-glucan glucanohydrolase), maltogenic amylase (EC 3.2.1.33), β-amylase (EC 3.2.1.2), amyloglucosidase (also named glucoamylase, EC 3.2.1.3), pullulanase (EC 3.2.1.41), and isoamylase (EC 3.2.1.68) (Ahlawat et al., 2018; van Oort, 2010). Amylases marketed and used in industry are mainly from microbial sources (Frantz et al., 2019), due to their biochemical versatility, higher production rate, stability, and easy availability of a huge number of microbial strains (Simair et al., 2017).

According to Zhang et al. (2017), a variety of microbial α-amylases have been extensively studied and applied since the earliest reports in the 1920s of industrial bacterial amylase. In the enzyme market, amylases represent the largest part (25%–30%); these are among the most important enzymes with industrial applications (Frantz et al., 2019). There are many commercial applications of amylases, such as in the food and feed industries (e.g., the saccharification and liquefaction of starch, cheese ripening, flavoring, flour, infant cereal, animal feed, brewing industry, sugar induction by the production of sugar syrups), ethanol production, pharmaceuticals, cosmetics, detergents, textiles, and the paper industry (Paludo et al., 2018; Simair et al., 2017; de Souza and de Magalhães, 2010; Zhang et al., 2017).

Patil and Mahajan (2016) reported that among the essential enzymes found in microalgae, amylases are the least found because microalgae are autotrophic. However, in their study, the profile of extracellular microalgae enzymes of microalgae cultured in M342 (containing 1% starch) medium was investigated. The authors demonstrated that *Spirogyra* sp. showed an amylase activity of $0.042\,\mu mol\,min^{-1}\,mL^{-1}$, *Oedogonium* sp. ($0.035\,\mu mol\,min^{-1}\,mL^{-1}$), *Chlorella sorokiniana* ($0.027\,\mu mol\,min^{-1}\,mL^{-1}$), and *Scenedesmus obliquus* ($0.009\,\mu mol\,min^{-1}\,mL^{-1}$).

Amylase has also been localized in the chloroplast of *Chlamydomonas reinhardtii*, with an enzymatic activity of 7.3–42 $\mu mol\,h^{-1}$ $(10^9\ cells)^{-1}$, and an ideal pH to achieve maximum activity of 6.7–7.6 (Levi and Gibbs, 1984). Kombrink and Wöber (1980) identified amylase in

crude extracts of the unicellular green alga *Dunaliella marina*, with enzymatic activity and specific enzyme activity of 612.6 ηmol min^{-1} and 9.6 ηmol min^{-1} $mg_{protein}{}^{-1}$, respectively.

Amylase activity was also evaluated in terrestrial microalgae such as *Rhizoclonium* sp. (30.620 U $mg_{maltose}$), *Spirogyra* sp. (30.217 U $mg_{maltose}$), *Klebsormidium* sp. (26.386 U $mg_{maltose}$), *Rhizoclonium hieroglyphicum* (30.553 U $mg_{maltose}$), and *Oedogonium* sp. (30.116 U $mg_{maltose}$) (Manoj et al., 2018).

25.2.2 Peroxidases

Peroxidases (EC 1.11.1.7) use hydrogen peroxide to catalyze the oxidation of a variety of organic and inorganic compounds. Plant peroxidases, animal peroxidases, and catalases are the three categories of peroxidases (Regalado et al., 2004; Shivakumar et al., 2017).

Peroxidase is found in nature and is used for the degradation of pollutants and pesticides, discoloration of dyes, as biosensors, in the pulp-paper industry, and in sewage treatment (Bansal and Kanwar, 2013; Regalado et al., 2004). An example of its application can be seen in the process water of textile industries, which normally have a strong coloration due to the presence of rhodamine dyes, which are resistant to the traditional bleaching process and can be degraded by peroxidase (Huber and Carré, 2012). The unique ability of white rot fungi to degrade lignin is largely associated with nonspecific oxidative reactions mediated by free radicals carrying out their extracellular peroxidases (Lundell et al., 2010).

Baldev et al. (2013) evaluated the peroxidase produced by the microalgae *Coelastrella* sp. for discoloration of the synthetic dye Rhodamine B. The cell-free supernatant presented the enzymatic activity of 2.1 μmol min^{-1} $mg_{protein}{}^{-1}$ under optimal operating conditions with pH 8 at 30°C. The activity of 7.9 U $mg_{protein}{}^{-1}$ was verified in *Galdieria sulphuraria* and studies have shown that this enzyme is stable to heat and acid conditions, with a molecular mass of 40 kDa (Oesterhelt et al., 2008). Manoj et al. (2018) evaluated the peroxidase activity for several microalgae; the ones with the highest enzymatic activity were *Rhizoclonium* sp. (Dandiganahalli) and *Oedogonium* sp. with activities of 0.21 μmol min^{-1} g^{-1}, whereas *Rhizoclonium* sp. (*Happanadka*) obtained the lowest activity (0.01 μmol min^{-1} g^{-1}) .

Murphy et al. (2000) analyzed the enzymatic production for *Porphyridium purpureum*, *Phaeodactylum tricornutum*, and *Dunaliella tertiolecta*, with specific activities of 5.2, 1.5, and 0.17 μmol min^{-1} $mg_{protein}{}^{-1}$, respectively. *Euglena gracilis Z.*, *E. gracilis* var. *bacillaris*, and *E. gracilis* var. *bacillaris* W3BUL were evaluated by Overbaugh and Fall (1985) and the activities ranged from 42 to 80 mU $mg_{protein}{}^{-1}$. Overbaugh and Fall (1982) evaluated the glutathione peroxidases of microalgae of the species *E. gracilis Z*, *E. gracilis* var. *bacillaris* UTEX884, W3BUL, and *Astasia longa*, obtaining specific activities of 13–400 μmol min^{-1} mg^{-1} of proteins.

25.2.3 Proteases

Proteases refer to enzymes that hydrolyze proteins (Parkin, 2017). They are classified as either *endo-* or exopeptidases, depending on their preferred site of action: while exopeptidases cleave at either the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) ends of a substrate, endopeptidases perform cuts within substrates (Kaiser et al., 2017). Due to their catalytic properties, proteases are used in various industrial processes, such as food, chemicals, and pharmaceuticals (Aguilar and Sato, 2018).

Silva et al. (2017) evaluated the production of protease in *Chlorella vulgaris* cultivated in Bold's Basal Medium (BBM) supplemented with low-cost agro-industrial residue. The maximum enzyme activity was $292\,U\,mL^{-1}$ in medium with 0.5% of corn steep liquor.

Niven (1995) separated by filtration in gel chromatography two proteases from *A. variabilis*, with different substrate specificities. One of the proteases has a molecular weight of 188 kDa and hydrolyzes tri- and di-peptides, while the other has 59 kDa and realizes hydrolysis of oligopeptides that contain a greater chain.

Anabaena variabilis produces a protease that is calcium-dependent, similar to trypsin (Lockau et al., 1988). In turn, Strohmeier et al. (1994) have studied the same species, but a mutant that lacks the calcium-dependent protease. The synthesis of a second soluble protease, like trypsin, and a prolyl endopeptidase was observed.

Nanni et al. (2001) and Yada et al. (2005) carried out studies on protease production by the specie *A. platensis*. Purifications in both studies did not require calcium for enzymatic activity and have 80 kDa molecular weight. In the studies carried out by Yada et al. (2005), a protease produced hydration of fibrin and gelatin, this enzyme is an expression of arginine, known as a protease, known as serine, which performs selective proteolysis of phycobiliproteins.

25.2.4 Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are responsible for the hydrolysis of triacylglycerols having long chains (Guerrand, 2017). These enzymes can be used in various industries, such as food, detergents, pharmaceuticals, and bioremediation, among others (Guerrand, 2017; Hasan et al., 2006). Lipase activity is detected, for example, in milk, oilseeds (soybean, peanut), cereals (oats, wheat), fruits, and vegetables, and in the digestive tract of mammals.

Microbial lipases have attracted industrial attention due to their stability, selectivity, and broad substrate specificity (Dutra et al., 2008; Griebeler et al., 2011). Many microorganisms have been identified as potential producers of extracellular lipases, including bacteria, yeasts, and fungi (Abada, 2008; Treichel et al., 2010).

Demir and Tükel (2010) studied for the first time the purification and characterization of lipase from *A. platensis*. In the crude enzyme solution, total activity was 0.23 U mL^{-1} with specific activity of 0.12 U $mg_{protein}^{-1}$. The lipase was purified about 375-fold with a specific activity of 45 U $mg_{protein}^{-1}$. The molecular weight and isoelectric point of purified lipase were found to be 45 kDa and 5.9, respectively.

According to Yong et al. (2016), the extracellular lipase obtained from *Botryococcus sudeticus* presents wide ranges of temperature resistance (40–70°C), pH tolerance (pH 5–11) and substrate specificities—promising characteristics for commercial application. Hubert et al. (2017) and Godet et al. (2012) investigated the production of lipolytic enzymes from the microalga *Isochrysis galbana*. According to Godet et al. (2012), lipase had a molecular mass of 49 kDa and the pI of 5.65, theoretically. Both studies indicated that microalgae are potential producers of lipolytic enzymes for biotechnological applications.

25.2.5 Phytases

Phytases (myo-inositol-hexakis-phosphate-phosphohydrolase) are enzymes that catalyze the hydrolysis of the antinutritional factor phytic acid and its salts (phytates), and release phosphorus and other minerals in inorganic form (Song et al., 2019; Spier et al., 2018). Phytic acid is the main form (80%) of phosphorus predominantly in cereal grains, legumes, oilseeds, and their by-products (Spier et al., 2018, 2015; Vasudevan et al., 2019). Phytase has a greater application in industry because it is an important enzyme for human and animal health, as it acts in the digestive process. Due to its ability to prevent the antinutritional effects of phytic acid, it increases the bioavailability of important nutrients that positively reflect on overall health (Haefner et al., 2005; Song et al., 2019). Plants, animals, and microorganisms (e.g., yeast, fungi), among others, are sources for phytases production (Spier et al., 2018; Vohra and Satyanarayana, 2003).

Four species of blue-green algae were studied by Klanbut et al. (2002); the species *Synechococcus lividus*, *S. bigranulatus Skuja*, and *Chroococcidiopsis thermalis Geitler* showed only intracellular phytase activity. *S. lividus* was the species that presented the highest enzymatic activity of 1.83 mU mL^{-1}.

25.2.6 Superoxide dismutases (SODs)

Superoxide dismutases (SODs; EC 1.15.1.1) are metalloenzymes that may contain iron, manganese, or copper/zinc and act against free radical cytotoxic superoxides as antioxidants, which defend cells against oxidative stress (de Raposo et al., 2013).

Currently, five isoforms of SOD have been detailed, in which metallic cofactors such as Cu/ZnSOD, MnSOD, FeSOD, NiSOD, and Fe/ZnSOD are found, being considered isoforms due to diverse differential analysis results (Ismaiel et al., 2014). SOD is one of the most important

enzymes involved in the conversion of superoxide radicals O_2^- into O_2 and H_2O_2, protecting cells and tissues from oxidative damage (Fridovich, 1995). In microalgae, SOD have an important role in protecting cells against oxidative stress caused by various methods, including chemical and biological stresses, heavy metals, and radiations (Sannasimuthu et al., 2018).

Synechococcus nidulans, *Pseudanabaena* sp., and *A. platensis* showed specific activities of superoxide dismutases of 50.4, 18.4, and 30.0 U mg^{-1}, respectively (Gunes et al., 2015). The production of SOD by *Phaeodactylum tricornutum* was similar to the production of the yeast *Debaryomyces hansenii*, by testing different substrate compositions for enzymatic production (Guzmán-Murillo et al., 2007). *Porphyridium cruentum* showed a stable Mn-SOD in thawing and freezing at 40 kDa molecular weight (Misra and Fridovich, 1977). Manoj et al. (2018) investigated and compared SOD activities in *Rhizoclonium* sp., *Spirogyra* sp., *Klebsormidium* sp., *Rhizoclonium hieroglyphicum*, and *Oedogonium* sp., and found the specific activities of 124.40, 186.2, 168.66, 178.91, and 92.23 U $mg_{protein}^{-1}$, respectively.

SOD is a primary antioxidant that defends against reactive oxygen species induced by environmental stress. Antioxidants can be found in either enzymatic or nonenzymatic form, the latter not being considered as effective a detoxifying mediator as the enzymatic group (Wolfe-Simon et al., 2005). The enzymatic group includes catalases, peroxidases, and SOD, the latter being valued as the main defense column against investigations to expose the protective role of SODs of cyanobacteria (Herbert et al., 1992).

Sannasimuthu et al. (2018) stated that in living cells, enzymes such as SOD contribute to the mechanism of protection against reactive oxygen and catalyze the neutralization of the superoxide anion in H_2O_2. In addition, *A. platensis* was found to be a potential cyanobacteria that can tolerate an extreme condition of oxidative stress caused by a higher concentration of H_2O_2, and in turn, it was confirmed that SOD plays an important role in this antioxidant function.

Ismaiel et al. (2014) revealed that *A. platensis* MIYE 101 only regulated the isoform of FeSOD under environmental stress conditions (salinity and iron levels). The enzyme was overexpressed in *Escherichia coli* and a size of 23 kDa was reported. The recombinant enzyme showed high activity (530 U mg^{-1}) and pH and thermal stabilities.

25.2.7 Carbonic anhydrase

Carbonic anhydrases (CA) (EC 4.2.1.1) are zinc-containing enzymes that catalyze the hydration and dehydration reactions of CO_2 and carbonic acid (H_2CO_3). The hydration reaction produces bicarbonate ions (HCO_3^-) and hydrogen (H^+) from CO_2, while the dehydration reaction generates CO_2 from carbonic acid (H_2CO_3) as evidenced in the following reaction (Ozsoy, 2019):

$$CO_2 + H_2O \underset{\leftrightarrow}{CA} H_2CO_3 \leftrightarrow H^{++HCO_3^-}$$

CA is an important enzyme in photosynthetic reactions, being part of the carbon concentrating mechanism (Rigobello-Masini et al., 2003). CA is a metalloenzyme that has been investigated over the years in photosynthetic organisms for the production of biofuels (González and Fisher, 2014). This enzyme can be found in algae because this organism has mechanisms of carbon concentration when CO_2 levels are below 3% ($v\,v^{-1}$) (Huang et al., 2017).

One pioneering study that evaluated CA production by microalgae was in several species of *Chlamydomonas reinhardtii* (Amoroso et al., 1998; Badger and Price, 1994). *Chlorella* sp. also showed activity, with an intracellular carbonic anhydrase that converts high levels of CO_2 (Satoh et al., 2001). The effect of salt (NaCl) on the intracellular and extracellular enzymes of CA was studied for several marine microalgae strains. *Phaeodactylum tricornutum* had its activity inhibited by salt. On the other hand, NaCl prompted the activity of *Pleurochrysis carterae*. The extracellular enzymatic activity was affected by NaCl, while the internal activity was not affected in the species *Dunaliella salina* and *Dunaliella parva*; the opposite occurred for *Chlorella vulgaris* and *Dunaliella peircei*. *Dunaliella tertiolecta* species did not show a modification of the internal activity due to the presence of salt, but the external one was increased in the presence of NaCl (Dionisio-Sese and Miyachi, 1992).

The activity of CA in *Tetraselmis gracilis* increased with the inorganic carbon depletion of the substrate. The decrease in carbon dioxide resulted in an increase of CA intracellular activity ($0.58\ UA \times 10^6\ cell^{-1}$) and the decrease in bicarbonate increased the extracellular activity ($0.24\ UA \times 10^6\ cell^{-1}$). The culture medium had a pH of approximately 9 in most of the cultures for carbonic anhydrase production. The CO_2 concentration decreased from 33 µM on the first day to a concentration of 3 µM on the third day and thereafter remained at 0.2 µM. The bicarbonate had a fall of 1.38 mM to a concentration of 0.4 mM simultaneously with the increase in the extracellular carbonic anhydrase enzyme (Rigobello-Masini et al., 2003).

Sun et al. (2016) showed that the high concentration of CO_2 (>10%, $v\,v^{-1}$) in *C. sorokiniana* increased and improved lipid accumulation. It is evident that high CO_2 concentrations do not require CA, but at lower concentrations (1%, $v\,v^{-1}$) CA establishes a prominent role in biomass production.

Carbonic anhydrase was synthesized at high activity concentrations when microalgae of the genus *Acutodesmus*, *Kirchneriella*, and *Desmodesmus* were grown in photobioreactors with low CO_2 levels (0.03%) at room temperature. High CO_2 levels led to a decrease in enzymatic activity (Swarnalatha et al., 2015). It was possible to correlate the enzymatic activity with the CO_2 levels, where the increase of free carbon dioxide caused a decrease of the enzymatic activity and vice versa. In addition, the decrease in bicarbonate increased CA activity, suggesting the conversion of this compound into CO_2 by the enzyme. The authors also observed that the increased availability of inorganic carbon discharged into the environment is related to an environment with high levels of carbon dioxide, and this factor modulates the action of the enzyme (Swarnalatha et al., 2015).

25.2.8 Laccases

Laccases (EC 1.10.3.2) belong to a blue multicopper oxidase family and are monomeric extracellular glycoproteins (Maté et al., 2011). They can be found in lichens, microorganisms, plants, and insects (Lisov et al., 2012). This glycoprotein has the ability to capture the O_2 from the air, using it as an electron acceptor to oxidize a variety of compounds and produce water, as well as oxidize complex polymers in phenolic compounds such as lignin. This ability to be "green catalysts" makes them suitable for use in a variety of industries, from bioremediation in the food industry (brewing) to the pulp industry (Baldrian, 2006; Giardina and Sannia, 2015; Mate and Alcalde, 2015; Piscitelli et al., 2011).

The activity of laccases ($130\,U\,L^{-1}$) was confirmed in the algae *Tetracystis aeria*, and with an optimum pH of 7.0 and 9.0 (Otto et al., 2015, 2010). The laccase was detected in *A. platensis* and discolored 96% of anthraquinonic dye reactive blue in 4 h. This enzyme was purified using anion exchange and exclusion chromatography. The protein presents an approximate size of 66 kDa, with optimal pH and temperature for the enzyme activity of 3.0°C and 30°C, respectively. It also showed high stability in alkaline pH (8) and high temperatures (50°C) (Afreen et al., 2017). *Chlamydomonas moewusii* and *Tetracystis aeria* were used for the degradation of phenolic biophilic pollutants, with excellent performance in neutral and alkaline pH (Otto et al., 2015).

In addition, algae laccases can be used to treat effluents from the pulp and paper industry (Tarlan et al., 2002), bio-transform various natural xenobiotic and aromatic compounds (Otto et al., 2015), reduce iron oxide and dissimilar manganese (Fredrickson et al., 2008; Nealson et al., 2002; Shi et al., 2012), and degrade phenolic components, textile dyes, and xenobiotics (Acuner and Dilek, 2004; Daneshvar et al., 2007; Lima et al., 2003; Safonova et al., 2005).

25.2.9 Galactosidases

α-Galactosidase (α-D-galactoside galacto hydrolase, EC 3.2.1.22) is an enzyme responsible for the release of α-D-galactose by the hydrolysis of α-1.6-linked galactose residues to polysaccharides and oligosaccharides of galactomannan, raffinose, galactans, and stachyose (Katrolia et al., 2014). This enzyme is widely used in pulp and paper, medicine, and the sugar industry (Weignerová et al., 2009).

The microalgae *Poterioochromonas malhamensis* show α-galactosidase (α-Gal) activity intracellularly, which increases when the external osmotic pressure is high. To recover the cells that undergo shrinkage as a result of this high pressure, isofloridoside biosynthesis occurs internally. The high external osmotic pressure induces the enzymatic production so that it reduces isofloridoside concentration intracellularly, releasing galactose and glycerol as the

product. This occurs due to the osmotic regulation mechanism present in this microalga (Dey and Kauss, 1981). α-Galactosidase is apparently induced by isofloridoid, which acts as a substrate when internal osmotic pressure levels are rapidly decreased by external pressure. Galactose and glycerol accumulate within the cell as an osmoregulatory mechanism to maintain vital cellular activities (Brasil et al., 2017b).

Lactase (β-galactosidase or β-D-galactohydrolase, EC 3.2.1.23) degrades the D-galactosyl residues bound from oligosaccharides and polymers (Husain, 2010). The dairy industries use this enzyme for the hydrolysis of lactose and the production of lactose-free foods, to improve flavor, solubility, and sweetness. Lactase is also currently used for the synthesis of food probiotics known as galactooligosaccharides among other applications (Husain, 2010; Oliveira et al., 2011).

Suwal et al. (2019) reported β-galactosidase syntheses by *Tetradesmus obliquus* under mixotrophic cultivation using cheese whey permeate as the medium. The optimal enzyme production (78 U g cells^{-1}) was recorded after 14 days of cultivation. In the same study, the production of a galactooligosaccharide was observed for the first time in microalgae. *Dunaliella tertiolecta*, *Chlorella minutissima*, and *Nannochloropsis oculata* were cultivated mixotrophically with lactose as the sole source of carbon. The cultures showed the production of extracellular β-galactosidase and presented growth rates and biomass production superior to photoautotrophic cultures. The species *D. tertiolecta* presented a β-galactosidase activity of 33.5 U L^{-1} and the species *N. oculata* and *C. minutissima* showed activities of 29.6 and 11.02 U L^{-1}, respectively (Zanette et al., 2019). Bentahar et al. (2018) evaluated the production of lactose by *Tetradesmus obliquus* under different conditions and culture media; the microtrophic culture showed an activity of 12.35 U L^{-1} day^{-1} in 7 days.

The microalgae *Scenedesmus obliquus* presented β-galactosidase production. The lactose was reduced by more than 50% throughout the culture, resulting in the accumulation of galactose and glucose in the medium, which in turn indicates the extracellular production of this enzyme (Girard et al., 2014). Nine microalgae and macroalgae of the species *Chlorophyceae* and the family *Cyanophyceae* were evaluated for the production of β-D-galactosidase, of which eight presented some activity. *Acutodesmus obliquus* and *Cosmarium* sp. presented the highest enzymatic activity (Davies et al., 1994).

25.2.10 L-asparaginase

Asparaginase (EC 3.5.1.1, L-asparagine amidohydrolase) is an enzyme with applications in the food and pharmaceutical industries (Cachumba et al., 2016; Shakambari et al., 2019), which hydrolyzes L-asparagine to L-aspartic acid and ammonia (Hosamani and Kaliwal, 2011). Generally, microbial L-asparaginase is intracellular.

In recent years, due to its important applications, L-asparaginase has gained more attention. In the pharmaceutical industry it is applied for the treatment of different diseases (e.g., cancers, acute lymphoblastic leukemia, malignant diseases of the lymphoid system, and Hodgkin's lymphoma). In addition, in the food industry, this enzyme is used to prevent the formation of acrylamide when food is processed at high temperatures (Cachumba et al., 2016; Shakambari et al., 2019).

L-asparaginases may be obtained from plants, mammals, and microbial (bacteria, fungi, algae) sources (Shakambari et al., 2019). Paul and Cooksey (1979) report the first description of a microalgal L-asparaginase; cell-free extracts contained $1.5\,IU\,mL^{-1}$ asparaginase activity.

According to Ebrahiminezhad et al. (2014) microalgae are viable economically for drug production on a large scale. In their study, *C. vulgaris* showed an intracellular L-asparaginase activity of $10\,IU\,g^{-1}$ dry cell weight. Therefore, they concluded that *C. vulgaris* is probably a new, safe, and economical source for L-asparaginase production.

In a medium containing $NaNO_3$, NaCl, and $FeSO_4{\cdot}7H_2O$, *A. platensis* showed the highest production of L-asparaginase of 0.275 U. The highest specific activity of L-asparaginase ($0.166\,U\,mg^{-1}$) was found in subcellular, cytoplasmic extracts. According to the authors, its production could be induced by using combined stress conditions (Prihanto and Wakayama, 2014).

Production, purification, and characterization of L-asparaginase from *S. maxima* were studied by El Baky and El Baroty (2016). It was observed that the L-asparaginase activity was directly proportional to the concentration of nitrogen present in the medium. The medium with $5\,g\,L^{-1}$ of $NaNO_3$ showed the highest activity of L-asparaginase (898 IU) and specific enzyme activity ($2.21\,IU\,mg_{protein}{}^{-1}$). Optimal pH and temperature of purified enzymes for the hydrolysate were 8.5°C and 37°C, respectively (El Baky and El Baroty, 2016).

Kebeish et al. (2016) studied the recombinant enzyme, L-asparaginase II (ASPII), cloned and expressed in *E. coli* BL21 (DE3) of the cyanobacterium *Synechococcus elongatus* PCC6803. The *S. elongatus* L-asparaginase showed an optimal reaction pH and temperature at 7.5°C and 37°C, respectively. The enzyme activity was decreased at extreme acidic and/or alkaline pH range. The maximum reaction velocity was reached at $80\,mM\,L^{-1}$ asparagine.

25.3 Final considerations

Microalgae can synthesize a wide variety of enzymes, demonstrating how these microorganisms are versatile for use as biofactories. This source is still little explored compared to other organisms, but a growing number of enzymes that can be applied in chemical, pharmaceutical, and food industries are being discovered. This shows the need to develop new strategies to optimize the production of microalgae of these enzymes at an industrial scale.

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PART 4

Engineering approaches applied to microalgal processes and products

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CHAPTER 26

Process integration applied to microalgae-based systems

Ihana Aguiar Severo, Mariany Costa Deprá, Rosangela Rodrigues Dias, Eduardo Jacob-Lopes

Bioprocess Intensification Group, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil

Chapter outline

26.1 Introduction 709
26.2 A general overview of microalgae-based processes and products 710
26.3 Process integration fundamentals 714
26.3.1 Energy integration 715
26.3.2 Mass integration 718
26.3.3 Water integration 720
26.4 Environmental and economic indicators of microalgae integrated systems 723
26.4.1 Life cycle assessment 723
26.4.2 Economic outlooks 726
26.5 Concluding remarks 728
References 730
Further reading 735

26.1 Introduction

The world energy crisis boosted advances in the industrial sector, resulting in considerable improvements in processes efficiency. At the same time, numerous technical, economic, environmental, and political challenges have arisen, which manufacturing facilities had—and still have—to face. Concerning prominent ecological crises—including global climate change, water scarcity, food raw materials, land misuse, and energy consumption—decision-makers are now thinking collaboratively for an even more intense wake-up call, inspiring sustainability ideas, driven by research and development (R&D) partnerships (FAO, 2017; D'Amato et al., 2019).

The chemical, petrochemical, energy, pharmaceutical, and food industries are based on fossil inputs, which exert some of the most profound impacts on the ecosystem. These facilities

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require high energy demands, mass, and water flows to operate, and generate a pollutant load, whether solid, liquid, or gaseous (Varbanov et al., 2018).

Today, it is imperative to adopt alternative technologies to optimize production chains, and global R&D activities provide much of the innovation. An example is the bioengineering and related fields for consumer goods production (Mattiasson, 2013). Microalgae-based processes, in particular, have a strong influence on current topics of study. These bioprocesses are unique because they associate the use of residual raw materials or not with the production of multiple valuable products that are in considerable demand in the market (Sathasivam et al., 2019). However, microalgae technologies have many bottlenecks, many of which are still far from being eliminated to become viable.

However, on the journey toward industrial competition, a trend that began in the 1970s was process integration, which is focused on connecting unit operations, equipment, or manufacturing techniques within a process. This approach would reduce costs, process time, workload, and product losses, and would increase productivity, efficiency, and sustainability (Walmsley et al., 2019).

Several process integration options have been proposed to enhance the cost-effectiveness of microalgae-based systems. Thus, three main types can be highlighted: mass, energy, and water integration. Moreover, when microalgae-based processes have low requirements for these inputs, they are undoubtedly preferable, since they provide more significant conservation of energy and mass. However, due to the complexity of integrations, there are still many bottlenecks that need to be elucidated, and these can only be well-explained by applying systematic tools for sustainability and cost assessments (Walmsley et al., 2018).

In this chapter, therefore, we display a comprehensive view of process integration applied to microalgae-based systems. In addition, we discuss the processes and products of interest in the microalgae market, as well as the fundamentals of integrated facilities with their respective integration elements. Finally, to assess all this potential, a study of environmental and economic insights is presented.

26.2 A general overview of microalgae-based processes and products

Microalgae is not a taxonomic term, but a merely commercial designation. The current situation of the species already identified is founded on a database and literature information (i.e., www.algaebase.org and Guiry, 2012). So what are microalgae and what is their application? As a brief answer, they constitute a vast group of microorganisms of very diverse morphology and physiology, which present simple culture requirements, developing rapidly and efficiently, inhabiting niches of extreme environments. Although photoautotrophic metabolism is preferable, microalgae can also grow under heterotrophic and mixotrophic conditions. Because of these peculiar traits, microalgae symbolize an exceptional matrix for the bioprospecting of

countless interesting metabolites. They derive from major molecules such as lipids, proteins, carbohydrates, and pigments (Borowitzka, 2018).

Given this general explanation, commercial exploitation of microalgae worldwide has evolved enormously over the years. The researchers, jointly with the industry, discovered the potential of these microorganisms for application in several sectors: food and feed (nutraceuticals and aquaculture nutrients), pharmaceuticals, cosmetics, chemicals (materials), and energy (biofuels, power, and heat). At the same time, these microorganisms are also promising for ecological uses, such as greenhouse gases mitigation and wastewater treatment (Severo et al., 2019).

As the advantages are many, start-ups and companies were founded in the past initially to manufacture microalgal whole biomass using cyanobacteria such as *Spirulina* and green algae *Chlorella*. Currently, many other small/medium-sized facilities and also pilot/demonstrative operations have been created, and the value of microalgae-based products has been improved (Gaignard et al., 2019); examples include Algenol, PetroSun, Solix Biofuels, Solazyme, Cyanotech Corporation, Cellana Inc., Fermentalg, AlgoSource, Isua Biotechnologie & Compagnie, Euglena, Algatech International, Simris, Algenuity, and Microsynbiotix. In addition, some technologies have also been developed and patented. The patents that are found on access platforms claim applications of the bioproducts in many segments, some of which are Sepal Technologies Ltd., Heliae Development LLC, Ecopetrol SA, and Synthetic Genomics Inc. (Khanra et al., 2018; Gifuni et al., 2019).

Regardless of the numerous examples of progress, the timeline shows that technical and economic barriers concerning microalgae commercialization persist today. Biomass production and other bioproducts, in general, are very onerous and require a massive volume of culture to satisfy the process design priorities. Thus, the cultivation systems are the centerpiece that hinders the large-scale economic viability of these bioprocesses (see Box 26.1 for more details).

When considering industrial manufacturing of microalgae-based products, one should take into account the market into which they are being sold and the facility objectives. Therefore, innumerable products can be divided into two broad classes for a better understanding: (i) commodity or bulk products, which must be produced in large quantities and purchased based on chemical composition, purity, and selling price; and (ii) fine or specialty chemicals, which must be produced in small quantities and are purchased due to their impact or function rather than their chemical composition (Xiong et al., 2019).

Additionally, production scale also differs between these products classes in terms of larger or smaller production volume and high or low added value (Smith, 2005). Fig. 26.1 demonstrates an overview of the classes of microalgae-based products and their respective values, volumes, and downstream expenditures for manufacturing.

The classes of specialty and fine chemicals, including pharmaceuticals, cosmetics, nutrients, and some chemicals, are high value-added products obtained, but in low volumes. On the other

BOX 26.1 CULTIVATION SYSTEMS

There are the open and closed systems, being raceway ponds and photobioreactors or heterotrophic bioreactors (fermenters), respectively. These cultivation systems are often based on the same mode, requiring: (i) energy light input, whether solar or artificial (except the heterotrophy); (ii) a carbon source (organic or inorganic); and (iii) agitation, aeration, and other operating conditions particular to each system (Gaignard et al., 2019). Open systems were the pioneers for microalgae culture and are currently responsible for 95% of worldwide industrial biomass production. Despite low capital expense, raceway ponds require large land areas (0.2–0.5 ha). They exhibit inferior productivity (0.7 g/L) due to the high evaporation rate, contamination, and inefficient mixing. In addition, the geographical location influences final product costs and overall process viability (Verma and Srivastava, 2018). Closed systems have already overcome almost all these barriers. They have the additional benefit of being equipped with monitors and controllers for better productivity performance, which can reach up to 3.5 g/L. The designs of these reaction vessels are numerous and have varied configurations, as described in Mantzorou and Ververidis (2019), but only tubular photobioreactors are widely commercially exploited. Of the three main disadvantages of photobioreactors, including the intense energy demand and difficulty in scaling up, undoubtedly the high purchase cost of equipment is the most critical factor. The value can be up to 100 times larger than an open system (Kumar et al., 2015). Finally, heterotrophic bioreactors have better kinetic performance and are easily scalable. Although they are exempt from the light requirements, and some strains use low-cost organic substrates as a carbon source, bioreactors need greater asepsis to avoid competition with other microorganism heterotrophs (Ramírez-Mérida et al., 2017). Deprá et al. (2019) estimated the purchased-equipment cost for raceway ponds, tubular and flat plate photobioreactors, and fermenters, whose values were in the range of 4–6 kUSD/m^3, 40–50 kUSD/m^3, 150 kUSD/m^3, and 8.5 kUSD/m^3, respectively. All commercially available cultivation systems have a win-lose relation. However, the focus should remain on the target product and company business needs.

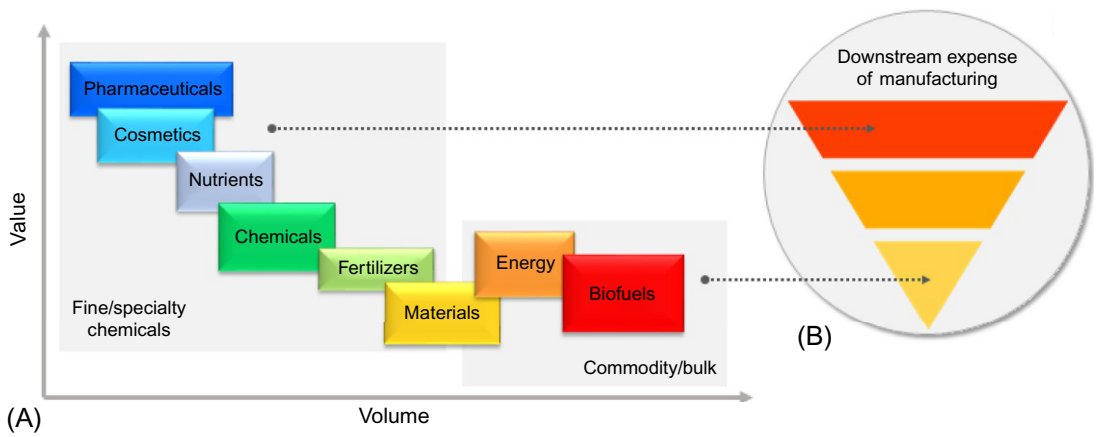

Fig. 26.1

Overview of microalgae-based products: (A) correlation between volume (x-axis) versus value (y-axis); and (B) hierarchy of downstream expenses for product classes manufacturing.

hand, commodities and bulk products, such as bioenergies and biofuels, are considered low value-added and are required in larger volumes (Fig. 26.1A) (Budzianowski, 2017).

For instance, pigments such as carotenoids, chlorophylls, and phycobiliproteins are found in microalgal biomass. However, the first commercial uses were from β-carotene and astaxanthin as food colorant agents. Although there are other products, pigments are predominant. The market share for these compounds expands to use as vitamins in food, animal feed, additives, products with antioxidant and anti-inflammatory properties, bioactive compounds to promote human health, and biomaterials (Jacob-Lopes et al., 2019). These biomolecules, despite being less competitive than traditional sources, are preferentially exploited because they attract the attention of niche consumers. However, they are not obtained in large quantities from the raw biomass at the factory gate due to intracellular compounds, which generate very low concentrations. In addition, pigments can be obtained at a relatively reasonable cost because some products are justified by their attributes, producing potential profitability for the manufacturing industry. For this reason, they reach sufficiently high sales prices, enabling the current market (Gerardo et al., 2015).

Nevertheless, it should not be overlooked that commercialization is based only on the extraction of a single compound, while the other co-products are depreciated or rejected. This does not take into account the sophisticated techniques of extraction and purification, which is the second hottest point of the process (further details in Box 26.2). In addition to the many unit operations, these steps are intense in materials, utilities, and energy, accounting for up to 90% of the product manufacturing expenses (Fig. 26.1B).

BOX 26.2 DOWNSTREAM PROCESSING

Another barrier to economic implementation of microalgae-based processes concerns downstream processing steps of biomass. Starting with harvesting methods, which include centrifugation, flocculation, flotation, filtration, and sedimentation, they are considered energy-intensive, expensive, and still inefficient for industrial use. This step also depends on the type of strain, cultivation condition, and cell density, affecting the final product yield. After that, drying, including methods such as spray-drying, convective drying, drum-drying, fluidized bed drying, and freeze-drying, may be efficient, but this will depend on the desired product, and the water content, which is often high, requiring a large surface to dry and the possibility of losing some compounds is high. In addition, cell disruption, which may be mechanical (bead mill, pressing, and high-pressure homogenization), biochemical (alkaline and acid treatment, enzymes, and osmotic shock), or physical (microwave, autoclave, pulsed electric field, and ultrasound), depends on the efficiency of the chosen process. This will require more or less energy and is also affected by the chemical composition and microalgae cell wall structure. Ultimately, the extraction method, including the use of solvents or supercritical fluid, has environmental demerits or requires expensive and sophisticated equipment, respectively (Kam et al., 2018; Gifuni et al., 2019). In this sense, it is imperative to streamline the unit operations that integrate the current downstream processing steps of microalgae-based processes.

Regardless of the bottlenecks inherent to the process, the marketing of microalgae-based products of high value-added continues to increase. It is expected by manufacturing companies that new market segments will be achieved, with duplicate sales prices exceeding production costs. Some pipeline bioproducts from the survey, whether commercialized or regulatory pipeline or at an advanced/early stage of development, will be introduced to the sales business, increasing the chances of successful large-scale applications (Enzing et al., 2014). However, these predictions are based on conservative assumptions, and the actual status of the pipeline product is unknown.

Because of this, the production of low-value commodities and bulk products would today be the highest priority to increase gross revenue from microalgae-based processes and boost the bioeconomy[a] of these facilities. Biodiesel, bioethanol, biogas, and bioelectricity, including heat generation, are the major, albeit distant, examples of what societies worldwide require and with selling prices as affordable as petroleum-derived fuel (Fig. 26.1A). In addition, the widespread protein supply in the human food chain or as an animal feed additive, and some chemicals, such as biopolymers and bioplastics, are also demanded but not forecasted for biomanufacturing in the coming years (Wijffels et al., 2010; Ruiz et al., 2016). This is because to make them successfully competitive, these products require massive production volumes. In fact, the technology is not available, among other aspects (i.e., microalgae strains selections and genetic engineering techniques), for processing biomass at the appropriate amount.

The key to the reported problems comes not from the attempt to optimize the overall plant design, but rather from an effort to streamline auxiliary operations. The integration and co- and sub-integration of other process units, whether partial or total, such as microalgae-based processes and products, can benefit all parties and are scenarios closer to real industrial exploitation. If subjected to these synergistic strategies and of smart interconnection, they could reduce the time and financial commitments involved, improving facilities' performance.

26.3 Process integration fundamentals

Process integration has experienced tremendous development, dating back more than 50 years, and is currently a well-established and mature methodology. Originally, the method was applied for heat recovery by thermal energy integration, reducing hot or cold utilities consumption. Over time, it has been used in many fields of research to minimize resource use, among other aspects. Therefore, it extended to mass integration through the materials usage, of which water integration was the most common branch. Today, water integration can be a separate branch, referring to the context of different water networks (Klemeš, 2013). In general,

[a] Bioeconomy: Despite numerous concepts, in a practical sense, the bioeconomy is based on the use of renewable biological resources as feedstock for the conversion and supply products, processes, and services of value, within a sustainable economic system.

to reduce resource demand, it is necessary to maximize internal recycling or recovery and the reuse of material and energy flows. Process integration improves the efficiency of these features, benefiting plant (see Box 26.3, which describes process integration).

Although process integration metrics are more often applied to traditional manufacturing industries (chemical sites), this approach has spread much more broadly covering microalgae-based systems. Therefore, the next sections discuss critically the different types of integration in these facilities.

26.3.1 Energy integration

Energy can be defined as the capacity to produce work, that is, of two systems interacting with each other. According to the conservation law, the total amount of energy is conserved; however, it can be transformed from one type to another. There are several types of energy and energetic resources that derive from thermal energy, electrical energy, chemical energy, kinetic energy, and mechanical energy. Already, energy integration is one of the process integration methodologies, which is fundamentally based on thermodynamic principles, from energy and exergy balance insights (Sinnott and Towler, 2020).

In microalgae facilities, energy integrations are used to define the heating, cooling, and power needs of given equipment, unit operations, thermal systems, or products. At an operational level, this will help show the energy usage pattern in the production chain and identify sites where there is a need to conserve and save energy forms. In this sense, the overall target is to achieve maximum integration levels for improved energetic efficiency and reduce costs (Aziz et al., 2014).

Based on these fundamentals, the starting point is to understand the location of energy-generating sources, as well as dissipating energy sources, in order to minimize external heating

BOX 26.3 WHAT IS PROCESS INTEGRATION?

Process integration is conceptualized as an engineering approach toward design and operation for application in industrial systems, which has been implemented in the most varied areas of knowledge (Klemeš et al., 2018). The central objective of process integration is to address a system holistically—that is, integrate individual operations, envisioning synergy to improve overall production chain efficiency. Originally, the use of the concept of integrated processes was in the field of heat recovery. However, it extended to many industrial complexes: materials, emissions, water, energy systems, energy, and logistics. As process integration adopts immense proportions, the installation design becomes more complex and, consequently, the overall benefits increase. In this sense, critical discussion of environmental policy issues, including resource conservation, sustainability metrics, and bioeconomy, is also a contribution of this methodology (Walmsley et al., 2019).

and cooling requirements. Typically, power and heat expenses are present in almost all downstream processing steps, which should not be treated as standalone operations at the end-of-pipe.

In this sense, the first form of energy integration may be at the reactor level. There is a specific persistent problem in the performance of the microalgal culture indirectly related to reactor design: the temperature. This dominant environmental factor requires substantial attention. The efficiency of microalgae cultivation systems to obtain a specific product depends on the optimal temperature, and activity of these microorganisms is in the mesophilic range, between 25°C and 35°C. Cell growth rates usually decrease at temperatures above 35°C and below 16°C, although the pre-adaptation of some strains in extreme temperature ranges (thermophilic conditions) has been considered in aiming the optimization of reactors for industrial-scale application (Huang et al., 2017).

Regardless, considerable variations are experienced by mass outdoor commercial cultivation due to daytime temperature fluctuations and seasonality. Thus, an economical temperature control system is essential to keep the culture temperature within a favorable range. In both cold and warm seasons, heat exchangers are usually installed next to the photobioreactor (Chang et al., 2017).

In winter, for example, these systems need to be heated; however, this operation increases the process power demand. Therefore, heat integration from solar energy would be a cheap method of temperature control. Of course, this would be possible with the installation of alternative devices, such as photovoltaic panels to generate electrical energy (Fresewinkel et al., 2014). Tredici et al. (2015) demonstrated photovoltaic integration and the potential energy gain exceeded 600 GJ ha/yr. Since this is a sustainable bioprocess, at first glance this strategy seems incoherent for microalgae, but reasonable to the extent that 15% of light-to-electricity conversion efficiency of this auxiliary source would cover conventional electrical power requirements.

On the other hand, in summer, photobioreactors present a problem of overheating. Solar radiation is so intense through the transparent walls, due to the small surface area, that it can cause damage to microalgae cells by photoinhibition. Depending on the geographical, weather conditions, including irradiation, can raise the temperature 10–30°C higher than the ambient temperature in summer (Huang et al., 2017). For this reason, the control and monitoring of reactor overheating are indispensable. Even in middle-latitude zones, cooling systems are adopted as a preventive measure. Some ideas for greener cooling control have been employed, but are often ineffective because they significantly reduce light irradiation. There is also submersion in water reservoirs, but with specific penalties. Spraying water to cool the reactor surface has been considered the most preferred option. However, spray-cooling capacity is deficient, and its effective use is only possible under certain environmental conditions

(temperature vs. humidity). A heat-exchange pump enables this procedure, yet incurs extra energy expenditure (Sierra et al., 2008).

It should be noted that the adoption of any temperature control system requires expensive components on a large scale. In this sense, energy integration through waste heat recirculation from other hot sources would be a promising approach. Song et al. (2015) proposed the optimization of an integrated process by coupling hot and cold streams of heat exchangers via exergetic recovery. Notably, these energy integration strategies from heating and cooling systems will only be viable if a suitable location for photobioreactor installation is chosen. The location should have available energy, waste heat, and water resources to maintain the temperature to the set point and then reduce energy consumption.

Going further, energy integration from the stages after cultivation is crucial. Depending on the product that the facility targets, downstream microalgal biomass processing goes through many operations, including equipment and thermal systems, which are often extremely energy-intensive. In the harvesting stage, the biomass dryer, for example, is a device that can consume up to 85% of the total energy required in the process due to the high moisture content (Aziz et al., 2014). Alternative minimal energy intensity drying routes are urgently needed. Several methodologies have been developed, but unfortunately the options to integrate or co-integrate energy from other sources are not yet viable. To date, no technology is applicable due to the low level of heat recovery and the enormous exergy destruction in equipment.

Furthermore, as a bioresource of varied chemical composition, microalgae biomass can be converted and processed by thermochemical and biochemical routes, which generate different types of bioenergy. Through these routes, energy integration can be done during gasification, pyrolysis, liquefaction, hydrogenation, fermentation, transesterification, or direct thermal combustion, whose steps produce heat, syngas, and biofuels, with substantial energy content (Lee et al., 2019). After that, the integration of these products in other burning processes, either by co-combustion (with other fossil fuels) or independently (biomass direct combustion or association of conversion routes and power generation, such as integrated gasification combined cycle) can be considered for enhancing energetic efficiency (IEA, 2017). Waste heat utilities could be collected and regenerated in these procedures that provide thermal streams.

Finally, other biomass-independent products, such as volatile organic compounds, oxygen, and partial carbon dioxide, which are released into photobioreactor exhaust gases, can be integrated as an energy source into combustion systems. Severo et al. (2018a,b) demonstrated that this energy integration approach has the potential to improve equipment thermal performance by more than 40%. It is worth mentioning that this process is performed in a single operation because the bioproducts are excreted by the microalgal cells, eliminating the biomass processing intermediate steps. Despite all the advantages, integration of gaseous molecules is

particularly challenging. The main issues are that these products are quite heterogeneous and in low concentrations, which would require an appropriate collection or recovery technique from exhaust stream to be commercially established (Lukin et al., 2018).

26.3.2 Mass integration

Mass is a property of an object, so mass integration techniques are based on the identification of the chemical constituents of a physical body. However, there are some problems related to the law of conservation of mass, which are dependent on material flows properties and not necessarily on chemical compositions (Klemeš, 2013). Analogous to what happens in heat integration, mass integration in industrial processes provides an understanding of global material flows, using mass balances, in order to track the best pathway of allocation, separation, recovery, and generation stream of the mass species (El-Halwagi, 2017).

Typical industrial microalgae plant designs require different types of masses, including carbon, nitrogen, phosphorus, and sulfur. They can be integrated in culture systems, since microalgae are able to assimilate these elements in both organic and inorganic form, aiming at the maintenance of cellular structures.

Carbon is the predominant element in microalgal biomass composition, with an average proportion of 50%, being considered the basis for robust bioproducts production. In the inorganic form, the main carbon integration route is from carbon dioxide (CO_2). Metabolically, microalgae bioconvert free CO_2 during photosynthesis, which is transported across the plasma membrane, being stored in cells, as a kind of reservoir. This procedure is known as the carbon concentration mechanism, through six distinct routes. High-efficiency enzymes catalyze these reactions, carbonic anhydrase and ribulose 1,5 bisphosphate carboxylase/dehydrogenase, which accumulate CO_2 concentrations up to 1000 times greater than that of the external circulating flows (Cheng et al., 2019).

Given the above, free CO_2 integration has been widely envisioned for environmental management reasons. Rather than integrating CO_2 from the atmosphere, which is inefficient for sustaining intensive cultivations due to the low CO_2 level in the air (380 ppmv), direct CO_2 integration from highly concentrated stationary sources is a promising strategy for balancing the economic interests of microalgae-based processes. The flue gases concentrations can usually reach 30%, allowing adjustment to values between 3% and 15% for input in photobioreactors (Van Den Hende et al., 2012). Typical examples are the use of off-gas streams from clinker and lime kilns, coal-fired boilers, exhaust pipes, internal combustion engines, as well as flue gases from chemical and petrochemical plants and other available CO_2 emitters (Anbalagan et al., 2017; Aslam et al., 2019). Integrating CO_2 from these sources would avoid, in principle, logistical problems. This attractive carbon mass integration strategy is often

proposed for input into microalgae photobioreactors; however, it is seldom implemented at the demanded scale. Firstly, these flue gases contain not only CO_2, but also hundreds of substances, most of which are toxic to cells (CO, CH_4, NO_x, SO_x, H_2, heavy metals, halogen acids, and particulate matter). Secondly, flue gas temperature is above 1200°C (Jacob-Lopes and Franco, 2013). These two problems are enough to inhibit photosynthetic activity.

In contrast, microalgae heterotrophic cultivation systems require an organic carbon exogenous source. However, to overcome the money-consuming processes, the use of low-cost organic carbon is interesting. The integration of cheap substrates including agricultural waste, municipal waste, molasses, fruit extracts, vinasse, and glycerol have been reported as efficient resources for this purpose (Katiyar et al., 2017). High lipid productivity is one of the advantages. Crude glycerol, for example, a by-product of biodiesel production, would be a potential integration form, since it is a particularly abundant feedstock in some regions and of difficult final disposal (Klein et al., 2018). Of course, any of these integrations will depend upon the physicochemical composition of the compounds, which later may or may not impair microalgae uptake. This option is by far a merely economic criterion.

After carbon, nitrogen is the most important element for microalgae cultivation. These microorganisms are able to metabolize various nitrogenous compounds to support cell growth and maintenance, such as ionized ammonia (NH_4^+), free ammonia (NH_3^-), nitrate (NO_3^-), and nitrite (NO_2^-) (Van Den Hende et al., 2012). For this reason, industrial effluents integration (i.e., dairy effluent, poultry, and swine slaughterhouse effluent), has been commonly suggested as an efficient method to supply organic nitrogen, and to reduce nutrient discharge, eutrophication in receptor streams, and downstream biomass processing costs. This source is rich in amino acids and urea, which have entry within the cell by active transport. In terms of amino acids, some of them have been used as carbonaceous and nitrogenous substrates for microalgal growth in the dark. However, the most common source is still urea, which is hydrolyzed to NH_3^- and CO_2, whose generated compounds can be co-used in cultivations. Another target of these microalgae-based processes is to reduce the purchase and degradation of nitrogen-based substances, such as fertilizers and proteins, for use in the effluent treatment plant itself. But what comes up is that the neutral form of NH_3^-, even at low concentrations (1.2 mM/20 mg/L), has detrimental effects on microalgae due to its toxicity (Peccia et al., 2013).

Additionally, other nitrogen compounds in inorganic form, also derived from industrial flue gases, can be integrated into the cultivation systems. In addition to air N_2, NO and NO_2 are the main NO_x species, while N_2O, NO_3, and other trace-level forms can be used by microalgae (Singh et al., 2019). However, research on NO_x integration into photobioreactors is progressing at a slow pace. If on the one hand there are questions about tolerance and effects on microalgae species, on the other there is the limitation on the dissolution of these nitrogen compounds in the culture medium.

Phosphorus is the third most in-demand element in these systems. Although the microalgae metabolize this essential macronutrient at deficient concentrations (1% by weight), reactive phosphorus is the most straightforward form to assimilate, despite hydrolysable acid phosphorus and organic phosphorus being efficiently used (Solovchenko et al., 2016). Similarly to the other elements, the integration of phosphorus-rich industrial effluents has been widely required for microalgal cultivation, since it is the second-largest contaminant in these sources—typical total phosphorus concentrations in swine effluents range from 100 to 620 mg/L (Nagarajan et al., 2019). However, besides the regulation and assimilation mechanism not yet being well understood, there is another considerable obstacle for the integration: microalgae strains are not yet able to metabolize phosphorus so quickly, as well as other pollutants present in the medium, and withstand high loads of real-world effluent.

Finally, sulfur integration also has the potential for application to microalgae cultivation systems. Although some sulfur compounds, such as dimethyl sulfonic propionate (DMSP) and dimethyl sulfide (DMS), are released by some species under specific conditions, this element is a component of the amino acids (cysteine and methionine) of microalgae, and is present in the thylakoid membrane (anionic sulfolipid) (Giordano et al., 2005). Regardless, in the same case of CO_2 and NO_x, flue gases simultaneously release sulfur oxides (SO_x), in the forms of SO_2 and SO_3, depending on the chemical reaction with the fuel. Therefore, some studies have shown that these compounds could be integrated into photosynthetic cultivations (Van Den Hende et al., 2012). However, as far as we know, very few strains can tolerate the sulfur compounds formation, due to the high solubility in aqueous media, and the mechanism leading to this is unknown.

In this sense, the different mass integration possibilities seem very attractive at first sight, because they would be a cheap source of nutrient enrichment to cultures. In practical terms, the biggest problem inherent to these integrations is that coupling of reactors for microalgae processing near industrial areas for the surpluses reuse is very complicated. Generally, there is no land available around these facilities to integrate the supplied effluents.

26.3.3 Water integration

Water is an essential natural resource for the survival of all living beings that inhabit the Earth. It is abundant, covering most of the terrestrial surface. However, effects on the quality and quantity of available water (surface and groundwater) are already evident in many parts of the world. The threat of its scarcity and impacts on ecosystems may seem exaggerated, but it is not (Damerau et al., 2019).

The industrial sector is the second largest water consumer, accounting for about 22% of worldwide consumption, below only agriculture. Regardless, all industrial activities need to be

supported by these limited water resources to transform feedstock into products. In addition, the rising price of freshwater, its scarcity, pollution, stricter environmental regulations, and wastewater treatment costs raise the need for better management and distribution in a process. Therefore, routes should be sought out to minimize water requirements in industrial facilities (De-León Almaraz et al., 2016).

Following this trend, microalgae-based processes are water-intensive, which makes this a strong constraint for marketing them. This critical issue is fundamentally related to water expenditure in the cultivation stage, alongside the substantial evaporation losses due to aeration rate, and secondarily in some downstream processing steps of the biomass, such as harvesting. For biodiesel production in photobioreactors, for example, it is estimated that freshwater requirements range from 80 to 291 m^3/GJ (Batan et al., 2013). Yet, according to these same authors, it has been shown that the global water volume for the manufacture of other biofuels is between 90 and 420 billion m^3 of water, equivalent to the additional direct consumption of up to three times the volume of water spent to support agricultural irrigation activity. Chinnasamy et al. (2010) demonstrated that microalgae biomass production in open ponds demands 11–13 million L/ha/yr of water. However, these water withdrawal numbers fluctuate due to the geographic location and climate resolution for a stable microalgae process.

The most obvious solution to this barrier is to integrate seawater to offset water requirements, since the oceans are considered as abundant natural resources. The use of seawater can significantly minimize the amount of demanded drinking water for cultivation preparation. This type of integration has a double benefit: in addition to volumetric water input, mass integration is possible. Seawater is composed of all the chemical elements supporting marine biology, such as carbonates, nitrates, phosphates, minerals, and other dissolved ions (Na^+, Cl^-, Mg_2^+, SO_4^{2-}, Ca_2^+, and K^+). Nutrient-enriched seawater supply can be an efficient way to replace partially some of the key elements of synthetic culture media. $MgSO_4$, $CaCl_2$, and $NaCO_3$ are examples that constitute the BG-11 medium, considered the universal broth for microalgae cell maintenance (Jung et al., 2015).

Additionally, square-kilometer-scale photobioreactors for outdoor marine microalgae cultivation are available in some coastal areas near the sea (i.e., Cyanotech Corporation, Hawaii, USA). A considerable number of practical investigations have been made using seawater integration, as is the case of the plant made of "Green Wall Panel-II" (GWP-II) photobioreactors (Tredici et al., 2016) and the emerging offshore cultivation photobioreactors floating on the ocean surface (Maeda et al., 2018). On the other hand, there are also laboratory studies addressing the cultivation of individual species in artificially manipulated brackish water (Sheets et al., 2014). Although some halophilic microalgae thrive in high saline concentrations and even present exceptional biodesalination potential, the number of domesticated salt-tolerant strains is very limited (Sahle-Demessie et al., 2019). Further

evaluations of microalgae gene modifications for screening individual mutant strains are needed in this field.

In addition to the implications mentioned above, there are other restrictions on seawater integration. Firstly, to supply a commercial microalgae plant, the use of seawater would be intensive. The reality here is that it would necessary to consider desalination as an alternative source for water security. But for industrial purposes, this is burdensome, and therefore still regarded as something remote (Förster, 2014). Secondly, as regards the guardianship of natural water resources, the legislation is aimed at protecting freshwater bodies, such as rivers, lakes, and underground reservoirs (Cosgrove and Loucks, 2015). For example, the European Water Policy, declared in the Water Framework Directive (2000/60/EC), exerts pressure on water bodies by estimating water abstraction and pollution from industrial activities. Perhaps to be taken as an inexhaustibility factor or the high cost of the desalination process, seawater is not treated by law as an isolated natural resource. In general, in this regard, the legislation is flawed in that fully effective measures cannot be implemented in the qualitative and quantitative management of seawater withdrawal and allocation. It is worth mentioning, however, that conflicts may be generated in the future about seawater integration because, as no universal standard exists, it is not known to what regime the use of this resource will be subject. On the one hand, there may be a requirement for a legal grant; on the other, fees may be charged for its use.

Another attractive option for water integration to offset expenses is through reuse of water and its coupling in microalgae-based processes. The amount of reused water is proportional to water demand in the process; that is, it reduces the need for further exploitation of this resource (Gude, 2015). Similarly to what happens with mass effluent integration, instead of nutrient cycling to cultures, the residual aqueous fraction of this material, from a given industrial process, can be incorporated into other operations. Water reuse, sometimes referred to as water recycling, can be viable for various applications, depending on site-specific conditions (EPA, 2019). For the microalgae industry, the destination of this "purified water" may be for incorporation into the cultivation reactor itself, or as cooling and heating water for auxiliary equipment (i.e., boiler makeup water in a process to generate bioenergy or biofuels) (Mo and Zhang, 2013). For this, the reused water usually goes through a wastewater treatment plant, considering some quality parameters (solids, color, turbidity, alkalinity, etc.) to fit the standards established by legislation for the intended use (EPA, 2012).

As mentioned above, although some microalgae species can assimilate the pollutants contained in the recycled liquid medium, the growth of the vast majority is often affected due to the toxicity of certain compounds that are not eliminated in treatment (Farooq et al., 2015). Waters from industrial activities include oils, pesticides, and heavy metals, among other constituents, whose toxic organic compounds often combine with the persistence and bioaccumulation potential (Priyadarshani et al., 2011). Another significant limitation of this type of reuse water

integration is related to locality issues (Fresewinkel et al., 2014). For example, the installation of a microalgae mass cultivation system requires a vast area of land and it is usually located far from the large industrialization sites to supply this water source, and its transport in tanks would be costly. This fact alone makes integration unfeasible. The integrations of the different types of water shown here are quite promising, but present real challenges to microalgae-based processes.

26.4 Environmental and economic indicators of microalgae integrated systems

26.4.1 Life cycle assessment

The challenge of understanding mass, energy, and water integrations and approaching them under the sustainable aspect appear complex. However, process integration strategies associated with environmental assessment tools play a key role in the practical and sustainable development of microalgae-based industrial systems. This is because using systematic methodologies, such as a life cycle assessment, presents significant potential to address and quantify the inherent environmental burdens (Mongkhonsiri et al., 2018). This methodology simultaneously enables costs improvements that are also achieved through the waste hierarchy, which consists of the three R's: reuse, recovery, and recycling, making them fundamental tools for implementation in industrial systems (Johnson, 2018).

An established consensus reports energy demands as a critical bottleneck in the development of any microalgae-based process. In this way, energy integration has become an issue widely discussed in energy management in industrial processes. Therefore, reducing energy requirements (conversion, supply, and consumption) should be investigated further for resource efficiency and the economy as well as environmental footprints (Chen et al., 2019). For this reason, the global energy and associated environmental impacts have been explored in detail in an attempt to optimize more sustainable technological routes by verifying the process steps that can be integrated (Banerjee et al., 2019).

Given this scenario, initial studies on integration strategies promoted using the residual heat of the combustion gases of the plants for the drying of algae (Chowdhury et al., 2012). About 8% of the energy used in coal plants is lost with the flue gases. Thus, when integrating the exhaust gases of coal plants with the unitary microalgal drying operation, it would result in approximately 10% less direct environmental impacts associated with the energy resource category. Moreover, since we would probably consider fossil energy as a source for the operating system, we would be indirectly interfering in the categories of ozone depletion, global warming, smog, acidification, eutrophication, and ecotoxicity. Therefore, the most efficient method to reduce environmental impacts is by minimizing external inputs of fossil energy as well as fossil-derived feedstock materials.

Another possibility for heat integration is wet algal biomass to biofuel production (Kouhia et al., 2019). Studies report that the direct combustion of biomass results in a gas mixture with substantial heat potential. This, in turn, can be used in industrial heating systems or can also be used for the production of steam and consequently applied in the production of electricity. It is estimated that the energy content of the biomass represents about 60%–85% of the total heat of the steam in a boiler (Kumar and Singh, 2017). In addition, the energy integration from biomass ensures decreased demand for fossil energy required for the system, providing a reduction of energy resources. Still, once energy is related indirectly to the categories of global warming and ecotoxicity potentials, this will be slightly minimized since, for the obtaining of energy, environmental factors of extraction of natural resources influence them.

In the same way, the VOCs generated in microalgal photobioreactors have been reported as promising heat sources to be integrated into a bio-combustion process (Jacob-Lopes et al., 2017; Severo et al., 2020). The VOCs produced in this bioprocess were considered as a supplementary biofuel, presenting energy potentials and a power generation rate of 86,320 kJ/kg and 15,247.78 $kJ/m^3/d$, respectively, contributing to a better combustion performance (Severo et al., 2018a,b). From an environmental point of view, the results showed improvements in the thermal efficiency of 30.5% compared to conventional combustion and about 25% of total energy, indicating energy resource potential proceeds. Moreover, greenhouse gas emissions represented a reduction of about 80% in global warming potential.

Simultaneously, as well as the depletion of energy resources, the persistent volumes of freshwater used in industrial processes have contributed to the establishment of legislative and policy requirements for effluent reduction (Ramos et al., 2016). As a consequence, water integration strategies have been designed to ensure the minimum demand for natural resources. In addition, numerous aspects can be considered as real advantages of integrating water in microalgae-based industrial processes such as minimizing water footprint through the use of seawater and freshwater as well as the reuse of effluents and consequently the reduction of effluents sent to treatment (Klein et al., 2018).

The considerable demand for limited natural resources is one of the main obstacles to the economic viability of microalgal products (Ishika et al., 2017). Given this aspect, studies employing seawater integration as an alternative to freshwater have been presented (Ishika et al., 2019). This resource could replace losses by evaporation, or cultivations could be filled exclusively with seawater. In contrast, another alternative for water integration occurs through water resulting from centrifugation processes (Förster, 2014). In the freshwater integration scenarios, the water footprints associated with the evaporation and recirculation system of water in the centrifugation and drying procedures would allow recirculation of 75% of the blue water footprint. In addition, 90% of the gray water footprint would be avoided, since there would be no wastewater to be treated. Therefore, the possibility of recovery of water volumes can reach about 80% of the total water footprint required in microalgae cultivation systems.

The wastewater integration strategy is the primary alternative for minimizing environmental indicators. In this context, Guldhe et al. (2017) proposed microalgae cultivation in wastewater. Thus, the wastewater requires about 90% less freshwater. It is essential to note that in addition to the integration of a substantial fraction of water, in parallel there is a considerable amount of mass integration, resulting in the reduction of the nitrogen requirement by up to 94%. This is because, depending on the composition of the effluent, the microalgae can remove approximately 85% of nitrates and 75% of ammonia, besides other nutrients such as phosphates and organic carbon demand (Rawat et al., 2013). Moreover, if we consider the energy resources and greenhouse gas emissions related to chemical fertilization, when integrating wastewater, a reduction of up to 50% is possible in these categories (Lam and Lee, 2012). Beyond the significant decrease of these impact categories, considerable values of acidification and potential eutrophication are minimized, since these categories are directly related to ammonia, nitrate, and phosphate emissions in water and terrestrial ecosystems.

Where the use of mass integration strategies is necessary to control excess waste in industrial practices, Deprá et al. (2019), simulating combustion gases, provided carbon concentrations of 15% as the sole source of carbon in a hybrid photobioreactor. The results showed maximum conversion efficiencies of 45.32 $kgCO_2/m^3/d$, resulting in a global carbon conversion of 30%. As an environmental indicator, the energy relation to the energy potential of the biomass produced and the required operational energy demand was determined, and the ecological viability was therefore achieved, resulting in a net energy ratio of 2.49. In addition, the carbon footprint attributed to this system can offer a reduction of approximately 41% of global warming potential.

Alternatively, among a wide range of possible uses of mass integration, glycerol is an attractive alternative in terms of environmental aspects (Ren et al., 2017). The results found in the literature show that microalgal growth and lipid content, 16.7 $g/m^2/d$ and 23.6%, respectively, were improved with the integration of glycerol in the culture. Moreover, the removal of nutrients such as nitrogen and phosphorus were increased when compared to the control treatment (without glycerol). Under a sustainability assessment, this integration has advantages over environmental equilibrium, since the reuse of a by-product can be reintroduced into the production of renewable energy (biodiesel) (Ma et al., 2016). Acidification and eutrophication potentials can be significantly reduced as they are directly related to organic carbon demand.

Besides carbon mass, nitrogen is the second most abundant element in biomass and, consequently, also represents a strong economic influence on nutrient demand during cultivation (Gao et al., 2019). Given this aspect, organic nitrogen, such as urea, was integrated into a microalgal system to raise biomass and lipid production when compared to the preferential sources of nitrogen (ammonia) (Batista et al., 2019). The results demonstrated that the use of urea did not modify cell growth rates. At the same time, the integration of nitrogenous

nutrients has a relevant character regarding the impact reduction associated with terrestrial and mainly aquatic eutrophication categories.

Likewise, nitrogen oxides (NO_x) may be used as nutrients during cultivation (Vuppaladadiyam et al., 2018). It is known that coal-fired flue gases present 90% NO_x as the major compound (Van Den Hende et al., 2012). Because of the above, microalgal strains were exposed under-treated with flue gas as a strategy to reduce greenhouse gases to the atmosphere. The microalgae presented NO removal of about 2.86 mg/L/d, resulting in global values of 96% of the converted nitrogen (Ma et al., 2019). However, it is important to note that the environmental footprint associated with photochemical oxidation, eutrophication, and acidification, according to these results, shows a drastic reduction in these impact categories. Furthermore, indirectly, the human toxicity category can be decreased since the excessive emission of these compounds poses a threat to respiratory health (Zhao et al., 2018).

Similarly, sulfur oxide (SO_x) emissions negatively influence the health of both biotic and human flora and fauna (Singh et al., 2019). The concentration levels of these compounds vary according to the combustion processes; however, values of the order of 200%–1500% are found in waste incineration systems. In view of this, gaseous effluents require proper treatment before being disposed to the atmosphere. Studies by Kumar et al. (2019), proposed the incorporation of exhaust gases from a coal-burning boiler composed of 180 ppm SO_x into microalgal cultures in sewage wastewater. The results showed that the SO_x removal efficiency was 45%, resulting in biomass productivities of 0.6 g/L. However, attention should be paid when exposing microalgal strains to high concentrations of SO_x, as this may inhibit their growth (Choi et al., 2019). Environmentally, by reducing the emitted levels, it is suggested that the acidification potentials be minimized by approximately 50% since sulfur compounds are directly related to acid rain. Furthermore, indirectly, this pollutant participates in photochemical smog, and consequently impacts on the category of photochemical ozone formation at ground level, which results in undesirable effects on human health.

Finally, several scenarios of partial mass, water, and energy integrations have been proposed to reduce the environmental impacts associated with the high demand of materials essential for the production and processing of microalgae processes. Therefore, the reuse of these surpluses could be a strategy to strengthen the non-generation of new wastes. However, although efforts have focused on determining key environmental indicators, these in turn represent only part of the sustainability metrics. The joint evaluation of economic aspects is crucial to try to reach a common denominator in the future.

26.4.2 Economic outlooks

From an economic point of view, the numerous evaluations of microalgae-based processes indicate a generally unfavorable economy (Doshi et al., 2016). Crucially, among ostensible

factors related to the processes viability are those associated with the costs of nutrient and energy supply. In addition, basic principles of process engineering such as the design and location of the plant, as well as economy of scale, make all these factors of a complex nature (Judd et al., 2017).

Since energy demand in microalgal processes is accentuated, metrics related to energy efficiencies and requirements are often accounted for in order to reduce the costs of this nature (Arcigni et al., 2019). Thus, through energy return on investment (EROI), this indicator can be an auxiliary measure of economic viability, since it quantifies energy inputs and outputs (Brigagão et al., 2019). An ideal option for microalgae production, EROI > 3, would be able to supply the entire production chain (Medeiros et al., 2015). Therefore, valuation strategies of the energy potential of biomass, integrating the co-product for the energy supply system, decrease the input demands of energy resource. Still, studies report that the use of renewable energy as a source of energy significantly increases EROI values to 8.35, while fossil energies are reduced to 1.25 and 2.13 (Beal et al., 2015).

Additionally, carbon dioxide is considered the most expensive consumable commodity (Wu et al., 2019). Theoretically, to produce 1 kg of biomass, about 1.83 kg of carbon dioxide is needed (Sepulveda et al., 2019). However, when considering microalgal processes where the supply of pure carbon dioxide values of 125.00 USD/kgCO_2 are estimated, this results in a cost of 228.75 USD/kg of biomass. In contrast, to integrate exhaust gases associated with a carbon capture and storage system, the Carbon Capture and Storage Association (CCSA) estimates that costs per ton of carbon dioxide are equivalent to around 95.00 USD/ton (Service, 2016). However, although the value is lower when compared to pure carbon dioxide, this value is still onerous. Furthermore, according to the Department of Energy, technological advances will make it possible in the short term to reduce to 20.00 USD/ton by 2025 (DOE, 2019).

Still, associated with carbon pricing, a strategy accessible to financial markets is related to carbon credits (Settre et al., 2019). It is estimated that around 16.1 million carbon credits have been sold in voluntary carbon markets in recent years (CER, 2018). In this sense, the possibility of acquiring additional carbon credits to affect the emission limit may reduce emissions. Moreover, the growing interest in carbon emissions indicates a substantial number of investors paying higher prices for carbon-neutral projects and technologies for the ease of selling surplus credits as it can be used to subsidize future projects in the plant (Günther et al., 2018).

Likewise, fertilization with nitrates and phosphates is indispensable to supply microalgal productivities. Currently, according to the United States Department of Agriculture, it is estimated that the costs of ammonium nitrate and superphosphates are approximately USD/ton 550 and 300, respectively (USDA, 2019). In addition, fertilizer production accounts for 1%–2% of global energy consumption (Winkler and Straka, 2019). Studies conducted by Zhang and

Kendall (2019) report requirements of ammonium nitrate 0.15 kg/kg of biomass and superphosphate in the order of 0.10 kg/kg of biomass. Therefore, in an estimated production of 1 ton of microalgal biomass, the required costs involving nutrient demand would be around 82.5 USD for nitrates and USD 30.00 for phosphates. Thus, a viable cost-reduction strategy is presented through the partial integration of nutrients. Besides, according to De Bhowmick et al. (2018), the cultivation of microalgae using liquid effluent nutrients in a closed circular biorefinery model, that is, total integration, results in a cost reduction of 35%–86%.

Microalgae cultivation requires large volumes of water. However, studies show that in the process of obtaining 1 kg of biomass without water recycling, values of the order of 1.08 m^3 are required. On the other hand, if there is total water integration, about 0.24 m^3 is required (Mayers et al., 2016). From an economic point of view, it is estimated that water prices in the world show values of approximately 1.50 USD/m^3. However, the values associated with wastewater treatment are around 3.0–4.0 USD/m^3 for sewage discharge, resulting in an approximate direct cost of 5.00 USD/m^3 (Clere, 2016). Since the water volumes represent a capital demand of approximately 1.62 USD/kg of biomass, with 100% of the recycled water, the values can reduce by up to 22% of the costs. In addition, considering that wastewater can be included integrally to microalgae cultures, in a hypothetical scenario, a medium-sized industry has about 16 m^3/d. If we think of the integration of this wastewater to the microalgae cultivation, it is possible to avoid spending about 26.4 million USD/year.

Thereby, it is believed that entire processes integration is aimed at reducing greenhouse gas emissions, as well as the remediation of wastewater associated with energy generation and high value-added co-products. In addition, other benefits are expected through carbon capture and carbon storage of carbon credits as an integral part of a process. Therefore, the microalgae-based systems would become economically viable and environmentally sustainable from the point of view of the circular biorefinery (Fig. 26.2.). In this way, it is suggested that there should be efficient recycling of the products generated within the integrated system. Exhaust gases would serve the nutrients for the development of biomass microalgae, in addition to wastewater with a high concentration of nutrients. Thus, the products and co-products generated could return to the industry in a closed loop. Therefore, this concept should be considered as a potential strategy to solve and to establish new green engineering associated with the environmental and economic benefits related to microalgal processes.

26.5 Concluding remarks

When considering that microalgae-based processes and products still face barriers to full market competitiveness, the process integration strategy seems, at first glance, to be a promising solution. However, by looking more closely at the reality of the facts, it is not enough

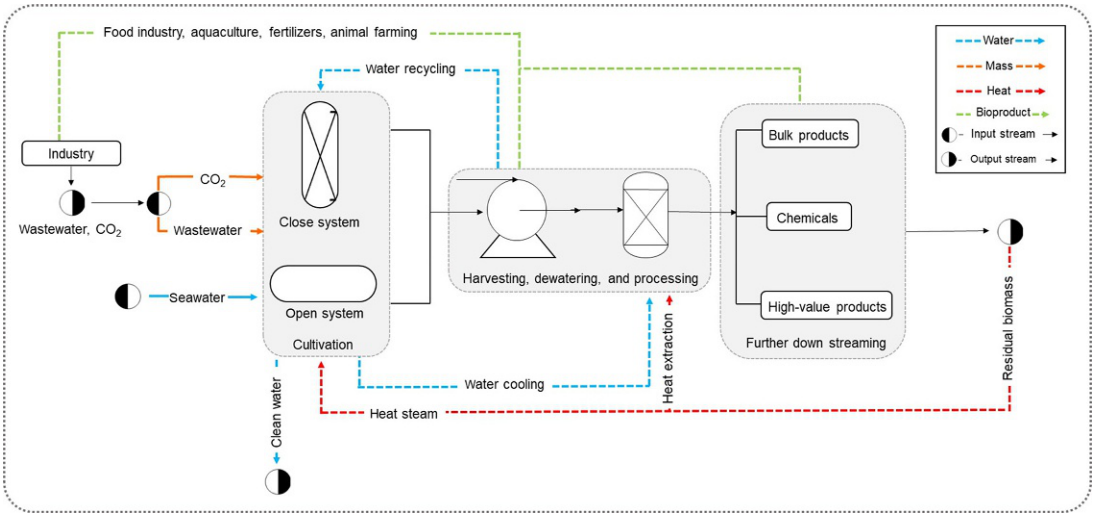

Fig. 26.2
An ideal process integration model in a microalgae-based biorefinery.

to integrate and co-integrate energy, mass, or water, as discussed here. The most important point now is to consider synergistically environmental and economic assessments, as well as the biorefinery approach, in an attempt to find a scenario for industrial deployment.

In this sense, for a better understanding, we separated the pros and cons of the different types of integration in Table 26.1, along with open questions for each of the categories. In general, it is complex to find a balance in each type of process integration category. All of them depend on seasonality and geographical conditions, which are considered the main factors for a stable microalgae system. Tools for strategically exploring the optimal sites for large-scale cultivation (e.g., the geographic information system, or GIS) are today one of the priorities of process integration; they consider global and local mapping, including several parameters, such as ideal temperature, solar irradiation, availability of land and inputs near industrial areas (CO_2 or wastewater supply), groundwater salinity, and rainfall. In addition to this information, many other vital factors must be addressed: the inherent requirements of microalgae strains under genetic engineering perspectives, and the technical, economic, social, governmental, and environmental aspects of the selected potential site.

Once adjusted, the progress of process integration applied to microalgae-based systems on a commercial scale will considerably reduce costly demands and then will result in inexpensive, safe, and sustainable technologies.

Table 26.1: Pros and cons of different types of integration and open questions for each category in microalgae-based systems.

Type of integration	Pros	Cons	Open questions
Energy	• Reduced electricity expense • Waste heat recovery from thermal operations	• Nonexistence of a cheap temperature control system • Poor heat recovery techniques	• What would be the best geographical location to adopt as the ideal climatic model for microalgae-based processes temperature control? • What recovery technique would be more suitable to optimize for application in large-scale manufacturing processes?
Mass	• Ability to remove surplus compounds from indus trial activities	• Limited assimilation of certain substances • Availability of geographically appropriate sites to introduce a microalgae plant	• How can we improve both strains' performance and cultivation systems to support the high abundance of effluents from industries?
Water	• Reduced water footprint • Combination of integration approaches (water vs. mass)	• Insufficient environmental regulation • Characteristics of cell toxicity and bioaccumulation	• How should genome editing techniques be engineered to improve the metabolism required for different types of water use?

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CHAPTER 27

Process intensification applied to microalgae-based processes and products

Rajshree Amrut Patil[a], **Shankar Balajirao Kausley**[b], **Saurabh Milind Joshi**[a], **Aniruddha Bhalchandra Pandit**[a]

[a]*Department of Chemical Engineering, Institute of Chemical Technology, Mumbai, India* [b]*Physical Sciences Research Area, TCS Research, TRDDC, Tata Consultancy Services, Pune, India*

Chapter outline

27.1 Introduction 737
27.2 Process intensification at different phases of bulk production of microalgae 738
27.2.1 Selection of microalgae strain 738
27.2.2 Cultivation 740
27.2.3 Harvesting 741
27.2.4 Cell drying 748
27.2.5 Cell disruption methods 749
27.3 Process intensification methods applied to different microalgae-based products 752
27.3.1 Biodiesel 752
27.3.2 Bioethanol 756
27.3.3 Biohydrogen/biogas 759
27.3.4 Process intensification in wastewater treatment using microalgae 760
27.3.5 Process intensification in carbon dioxide capture using microalgae 761
27.3.6 Process intensification in microalgal pigments extraction 762
27.3.7 Single-cell protein for food application 763
27.4 Conclusions 763
Acknowledgments 765
References 765

27.1 Introduction

Innovative use of biological resources to fulfill the growing demands of food and energy is a current need. In this regard, microalgae-based products are gaining considerable interest as a promising source of different high- to low-value products such as nutraceuticals, pharmaceuticals, biofuels, bio-feeds for animals, and bio-fertilizers (Borowitzka, 2013). By 2025, the global market for algae-based products is expected to reach USD 3.5 billion with a

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compound annual growth rate (CAGR) of 4.2% from 2018 to 2025 (Kadam, 2018). In response to the increasing trend of these products, the industry has already initiated a proactive effort to formulate microalgae-based consumable products with good quality in terms of nutritional value, texture, and safety. However, although research activities on microalgae-based products are promising, the products currently in the market are still limited and expensive, and face technological challenges in terms of cost reduction, reducing energy requirements, and reducing the waste generated during the process. In order to come up with competitive products, crucial issues in both upstream and downstream production processes need to be addressed during large-scale manufacturing by employing different process intensification (PI) approaches. Considerable progress is being made in employing different PI approaches to achieve maximum economic benefits to the manufacturer.

The major objective of PI is to develop cleaner, safer, smaller, and more energy-efficient processes (Stankiewicz and Drinkenburg, 2018; Vaghari et al., 2015). Technically, this means an improvement or increase in the rates of heat and mass transfer of the reaction through a combination of different methods/processes and equipment. This chapter reviews different technological approaches that can be employed to intensify production processes of different microalgae-based products at different phases of production. In the initial part of the chapter, a brief description of different features of methods/technologies used during different stages (cultivation, harvesting, and product extraction) of product formation are described. The latter part of the chapter describes PI strategies currently used as well as those that could possibly be employed during the manufacturing of a selected set of microalgae-based products.

27.2 Process intensification at different phases of bulk production of microalgae

Fig. 27.1 illustrates a process flow scheme that most microalgae-based products undergo. It involves a series of processes including bulk cultivation of appropriate microalgae strains in a suitable growth media, harvesting of biomass, processing of biomass to extract the desired component, and finally modification and processing of final products. Each phase has its own independent requirements in terms of nutrients and energy. Effective recycling of by-products produced during the process, such as wastewater, spent organic solvents, and biomass sludge, is also a key requirement of the PI measures.

27.2.1 Selection of microalgae strain

Employing specific microalgae strains with high production ability of the desired component will help in a significant increase in the yield of the product. Fig. 27.2 illustrates the selection criteria of microalgae strains for employment in the production process. The selection of a strain should be based on its advantage at different phases of process flow of product formation in upstream as well as downstream processes to make it economically viable.

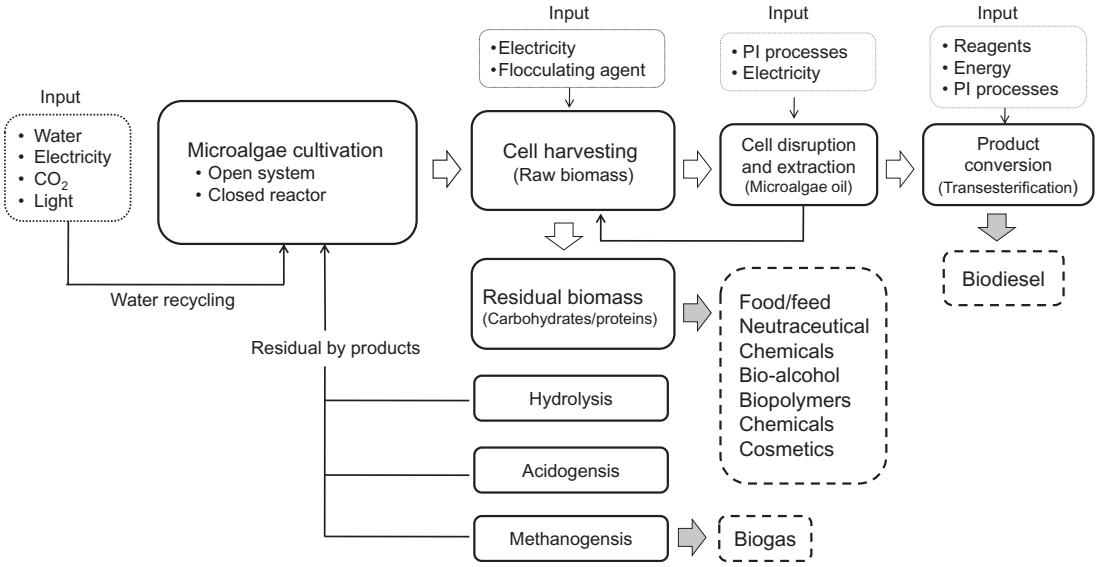

Fig. 27.1
Process flow scheme of the microalgae-based production system.

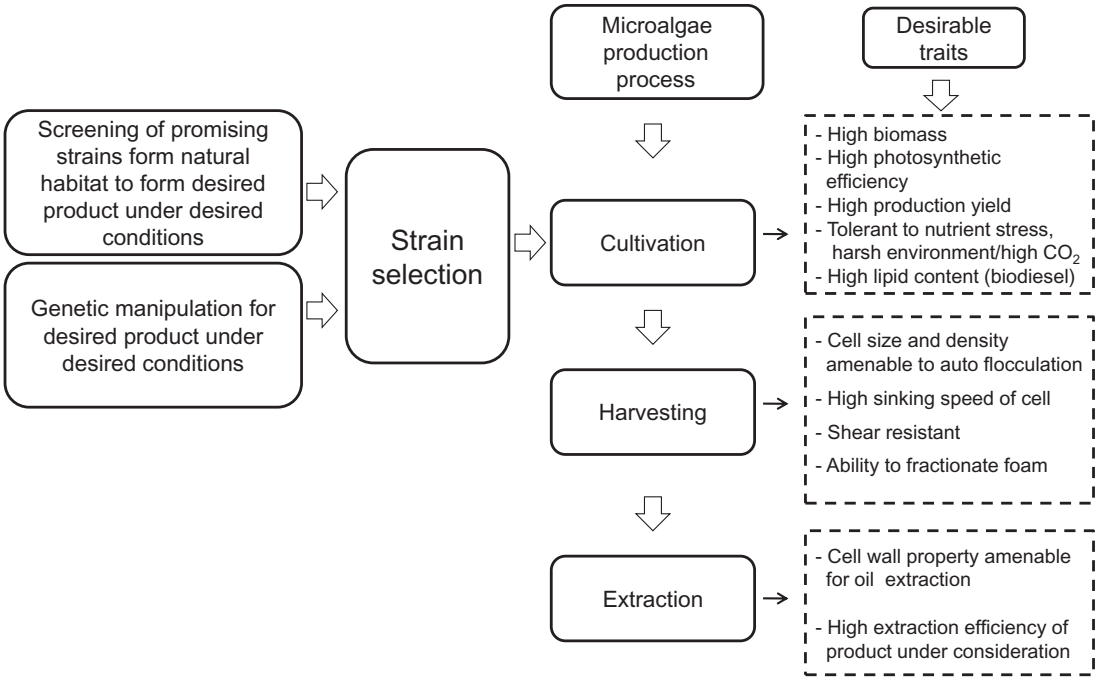

Fig. 27.2
Criteria for selection of microalgae strains for enhanced production.

The selected strain should possess high photosynthetic efficiency, carbon dioxide consumption rate, ability to grow in different kinds of water, and rapid growth rate. It should also be robust and flexible in order to tolerate different environmental conditions. In addition, it is desirable that the strain possesses cellular characteristics, making it easily amenable (controlled) and processable during harvesting and extraction of products under consideration. Isolation of appropriate microalgae strains for producing respective compounds is usually done from their natural habitat. Recently, significant research has been undertaken in the genetic manipulation of microalgae species for improving their photosynthesis ability and CO_2 utilization potential, accumulation of desired component at higher concentrations, and ability to grow at extreme environmental conditions (Wan et al., 2014). However, the technology still faces challenges in order to be suitable for large-scale commercial production.

27.2.2 Cultivation

The main goal of cultivation is to achieve high production of good-quality biomass with desired characteristics. The control of microalgae growth is complex due to the effect of photosynthesis, CO_2 concentration, and different environmental conditions. Knowledge about specific growth requirements in terms of light, temperature, and nutrients of photoautotrophic organisms is key in employing different PI strategies for achieving maximum biomass output at the cultivation stage. Microalgae cultivation systems are categorized as open and closed systems. In an open system, open ponds and lagoons are used for the cultivation of microalgae. The system is easy to construct, maintained at a low operating cost, and ideal for large-scale cultivation. However, since the system is open it faces significant drawbacks such as requirements of large tracts of space, loss of water due to evaporation, loss of CO_2 into the atmosphere, less control on the environmental barrier, and high risk of contamination from other microbial strains or predators. However, due to its cost-effectiveness, most commercial operations for the large-scale production of algal biomass are based on open-pond systems.

The open-pond system can be of different types including unstirred ponds, circular ponds, and raceway ponds; the latter are the most commonly used. The mixing of cultivation media in an open-pond system is performed using impellers, paddle wheels, and rotating arms. This helps in increasing the production through the uniform distribution of nutrients and prevention of dead zones. Many times CO_2 rich air is used for mixing where it also serves as a nutrient source. The major focus of PI methods is to optimize the configuration of raceway ponds to reduce the energy consumption required during the mixing operation. Sompech et al. (2012) reported modification in the design of the standard configuration of raceway ponds by installing three flow-deflector baffles at each end; this is relatively inexpensive and can effectively prevent the formation of dead zones. The baffles reduce energy consumption (by 20%) relative to the standard configuration from 3464 to 2852 W (Sompech et al., 2012).

The limitations of an open system can be addressed by the use of a closed system for cultivation of microalgae. Closed systems are generally considered as closed photobioreactors (CPBRs), where the cultivation of algae is carried out in a closed system to prevent contact between the enclosed algae and the environment. The closed system provides efficient capture and utilization of light as well as control of different environmental parameters, and can be operated outdoors where solar radiation is used as a source of light. The most popular systems are tubular, flat-plate, bubble-column, and vertical photobioreactors. The PI of a photobioreactor focuses on efficient use of light distribution inside the reactor, better gas exchange (CO_2 supply and oxygen removal), uniform distribution of nutrients within the cultivation medium, optimizing the size of the reactor for scale-up, and increase in biomass production. However, the high construction, maintenance, and operational costs of a PBR usually limit its application to laboratory scales.

Use of hybrid systems or two-stage cultivation is a recent approach to address the limitations associated with open and closed cultivation systems. This hybrid system combines exponential biomass production in closed bioreactors under controlled conditions followed by a transfer of this biomass to nutrient-depleted open raceway ponds, where the conditions stimulate accumulation of the desired compound at a high rate. Since the biomass is kept in the open raceway ponds for only a few days, risk of contamination by different predators is reduced. The cost of cultivation of biomass can be further reduced by using low-cost substrates such as agro-industrial wastewater and flue gas as nutrients for the cultivation of microalgae. Such systems along with an increase in biomass content are useful for bioremediation of wastewater and fixation of greenhouse gases (Płaczek et al., 2017).

In addition to the above, one interesting strategy commonly employed at the cultivation stage involves the manipulation of nutritional (e.g., nitrogen and phosphorus), environmental (e.g., temperature and light), and osmotic (salinity) conditions, to create stress conditions, to induce metabolic pathways in microalgae which lead to the accumulation of the product under consideration in larger quantities (Chu, 2017). Addition of phytohormones and co-cultivation of microalgae with yeasts and bacteria are some recent strategies to enhance biomass production at the cultivation stage (Singh et al., 2016). A few examples of different cultivation systems developed for the enhancement of microalgae systems which have been reported in the literature are given in Table 27.1.

27.2.3 Harvesting

Harvesting is the process of separation of microalgae cells from the cultivation media to convert it further into the desired product by employing different processing steps depending on the type of the target product. The process of harvesting comprises a sequential operation of three main steps: biomass recovery, dewatering, and drying. Harvesting microalgae from a dilute suspension of growth media (concentration less than $1\,g\,L^{-1}$) requires high energy inputs and is considered to be a major challenge. Moreover, the small size of the algal cell with a

Table 27.1 Systems used for intensification of microalgae production.

Microalgae species involved	Geometry and the process intensification technique/method	Process conditions/ equipment details	Performance improvement	Advantages	Limitations
Chlorella vulgaris	Deflector baffles in raceway pond (Sompech et al., 2012)	Uniform mixing prevents dead zone, low energy consumption	Energy requirement reduced from 3464 to 2852 W	Large-scale production	Contamination risk, less control on environmental parameters
Chlorella sorokiniana IAM-C212	Stirred tank PBR (Singh and Sharma, 2012)	Mechanical agitator for mixing, heat exchanger, gas exchange through the sparger	Increase in biomass	Good heat and mass transfer, good light-diffusion, simple design	Heating issue, require extra energy, low surface to volume ratio
Phaeodactylum tricornutum *Thalassiosira pseudonana*	Flat-panel PBR (Singh and Sharma, 2012)	Bubbles used for mixing, gas exchange at the headspace of reactor control the temperature	Uniform distribution of nutrient due to good mixing, design helps to control temperature	High surface to volume ratio, low space requirements, high photosynthetic efficiency, cost-effective, low oxygen buildup	Short light penetration depth, many components required, frequent fouling, clean-up issue
Nannochloropsis sp. CCAP 211/78	Horizontal tubular PBR (De Vree et al., 2015)	Mixing by recirculation through a pump, temperature control by shading, overlapping, water spraying, gas exchanger, degassing unit	High surface to volume ratio	Low hydrodynamic stress, suitable for outdoor application, cost-effective	Buildup of dissolved oxygen, large space requirement, susceptible to photoinhibition
Scenedesmus Chlorella Arthrospira platensis	Thin-layer cascades (TLC) (Masojídek et al., 2015)	Culture flows from the top to the bottom over sloping platforms and pumped back to the top from retention tank.	Biomass production in the range of 0.5–1 g dry mass L^{-1}	Use of solar energy, simple cleaning, and maintenance, efficient degassing, high biomass densities	Risk of contamination
Tetraselimis	Floating PBR (Kim et al., 2016)	The system is allowed to float on the ocean	Biomass productivity 3.9 g m^{-2} d^{-1} and biodiesel productivity of 554.4 g m^{-2} d^{-1}	Natural temperature control, open ocean to provide low-cost space, increased mass transfer and mixing efficiency, use of ocean wave energy for mixing	No control on environmental variable

Haematococcus pluvialis *Chlorella vulgaris*	Submerged-light PBR (SL-PBR) (Murray et al., 2017)	Light emitting diode (LED) is used as a source of light	Increase in light delivery system at least 5-fold	Uniform light distribution, reduce dark zone, minimum shelf shading	Possibility of decrease in light intensity
Chlorella vulgaris	Solar-powered thin film PBR (Pruvost et al., 2017)	Cultivation in the form of thin film (1.5–2 mm)	High productivity: 5.7–7.07 $kg\,m^{-3}\,d^{-1}$	High volumetric and real productivity, large-scale cultivation of microalgae, high specific illuminated surface area	Broad variation in photon absorption during summer
Chlorella sp.	Twisted tubular PBR with swirl flow (Gómez-Pérez et al., 2017)	Swirl mixing to provide good to light-dark (LD) cycles, nutrients and dissolved CO_2	Increase in production of biomass	Lower energy demand	High operating cost
Chlorella sp.	Tubular PBR with helical mixer (Zhang et al., 2013)	Mixing with a static mixer	Increase in biomass production	Intensified mixing, creating light/dark cycle	Increase in operational cost
Haematococcus pluvialis	Flashing light PBR (Abu-Ghosh et al., 2016)	Increase in photosynthesis efficiency (activation of Photosystems I and II)	Fourfold increase in rate of astaxanthin production per photon	Controllable light/dark cycle	Not effective at low cell density, high O_2 production rate
Haematococcus pluvialis	Hybrid PBR (Płaczek et al., 2017)	Integration of various phases of microalgae growth in a single two-stage system	Increase in lipid productivity	High yield of product	Need precise control for change from one phase to other
Spirulina	Pyramid PBR (Płaczek et al., 2017)	Design allows increase in biomass	Fourfold increase in production yield	Design provides large area for biomass production	At experimental stage, requires large area for scale-up

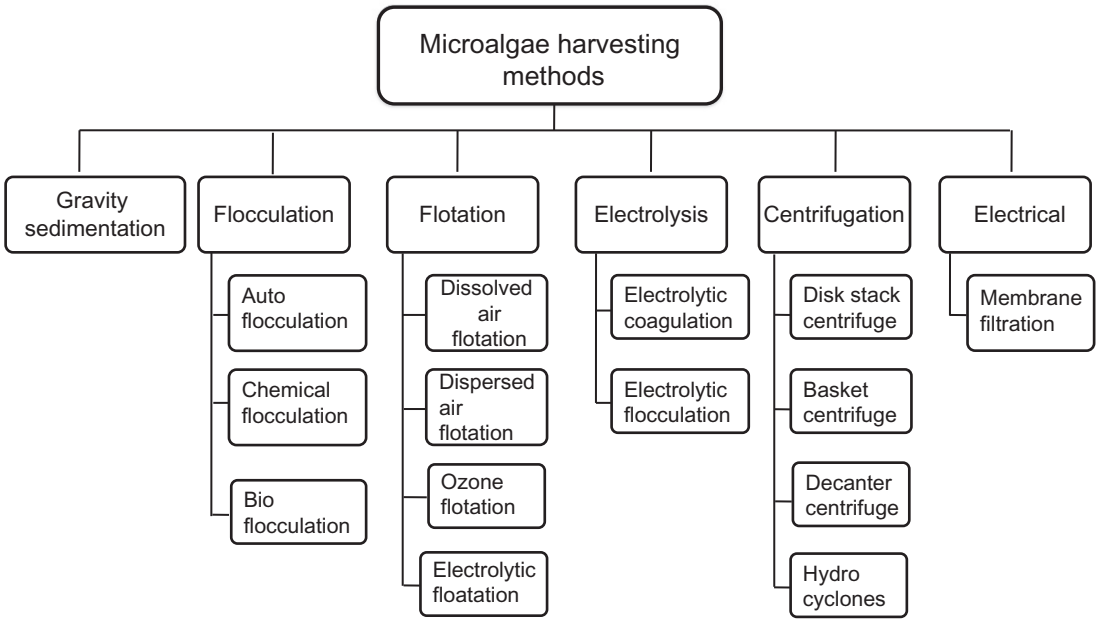

Fig. 27.3
Microalgae harvesting methods.

density nearly equivalent to the growth media, presence of negative charge, and presence of algogenic organic matter allows them to remain as a stable dispersant in the media (Danquah et al., 2009). Microalgae harvesting methods currently in use involve mechanical, chemical, biological, and electricity-based methods, as shown in Fig. 27.3.

The choice of harvesting technique depends on the properties of algae, their cell density, and the required quality of the desired product. The harvested biomass may also need further processing and thus the procedure must not be toxic to the biomass or add harmful contaminants. For developing a sustainable and economically viable operation, the method also needs to allow the recycling of culture media, wastewater, or any residual chemical left during the process. In most applications, harvesting is carried out as a two-step process comprising thickening and dewatering. Different techniques used for thickening include chemical methods like coagulation and flocculation, bioflocculation as a biological method, and gravity sedimentation, flotation, and electrophoresis as physical methods of separation. Dewatering is done by applying physical methods, mainly centrifugation and filtration. A brief discussion of each method is given in Sections 27.2.3.1 to 27.2.3.6, whereas these methods are compared in Table 27.2 in terms of their benefits and limitations.

27.2.3.1 Coagulation/flocculation

The algal biomass comes out of suspension in the form of floc, either spontaneously or after the addition of chemicals. Coagulation using chemicals is widely known and discussed in the previous literature (Branyikova et al., 2018). Use of magnetic flocculant is a more recently

Table 27.2: Comparison of different harvesting techniques.

Method	Advantages	Limitations	Dry solid output concentration (%)
Sedimentation (Milledge and Heaven, 2013)	• Simple • Inexpensive • First stage use reduces the energy input and cost of subsequent stages	• Time-consuming • Need high cell density • Low separation • Low final concentration	0.5–3
Flocculation (Branyikova et al., 2018)	• Simple • Rapid • Wide range of flocculants available • Low cost	• Need to remove flocculants as these may be toxic to microalgae • Issues with media recycling	3–8
Flotation (Singh and Patidar, 2018)	• Suitable for large-scale application • Faster than sedimentation • Possible to combine with gaseous transfer	• Algal specific • High capital and operational cost • Requires chemical flocculants • Not suitable for marine microalgae	7
Centrifugation (Al Hattab et al., 2015)	• Rapid • Suitable for small microalgae • Highly efficient	• High capital and operational costs • High shear force can damage cells • Time-consuming • Expensive for large-scale operation	10–22
Filtration (Al Hattab et al., 2015)	• No chemicals required • Cost-effective • Water recycle • Low shear stress • Less consumption of energy	• Requires pressure and vacuum • Slow • Not suitable for small algae • Membrane fouling • High maintenance and operation costs	2–27

Continued

Table 27.2: Comparison of different harvesting techniques—cont'd

Method	Advantages	Limitations	Dry solid output concentration (%)
Ultrafiltration (Al Hattab et al., 2015)	• Can handle delicate cells	• High capital and operational costs • Requires regular cleaning and periodic replacements	1.5–4
Electrical-based process (electro-flocculation + sedimentation or flotation) (Branyikova et al., 2018)	• Applicable to all microalgal species • No chemical requirement	• Requires metal electrode • Metal contamination • High operating cost	10 (sedimentation) 30–40 (flotation)
Magnetic separation of microalgae (Branyikova et al., 2018)	• More than 90% cell recovery • Rapid	• Requires magnetic modification of biomass • Contamination with magnetic particles	10–20

studied approach for the harvesting of microalgae. The process is carried out by using an external magnetic field to separate microalgal cells adsorbed with magnetic particles. By this process, the flocculation and separation can be carried out in a single step. The process is nondestructive and can be easily manipulated and regenerated. The magnetic particles used consist of uncoated magnetic iron oxide particles or as a functionalized composite with core coated with silica. However, the process may be expensive to apply to a large-scale operation (Cerff et al., 2012; Hu et al., 2014).

Flocculation can be also induced in the system by the process of auto-flocculation or bioflocculation. Auto-flocculation is usually caused by precipitation due to the action of the carbonic components present in the media with extracellular biopolymers secreted by the algae. The process of bioflocculation involves other microorganisms as a whole cell with or without extracellular polysaccharide (EPS) compound. During the process, the microalgae or bacterial species that flocculate readily can be mixed with other species to induce mutual flocculation. Recently, the *Skeletonema* species was reported to have been employed to induce flocculation. Flocculating fungi or bacteria, which produce EPS, can also be used to induce flocculation. A bioflocculant from *Paenibacillus polymyxa* in combination with cationic chemicals has been used for harvesting *Scenedesmus* sp. with an efficiency of 95% (Kim et al., 2011).

27.2.3.2 Sedimentation

Gravitational forces separate the solids and liquids from one another. The separation of the materials occurs based on their density. The method is well-suited for products such as biofuels. Generally, coagulation/flocculation is applied before sedimentation to enhance the microalgae settling (Al Hattab et al., 2015).

27.2.3.3 Flotation

The solid particles are allowed to float on the liquid surface by attaching them with air or gas bubbles. Based on air bubble size, the process of flotation is categorized as dissolved air flotation (10–100 μm) and dispersed air flotation (700–1500 μm). Recently, instead of atmospheric air, ozone has been used to produce charged bubbles. Ozone oxidizes the soluble organic content in the medium and the charged air bubble separates the microalgae. The biopolymers released during the cell lysis act as a coagulant and help in separating cells. The process is found to remove *Chlorella vulgaris* and *Scenedesmus obliquus* successfully, with 98% efficiency.

27.2.3.4 Electrical base process

An electric field is used to remove the charged microalgae from solution. During the process, water electrolysis generates hydrogen in the form of small bubbles which adhere to the microalgae flocs and carry them to the surface. The process is also referred to as electro-coagulation-flotation (ECF). The method is selective and does not require the addition of

any chemicals (Chen et al., 2011; Gao et al., 2010). However, it is difficult to scale-up and needs parameters such as pH of media, temperature, and type of electrode to be in an optimal range.

27.2.3.5 Centrifugation

The microalgae cells are retrieved from the cultivation media by applying centrifugal force. The cell separation takes place based on the difference in size and density of cells. The different types of centrifuge systems used for microalgae cell separation include disk stack centrifuge, basket centrifuge, decanter perforated basket centrifuge, and imperforated and hydro cyclones. The disk stack centrifuge and decanter centrifuge are most commonly used. Centrifugation is the preferred method for harvesting of high-value products from microalgae biomass. It offers a very high recovery rate and chemical-free biomass. However, for large-scale applications, energy consumption, treatment time, maintenance, and capital costs are very high.

27.2.3.6 Filtration

The microalgae broth is filtered through filtration media of a specific pore size under gravity or pressure, or by vacuum. Due to the small size of microalgae, conventional filtration systems are not suitable; instead, membrane filtration systems are preferred. The membrane holds the microalgae in a thick paste form, allowing water to pass through it. Classification of filtration system depends on the pore size of the membrane as microfiltration (0.1–10 μm), macrofiltration (>10 μm), ultrafiltration (0.005–0.1 μm), and reverse osmosis (<0.001 μm). Microfiltration and ultrafiltration are usually employed for separation of microalgae. The lower disruption of microalgae cells during membrane filtration compared to other filtration techniques results in a good quality of harvested biomass. Membrane filtration is usually not suitable for large-scale operation as maintenance, replacement, and pumping costs are very high.

As seen in Table 27.2, harvesting of microalgae using filtration and centrifugation has proven to be very efficient. However, these techniques are energy-intensive and consume around 20%–30% of the total processing cost. Thus, instead of using a single technology, applying preconcentration steps by using flocculation, sedimentation, or flotation can result in lower energy consumption. A reduction of nearly 10-fold in energy consumption can be achieved by preconcentration of the biomass by flocculation compared to applying only centrifugation (Al Hattab et al., 2015; Salim et al., 2012).

27.2.4 Cell drying

After harvesting the microalgae cells, the next step is to dry the dewatered slurry further, either for its end-use application or for extraction of different components such as algal oil. The drying process of algae should be designed to minimize the possible deterioration of biochemical

Table 27.3: Methods of cell drying.

Method	Advantages	Limitations
Rotary drying (0.077 kWh kg^{-1}) (Nappa et al., 2016)	Simultaneous sterilization and cell disruption	Consumes high energy
Spray-drying (0.1 kWh kg^{-1}) (Nappa et al., 2016)	Rapid and efficient drying, suitable for human consumption products	High-pressure atomization cause product degradation, expensive, low digestibility of dried algae
Solar drying (0.02–0.04 kWh kg^{-1}) (Nappa et al., 2016)	Cost-effective, feasible in a remote area lacking an electricity source	Depends on intensity of solar radiation, chances of airborne contamination hence not suitable for human consumption
Crossflow air drying	Low cost and rapid operation	Energy consumption is high
Vacuum-shelf drying	Highly efficient	Expensive installation and handling
Flash drying (0.032 kWh kg^{-1}) (Nappa et al., 2016)	Fast	Quality depends on source of flue gas
Incinerator drying	Suitable for management of sludge produced after wastewater treatment	Expensive and complex process

properties of algae cells or their components. The drying process is the major economic constraint in the scale-up of the process, as it accounts for around 75% of the overall cost. Major drying methods used for commercial application include rotary drying, spray-drying, cross-flow drying, solar drying, vacuum shelf drying, flash drying, and incinerator drying. A comparison of the different drying methods is given in Table 27.3.

27.2.5 Cell disruption methods

Cell disruption is an essential step to recover microalgae products such as lipids, carbohydrates, proteins, antioxidants, and pigments; these are typically located inside the cells and usually bound by internal organelles or are sometimes attached to the cell membrane. It is essential to maintain the functionality of active components while the biomass is undergoing the process of cell disruption. Cell disruption techniques are divided into two main groups based on the working mechanism, i.e., mechanical and nonmechanical methods, as shown in Fig. 27.4. Mechanical methods include bead milling, high-pressure homogenization, high-speed homogenization, ultrasonication, microwave treatment, and pulsed electric field treatment. Nonmechanical methods include enzymatic cell lysis, use of organic solvents, supercritical fluids, and freeze pressing.

27.2.5.1 Bead milling

Rapidly rotating notched discs are used to disrupt microalgae cells. Bead milling is generally used in combination with solvents to recover oil. The method is most effective and energy-effective for biomass concentrations in the range of 100–200 g L^{-1}. With

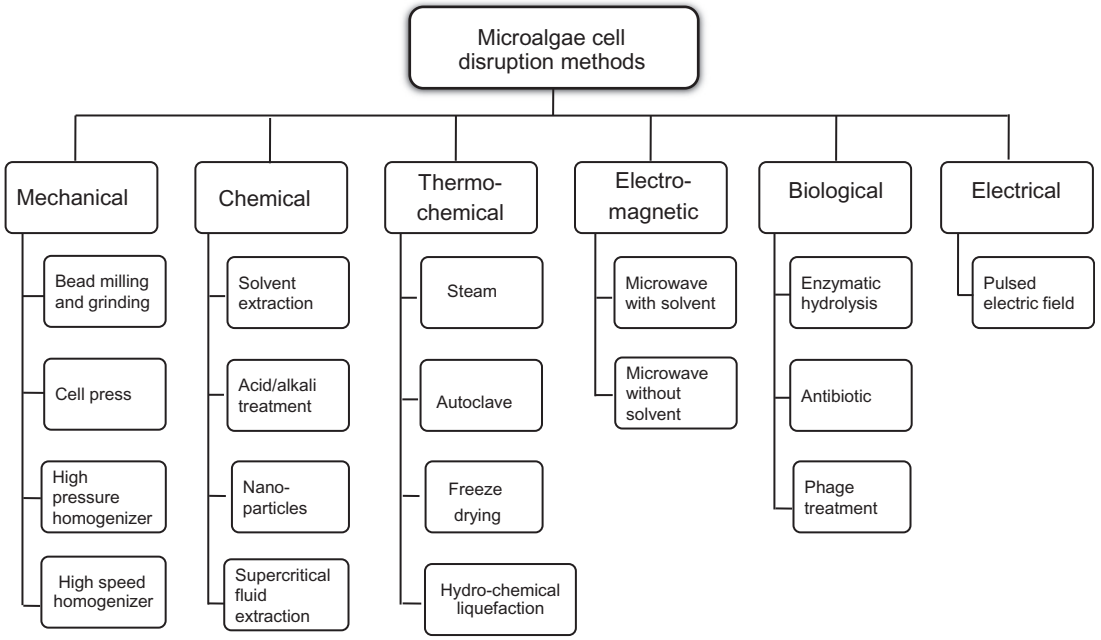

Fig. 27.4
Microalgae cell disruption methods.

bead milling, a high rate of cell disruption is possible; however, larger-scale operations require large amounts of energy.

27.2.5.2 High-pressure homogenization

Rupturing of microalgae cells is carried out under high pressure to release the cell content. The different configurations such as screw, piston, or expeller are used for commencing the pressing process. The most common instrument used is the French press machine, which can extract approximately 70%–75% oil from microalgae.

27.2.5.3 High-speed homogenization

This involves a device consisting of an impeller for stirring at high speeds. Cell disruption occurs due to hydrodynamic cavitation and shear forces at the solid liquid interface. However, it has been observed that it is difficult to obtain a high rate of cell destruction using high shear homogenization systems as the process consumes high amounts of energy. A combination of high-speed homogenizer with acid, alkali, and enzymatic treatment is widely used for the extraction of high-value products such as astaxanthin, which yields at least three times more product compared to processes carried out without a homogenizer.

27.2.5.4 Ultrasonication

High-power ultrasound could generate intensive microbubbles in a liquid medium. During ultrasonication, acoustic waves pass through the liquid medium causing the formation of microbubbles, their growth, and eventual collapse to create cavitating conditions. The violent collapse of bubbles leads to tremendous increase in local temperature over 5000 K and pressure by several hundred atmosphere. The process requires a very short time—as low as 5 min while operating at 10 kHz. It reduces the overall solvent consumption and allows greater penetration of solvent inside the cells. However, relatively low efficiency of cell disruption observed in only a few species is the major limitation of this technology. Moreover, the local heat generated during the process can lead to degeneration of heat-sensitive cellular components. Temperature control during treatment can improve product quality; however, the effectiveness of cell disruption decreases significantly. Ultrasonication can be used with different cell disruption methods to reduce the energy requirement and overall cost of the process.

27.2.5.5 Microwave

This treatment causes local heating due to frictional forces from inter- and intramolecular movements. This results in cell lysis, followed by release of the cell content. The treatment is more effective at a frequency of 2450 MHz (Dvoretsky et al., 2017).

However, the major disadvantage of this method is that since only a fraction of the water is held inside the cells, the majority of the radiation energy is absorbed by the surrounding medium and results in heat, which causes protein aggregation and denaturation. The advantages of microwave treatment are its robustness and easy scale-up due to its simplicity. However, the technique is limited to polar solvents and not suitable for volatile target compounds. Additionally, the rise in temperature and free radicals generated during the process can be harmful to sensitive molecules in the microalgae cells and thus it is not suitable for extraction of components, which comprise sensitive molecules.

27.2.5.6 Supercritical fluid extraction

This process involves the use of supercritical fluids such as CO_2, which show properties of both gas and liquid at specific temperatures and pressures. At the supercritical stage, the fluid extracts the components, and after returning to normal atmospheric conditions, it leaves the system with no residues. Carbon dioxide is the most common supercritical fluid used, sometimes in conjunction with cosolvents such as ethanol or methanol. The process yields highly purified products. However, high capital, maintenance, and operational costs prevent its application for large-scale use.

27.2.5.7 Biological extraction

Biological methods of cell disruption involve the application of enzymes, antibiotics, and phage treatment. Among these, the enzyme-based method is well-known in the literature. Use of cellulolytic enzymes to break down the cell wall of microalgae is one strategy to enhance the cell disruption of microalgae cells that are resistant to other methods of cell disruption. The enzyme can be used alone or in combination with other methods like solvent extraction or bead milling. Due to the high cost of enzymes, a process depending solely on enzymatic hydrolysis is expensive, and hence this method is viable only when combined with other cell disruption methods.

27.2.5.8 Pulsed electric field (PEF)

This is a low temperature, nonchemical, low impact process that induces electroporation of cell membranes. The process is carried out over a very short duration, usually microseconds, by applying high voltage pulses into the system, which result in a high voltage field ranging from 1 to $50\,kV\,cm^{-1}$, leading to release of cell content within the media. PEF processing has the potential to provide higher productivity for the production of biofuels, human and animal feed and food supplements, and high-value specialty chemicals. However, it is expensive to scale-up the operation. Table 27.4 provides a comparison of different cell disruption methods along with their advantages and limitations.

27.3 Process intensification methods applied to different microalgae-based products

27.3.1 Biodiesel

The oil derived from microalgae is a promising precursor for biodiesel formation due to its high lipid content compared to other bio-based feedstocks. Biodiesel chemically known as fatty acid methyl ester (FAME) is produced by esterification of triglycerides in lipids using acidic catalyst followed by transesterification under alkaline conditions by using methanol. The high production cost of biodiesel formation from microalgae in comparison with petroleum-based fuels is the limiting factor for its commercialization. The detailed process flow scheme for biodiesel production is shown in Fig. 27.5. The major process steps include selection of suitable microalgae strain for cultivation, maintaining suitable growth conditions during cultivation, harvesting, lipid extraction, and conversion of lipid to biodiesel through transesterification. The different parameters affecting each stage need to be optimized to achieve economically viable production of biodiesel.

The selection of microalgae strain with high lipid content and providing suitable growth conditions during cultivation is the first stage in achieving high yield of biodiesel. The lipid content in most microalgae species is generally 20%–50%. Examples of a few strains

Table 27.4: Comparison of different cell disruption methods.

Method	Principle mechanism	Advantages	Limitations
Bead milling (Günerken et al., 2015)	Mechanical compaction, shear stress	High rate cell disruption, suitable for large scale	Efficiency depends upon characteristics of beads, need high energy
Ultrasonication (Gerde et al., 2012)	Cavitation and free radical formation	Short extraction time, less solvent consumption, high extraction efficiency, reproducible result	High power consumption, difficulties in scale-up
High-pressure homogenizer (Kumar and Pandit, 1999)	Cavitation and shear stress	Suitable for large-scale application, high extraction yield, can be combined with acid, alkali, or enzyme treatment method	High energy consumption, hence used only for extraction of high-value product
Chemical method (Günerken et al., 2015)	Use of organic solvents	Inexpensive solvent, reproducible results	Toxic and flammable solvents, requires large volume of solvent, solvent recovery is expensive, energy-intensive
Enzyme extraction (Demuez et al., 2015)	Enzyme substrate interaction	Use in combination with another disruption method, faster, higher yield, use for organisms with resistant to disruption	Costly process
Osmotic shock (Yoo et al., 2012)	Sudden reduction in the movement or concentration of water across the algal cell membrane	Simple, extraction from wet biomass possible	Expensive, large-scale application is costly
Freeze press (Show et al., 2015)	Passing frozen paste of cells through a narrow opening under high pressure at temperatures less than zero	Simple operation, toxic solvents are not involved	High biomass content is required, expensive to scale-up

used for commercial application due to their rapid growth rate and high lipid content are *Tetradesmus* (*Scenedesmus*) *obliquus, Chlorella sorokiniana, C. vulgaris*, and *Ankistrodesmus*. Of the above, *C. vulgaris* accumulates the highest amount of lipid (up to 58.5% dry weight of cell).

Another requirement is selection of microalgae strain suitable for the desired environmental condition. For example, microalgae strains that survive at high temperatures can be employed for cultivation in regions having hotter climates. The thermotolerant microalgae strain *Desmosdesmus* sp. F2 is suitable for growth at 45–50°C and shows high lipid content at 35°C.

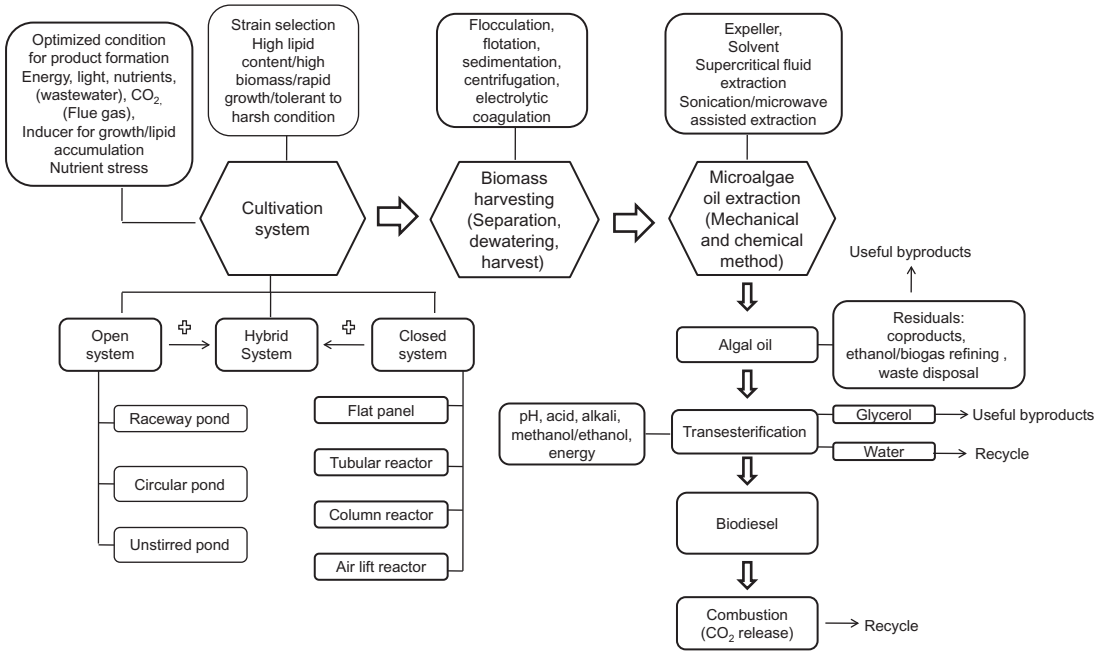

Fig. 27.5
Process flow of biodiesel production.

On the other hand, the microalgae *Tetraselmis* sp. can tolerate a wide range of salinity and hence is suitable for growth in seawater. The strain *Monoraphidium dybowskii* Y2, which is isolated from desert soil, is suitable for surviving under desert conditions with the capability to accumulate high lipid content (Chu, 2017).

Manipulation of environmental conditions such as light and temperature during cultivation also has a positive impact on increasing the yield of lipid content. The use of light-emitting diodes (LEDs), dyes, and paints are recent PI strategies to provide the desired wavelength of light for maximum growth. Light having wavelengths in the blue (450–475 nm) and red (630–675 nm) regions of the electromagnetic spectrum is useful to achieve maximum photosynthesis efficiency. A PBR with light emitting diodes (LEDs) as a light source has been reported to enhance microalgae cultivation. Modifying the light spectrum using organic dye such as rhodamine 101 and 9, 10-diphenylanthracene to obtain light of desirable wavelength are reported to yield a two to threefold increase in biomass (Seo et al., 2015). Addition of fluorescent paint in the media is shown to be helpful in absorbing excess solar radiation mainly in the nonuseful range, which ultimately leads to enhanced biomass production. The change in temperature of growth media is also found to affect the lipid production of cells. The study has shown that at low temperatures, unsaturated fatty acids dominate the cells, whereas lipid accumulation was found to increase with an increase in temperature.

After selecting a microalgae strain with high lipid content and suitable to the given environmental conditions, growth conditions during cultivation can be manipulated for enhanced lipid content. Nutrient deprivation is one such approach. For example, phosphorus limitation could result in the accumulation of lipid content up to 53% of cell biomass in *Scenedesmus* sp. LX1 (Xin et al., 2010) and sulfur starvation triggers triacylglycerols (TAG) accumulation in *Chlamydomonas reinhardtii* (Sato et al., 2014). The microalgae strain *Monoraphidium dybowskii* LB50 shows high lipid productivity under saline stress and thus is suitable for cultivation in water with high salt content (Chu, 2017). Addition of phytohormones such as melatonin, fluvic acid, and indol acetic acid in growth media is one recent approach to increase growth and lipid production. These phytohormones are usually inexpensive and are required in very small quantities, and hence they could indicate an attractive strategy to obtain high biomass with high lipid content (Lu and Xu, 2015).

Along with strategies for induction of lipids at a cellular level, a high amount of biomass could be achieved by employing a two-stage cultivation process. In the first phase, microalgae could be grown under optimal conditions to produce maximum biomass, and then subjected to nutrient starvation at the second stage, leading to an increase in lipid accumulation. For example, *Ankistrodesmus falcatus* shows an increase in lipid production up to 35%–45% of dry weight when grown under the two-stage cultivation process.

The selection of cultivation system is another important aspect in biodiesel production. Different PI approaches have been tried to increase the biomass production in both open and closed systems. These approaches are discussed in detail in Section 27.2.2. A few novel photobioreactor (PBR) design approaches which have been recently reported in the literature are floating PBR, pyramid type PBR, and submerged light PBR, which can be used as an economically feasible way to produce microalgae biomass for biodiesel production (Abu-Ghosh et al., 2016; Kim et al., 2016; Płaczek et al., 2017).

After the emergence of algae from the cultivation system, the most common methods of harvesting biomass from the cultivation media involve flocculation, which causes algae to aggregate and eventually increases efficiency of harvesting processes such as gravity sedimentation, filtration, and centrifugation.

The harvested biomass is then processed for lipid extraction. The extraction is one of the most costly processes of biodiesel production, accounting for 40%–60% of the production cost. Mechanical disruption techniques and solvent extraction have been used to extract algae oil after dewatering and drying biomass. Wet biomass harvesting is another approach where lipid can be extracted directly from wet biomass, thus reducing the processing steps of dewatering and cell drying. In this process, a nonpolar solvent such as n-hexane is used as an extraction solvent and a pH conditioner is used to adjust cell permeability. This results in separation of oil in the nonpolar solvent and residual biomass and other cellular impurities in an aqueous phase. Recently, supercritical fluids such as CO_2 have been used under mild

conditions to extract lipids from wet biomass. The optimized process has shown up to 92% recovery of lipids (Lorenzen et al., 2017). Another emerging PI approach is the application of pulsed electric field technology to enhance lipid extraction from wet biomass of microalgae using the green solvent ethyl acetate, resulting in 90% of cell disruption with significant enhancement in lipid extraction by avoiding the processes of dewatering and drying (Zbinden et al., 2013).

The lipids extracted from the microalgae are finally converted to biodiesel by the transesterification process. The most common problem in the transesterification reaction is the presence of free fatty acid in the microalgae oil as an impurity and the immiscibility of microalgae oil (triglycerides) in organic solvent such as methanol, which is required in the process. The use of a heterogeneous bifunctional solid catalyst such as Li/CaO is one of the PI approach to address this issue, as this catalyst helps to catalyze simultaneously the free fatty acid esterification and transesterification reactions. The transesterification reaction can also be intensified by modifying the reactor design to achieve higher mass transfer rates. Reactor designs based on different technologies such as microwave, ultrasonication, and hydrodynamic cavitation have been reported in the literature. Table 27.5 gives different PI measures reported in the literature for enhancing biodiesel production.

27.3.2 Bioethanol

Bioethanol is one of the commercially produced biofuel and is mainly obtained from corn and sugarcane, which are food crops. This has raised concerns regarding whether such crops should be used for food or to generate fuels. Currently, researchers are looking for possible alternatives to corn and sugarcane by employing agricultural waste, mainly crop residues. Microalgae is one of the promising potential alternatives. The process of hydrolysis plays a crucial role in ethanol production and development of a hydrolysis process which is cost-effective, easy to handle, and resulting in higher yields of reducing sugars (Shokrkar et al., 2017). The absence of lignin in microalgae makes it easily amenable to hydrolysis. Microalgae are capable of storing starch inside their cells and periodic collection of microalgal cells along with further disruption and starch extraction is required to produce reducing sugars. Extraction of starch is performed with water or a solvent after the cell disruption. Disruption of microalgae is generally performed with acid hydrolysis (dilute and concentrated). A study performed on the production of reducing sugars production from microalgal cells has reported a 83% yield of reducing sugars with 2% HCl along with 2.5% $MgCl_2$ (Zhou et al., 2011). Microalgae such as *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Scenedesmus* and *Spirulina* are known for their higher content of starch and glycogen, and can be utilized in bioethanol production.

A study performed by Onay (2019) reported a 11.2 $g\,L^{-1}$ yield of bioethanol from *Hindakia H. tetrachotoma* ME03 when pretreated with acid and by employing enzymatic hydrolysis. The

Table 27.5: Different PI measures used for enhanced biodiesel formation from microalgae.

PI strategy	Microalgae species	Performance improvement
Selection of strain Selection of indigenous strain from their natural habitat so that the culture is already well adapted to the local environment (Mutanda et al., 2011)	*Botryococcus braunii*	Accumulation of 25%–75% oil content
Nutrient stress Nitrogen stress (Chu, 2017)	*Tetradesmus (Scenedesmus) obliquus*	Lipid content increase from 26 to 43%
Nutrient stress Phosphorous stress (Chu, 2017)	*Tetradesmus obliquus*	Lipid content in cell increased up to 30%
Nutrient stress All element stress (Chu, 2017)	*Parachlorella kessleri*	Lipid content in cell increased from 0% to 29%
Growth stimulator To stimulate rapid growth and increased lipid production Addition of phytohormones (indole acetic acid (IAA)) (Singh et al., 2016)	*Scenedesmus obliquus*	2.5-fold increase in growth and 59% increase in fatty acid content
Cultivation condition Growth temperature at 20–25°C to promote rapid growth (Singh et al., 2016)	*Nannochloropsis oculata*	7.42%–14.9% increase in lipid content
Cultivation condition CO_2 level (12%) (El Baky et al., 2012)	*Scenedesmus obliquus*	33.14% increase in lipid content
Cultivation condition Salinity (1 M) (Takagi and Karseno, 2006)	*Dunaliella salina*	67% increase in lipid content
Genetic engineering Alteration in lipid metabolism, alteration in genes functioning for photosynthetic apparatus (Wan et al., 2014)	*Chlorella sorokiniana*	51% increase in lipid content
Cultivation system High rate algal ponds integrated with wastewater treatment (wastewater from municipal waste, piggery waste, tapioca wastewater) (Chu, 2017)	*Chlorella vulgaris*	Biomass productivity 2.3–12 $g\,m^{-2}\,day^{-1}$, with removal of 99% COD and 93% PO_4
Cultivation system CO_2 sequestering (Francisco et al., 2010)	*Chlorella vulgaris*	Rate of CO_2 sequestration 17.8 $mg\,L^{-1}\,min^{-1}$, biomass productivity 20.1 $mg\,L^{-1}\,h^{-1}$, lipid content of 27%, lipid productivity of 5.3 $mg\,L^{-1}\,h^{-1}$
Cultivation system Hybrid system (combination of open pond and PBR) (Chu, 2017)	*Arthrospira platensis*	Increase in lipid content from 19% to 36.6% dry weight of cell

Continued

Table 27.5: Different PI measures used for enhanced biodiesel formation from microalgae—cont'd

PI strategy	Microalgae species	Performance improvement
Harvesting Flocculation assisted by magnetic particles (Wang et al., 2013)	*Chlorella ellipsoidea*	>95% cell recovery within 10 min at a dosage of 125 mg L^{-1}
Lipid extraction and transesterification Use of hexane and ethanol at 200°C, for 2 h (Zhu et al., 2017)	*Nannochloropsis oculata*	Lipid yield 52%
Lipid extraction and transesterification Supercritical fluid extraction with CO_2 and ethanol at 40°C, 35 MPa, 30 min (Zhu et al., 2017)	*Schizochytrium limacinum*	Lipid yield 33.9%
Lipid extraction and transesterification Microwave-assisted (Patil et al., 2011)	*Nannochloropsis* sp.	80.1% (lipid conversion)
Lipid extraction and transesterification Sonication-assisted (Martinez-Guerra et al., 2014)	*Chlorella* sp.	Yield 95% (lipid conversion)
Lipid extraction and transesterification Hydrodynamic cavitation-assisted cell disruption and lipid extraction (Lee and Han, 2015)	*Nannochloropsis salina*	Lipid yield (25.9–99%)
Lipid extraction and transesterification Solid base heterogeneous catalyst (Umdu et al., 2009)	*Nannochloropsis*	Increase of yield up to 97.5%
Lipid extraction and transesterification Enzyme-assisted extraction (EAE) using high-pressure homogenization (Kumar et al., 2017)	*Neochloris oleoabundans*	92.6% lipid recovery
Lipid extraction and transesterification Surfactant-assisted extraction (SAE) (Salam et al., 2016)	*Nannochloropsis* *Nannochloropsis oculata*	98.3% FAME yield at 20% moisture in the algae

enzymatic hydrolysis with β-glucosidase/cellulose +α-amylase resulted in 92% saccharification. Another process with dilute acid hydrolysis was reported to produce 52% higher ethanol by employing 3% *v*/*v* sulfuric acid along with 10 mg mL^{-1} microalgae (*S. abundans*) at 160°C for 15 min (Guo et al., 2013). In one study, a comparison of pretreatment technologies was performed by using acidic, enzymatic, and alkaline hydrolysis, and the production of reducing sugars from mixed microalgal culture was studied. It was observed that $MgSO_4$ in combination with dilute sulfuric acid results in enhanced production compared to sulfuric acid and enzymatic hydrolysis (Shokrkar et al., 2017). A study performed by Choi et al. (2010) produced ethanol using *C. reinhardtii* biomass by employing α-amylase and amyloglucosidase in liquefaction and saccharification, respectively. The bioethanol yield obtained was 0.235 g g^{-1} of biomass, which was comparable to that obtained from cellulosic feedstocks. The lipid extracted from microalgae biomass can be further used in bioethanol production. A study performed by Harun et al. (2010) reported the usage of lipid extracted from microalgae *Chlorococum* sp. for bioethanol production and obtained 3.83 mg L^{-1} of ethanol yield from 10 mg L^{-1} of microalgae. Genetic engineering can also play a significant role in bioethanol production from microalgae and can be achieved with genetic manipulations in the cellular structure of microalgae. Increased activity of enzymes like alcohol dehydrogenase and pyruvate dehydrogenase can convert the stored carbon into ethanol. Currently, increased accumulation of carbohydrates in microalgae has been achieved, and further focused research is required to convert it directly to ethanol (De Farias Silva and Bertucco, 2016).

27.3.3 Biohydrogen/biogas

Microalgae are composed of lipids, carbohydrates, and proteins along with nitrogen, phosphorous, and micronutrients like iron, cobalt, and zinc. The composition of microalgae is expressed as $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Grobbelaar, 2004). The carbohydrate, protein, and lipid contents vary in the ranges of 5%–23%, 6%–52%, and 7%–23%, respectively, depending upon the species of microalgae. These components in microalgae can be converted in to biohydrogen or biogas during the anaerobic digestion process. Since microalgae cells are resistant to anaerobic environments, they need pretreatment before biogas production (Passos et al., 2018). Different mechanical, chemical, thermal, and biological methods are employed for pretreatment of microalgae. The thermal treatment is reported to be the most effective treatment in the continuous mode of operation. In one study, thermal treatment of microalgae in the range of 75–95°C for 10 h resulted in a 70% increase in biogas production while treatment at 120°C for 2 h resulted in a 108% increase (Passos and Ferrer, 2015; Schwede et al., 2013). In another study, enzymatic treatment of microalgae (*Scenedesmus* sp.) in the first step of an anaerobic membrane bioreactor (AnMBR) resulted in 0.203 L CH_4/g COD with 70% COD removal (Gómez-Pérez et al., 2017).

Use of low-cost chemicals like calcium oxide (CaO) is reported to be beneficial in the pretreatment of microalgae, with treatment at 72°C resulting in a 25% increase in biogas production (Solé-Bundó et al., 2017). There are various other upcoming technologies like ultrasound- and microwave-assisted irradiation processes, which give the desired effects with a lesser requirement of chemicals and energy, and in a shorter time. A study performed by Park et al. (2013) on replacing the pretreatment step by ultrasound-assisted biogas production reported an increase in yield from 0.230 to 0.440 L CH_4/g (Park et al., 2013). Another study employing ultrasound-assisted pretreatment on *Scenedesmus* sp. resulted in an increase in biogas yield from 0.082 to 0.154 L CH_4/g COD (González-Fernández et al., 2012). Commercialization of ultrasound- and microwave-assisted processes requires more detailed studies, and the focus of the study should be on the efficient breakage of the microalgal cell wall and solubilizing the organic matter for enhanced biogas production.

27.3.4 Process intensification in wastewater treatment using microalgae

Wastewater treatment is currently one of the major issues faced in the world. There is a need to develop cost-effective technologies to make the treatment process economically viable. Microalgae production can be performed utilizing the nutrients present in wastewater from different sources such as municipal, agricultural, and industrial. The combined approach of microalgae production along with wastewater treatment can result in a more efficient process compared to other available treatment processes. The nitrogen and phosphates present in the residual water after secondary treatment provide essential nutrients for the growth of algae (Olguín, 2012). A study performed by Lu et al. (2015), reported a reduction in nitrate, ammonia, phosphorus, and COD by 90%, 90%, 70%, and 60%, respectively, using *Chlamydomonas polypyrenoideum* during the treatment of dairy wastewater (Lu et al., 2015). Another study performed by Solovchenko et al. (2014) reported the usage of a 50 L photobioreactor consisting of *C. sorokiniana* to treat wastewater. A study performed by Solovchenko et al. (2014) showed that the microalgae were able to significantly reduce nitrates by 95%, phosphates by 77% and sulfate by 35% within 3 days of treatment time (Solovchenko et al., 2014). A study performed by Feng et al. (2011) on a column aeration photobioreactor reported the removal of COD and total phosphorous by 86% and 96%, respectively, using *C. vulgaris* with lipid productivity of 147 $mg\,L^{-1}d^{-1}$ (Feng et al., 2011).

Treatment of wastewater with microalgae can be employed as a tertiary process as they can also remove heavy metals along with nutrients from wastewater (Rai et al., 1981). *Chlorella*, *Botryococcus*, *Chlamydomonas*, *Scenedesmus*, *Phormidium*, and *Spirulina* are the most studied species in wastewater treatment using microalgae (Chinnasamy et al., 2010; Kong et al., 2010; Olguín, 2003; Wang et al., 2010). Microalgae can also treat emerging pollutants like cosmetics and pharmaceuticals to some extent, compared to other treatment processes. Microalgae have been reported to treat residual pharmaceutical chemicals, personal care products, endocrine-disrupting chemicals, and pesticides (Ahmed et al., 2017). The

efficiency of wastewater treatment by microalgae depends upon conditions like wastewater composition, light intensity, the available ratio of nitrogen:phosphorus, light/dark cycle, and the species of microalgae used for treatment.

27.3.5 Process intensification in carbon dioxide capture using microalgae

Increasing levels of carbon dioxide (CO_2) emissions from different sources such as power plants, steel, cement, oil, and automobiles are causing a dramatic change in climate, leading to global warming. CO_2, once released into the atmosphere, is very difficult to trap. Technologies that remove or capture carbon at its source are called carbon capture systems (CCSs), and prevent the release of CO_2 into the air. Microalgae are among the most efficient biological systems for capturing carbon in the form of biomass through photosynthesis. Microalgal cells can assimilate CO_2 from industrial flue gas within various ranges of concentrations from ambient (0.036%, *v/v*) to extremely high (100%, *v/v*). CO_2 fixation from industrial flue gas coupled with nutrient recycling from wastewater makes algae ideal organisms for reducing the problem of environmental pollution due to flue gas emissions as well as wastewater while producing useful by-products (Packer, 2009).

The first step toward developing an efficient CO_2 sequestering process is selecting strains suitable for surviving in a very harsh environment, specifically hot flue gases containing high levels of CO_2, NO_2, and SO_2. As reported by Yoo et al. (2010), different microalgae species, mainly strains of *C. vulgaris and Scenedesmus* sp., can assimilate very high concentrations of CO_2 (up to 10%) from flue gas. With this concentration of CO_2, *Scenedesmus* sp. can yield biomass up to $217.50\,mg\,L^{-1}\,d^{-1}$ and lipid production in the range of $20.65\,mg\,L^{-1}\,d^{-1}$ (9% of biomass) (Yoo et al., 2010).

However, for effective CO_2 capturing, it is essential to utilize a consortium of hyper-CO_2-tolerant microalgae strains instead of a single strain. Strains often favored are those that can directly utilize CO_2 from industrial flue gas, those that grow well under natural day-night cycles, strains with high productivities that are easy to harvest, and most importantly those that produce biomass that can be used in the production of desirable co-products. Using genetically modified species with the ability to tolerate high concentrations of CO_2 is also one of the PI approaches to enhance the rate of carbon capture. For example, *Chlorella* sp. AT1, a mutant strain developed with NTG (*N*-methyl-N′-nitro-N-nitrosoguanidine), survives well at 10% CO_2 with an accumulation of high concentrations of lipids and carbohydrates (Kuo et al., 2017; Vazquez-Villegas et al., 2018). However, the technology is limited to a few such species. Coupling wastewater treatment with CO_2 biofixation and conversion into biomass for utilization as a feedstock for biofuel extraction is the most economically viable model, which also helps to reduce the ecological footprint.

Raceway ponds integrated with CO_2 capturing systems is the most cost-effective strategy for obtaining biomass through CO_2 sequestering. The addition of CO_2 is done by

injecting it in the form of a stream, followed by mixing with a paddle wheel. One of the challenges in CO_2 sequestering is loss of gas to the atmosphere during mixing at ambient temperature.

27.3.6 Process intensification in microalgal pigments extraction

Microalgae are a potential source of different pigments that find application in the food, pharmaceutical, and cosmetic industries. The three main types of pigments obtained from microalgae are carotenoids, chlorophylls, and phycobiliproteins. Extraction of pigments from microalgae consists of disruption of cells followed by extraction of pigments. The extraction of pigments from microalgae is performed using processes like supercritical or subcritical solvent extraction, ultrasound, microwave, pulsed electric field, and high speed or pressure homogenization (Joshi and Gogate, 2018). Solvent extraction is the most common conventional process employed in pigment extraction. Supercritical fluid extraction (SFE) is one of the potential alternatives (Guedes et al., 2013) to the conventional solvent extraction process, as it is more environmentally friendly and does not involve any toxic solvents. Supercritical fluids can easily diffuse into cells under high pressure and temperature, resulting in increased mass transfer along with selective extraction of pigments. Carbon dioxide is the most common supercritical fluid used in these processes. Polarity modifiers such as ethanol, acetone, and vegetable oil are used along with supercritical fluids to enhance the pigment recovery (Nobre et al., 2006). Pigment extraction from *Synechococcus* sp. performed with supercritical fluid at 50°C along with 200 bar pressure was reported to be the optimum, with 1.51 $\mu g\,mg^{-1}$ of biomass as the pigment yield (Macías-Sánchez et al., 2007). In another study, using SFE along with olive oil (10%) at 70°C and 400 bar pressure increased the pigment yield by 51% (Krichnavaruk et al., 2008).

Ultrasound- and microwave-assisted processes are upcoming technologies and their utilization in microalgal pigment extraction has been studied by many researchers. These processes are also reported to require fewer chemicals and less processing time. A microwave-assisted process was reported to produce 629 $\mu g\,mg^{-1}$ yield of carotenoids from *Arthrospira platensis* with 1 bar pressure and 400 W power at 50°C for 15 min as operating conditions (Esquivel-Hernández et al., 2016). Similarly, in the case of an ultrasound-assisted process, using ethanol as the solvent resulted in 1.31 $mg\,g^{-1}$ of pigment yield from *Heterochlorella luteoviridis* (Jaeschke et al., 2017). There are many other studies reporting efficient performance of these processes, but the difficulty in scale-up and higher operating costs have resulted in their limited use. High-pressure homogenization (HPH) reported a yield of 4.21 $\mu g\,mg^{-1}$ from *H. pluvialis* at 1000 bar with three passes (Taucher et al., 2016) and pressurized liquid extraction (PLE) resulted in 4.28 $mg\,g^{-1}$ zeaxanthin extraction from *C. ellipsoidea* at 115.4°C for 23.3 min (Koo et al., 2012). All the above processes act as PI approaches to the conventional solvent extraction process, with a requirement of fewer chemicals and less time.

27.3.7 Single-cell protein for food application

Application of microalgae for the production of single-cell protein (SCP) has gained considerable attention because of its high protein content (60%–70%) and the presence of other essential nutritional supplements such as omega-3-fatty acid, vitamins, minerals, and chlorophyll. The low nucleic acid content of microalgae (3%–8%) gives it an added advantage as an SCP compared to other groups of microorganisms such as bacteria and fungi, which usually have a high content of nucleic acid (Putri et al., 2018). Additionally, microalgae have simple and inexpensive growth requirements along with a rapid growth rate. Spirulina is the most widely used algae as a source of SCP, with 57 g of protein content in 100 g of biomass. Similarly, biomass obtained from *Euglena gracilis*, *Scenedesmus*, and *Chlorella* have been harvested and used as a source of food in many parts of the world. Apart from freshwater microalgae, marine microalgae *Tetraselmis suecica*, *Isochrysis galbana*, *Dunaliella tertiolecta*, and *Chlorella stigmatophora* are also good sources of single-cell protein (SCP), with protein content in the range of 39.12%–54.20% of the dry matter. Among these, *D. tertiolecta* has the highest protein content, having lysine value in the range of 3.67 and 4.94 g/100 g of protein (Fábregas et al., 1989).

The most economical way to cultivate microalgae for SCP production is using wastewater preferably from food industries such as tofu, tempeh, and cheese, as the waste from these industries is rich in complex organic materials like carbohydrates, proteins, lipids, and several microelements. The protein content of the waste is around 60%, and thus after degradation provides a good source of macronutrients or micronutrients for the cultivation of microalgae. Therefore, the common trend is to combine algal feed protein production with waste treatment where a high-quality source of protein is required, especially for making animal feed. This waste is usually recovered in the process of upgrading waste oxidation pond effluents (Lu et al., 2015).

Drying of harvested biomass is the most crucial step during SCP formation. The drying process, in addition to increasing product shelf life by decreasing residual moisture below 10%, helps to pasteurize or sterilize the product. Drying also improves protein digestibility due to rupturing of the algal cell wall. Use of liquid concentrate directly as a feed can save on the drying cost. However, the liquid concentrate needs to be cooked to reduce contamination and make it palatable (Moraine et al., 1979).

27.4 Conclusions

Microalgae are a promising source of renewable feedstock for value-added products in different industries such as food, energy, cosmetics, and fertilizers. The simple and inexpensive nutritional requirements and their rapid growth rate have attracted the attention of researchers for establishing their use as an alternate to crop-based feedstock. However, products derived using microalgae are relatively more expensive than those made using alternate

chemical-based routes. There is an immense need to apply different PI measures throughout the process of microalgae-based product development, that is, from strain selection, cultivation, harvesting, and production to the extraction of desired products.

Generation of sufficient biomass of microalgae using specific microalgae species during cultivation is key in developing an economically feasible desired application. In this regard, selection of a highly efficient strain with a rapid growth rate can play a crucial role. Application of genetic engineering, manipulation of nutritional supply, environmental conditions, addition of growth stimulators, and co-cultivation with other microbial species are among the various strategies that can be applied to enhance cultivation of algal biomass.

The design of the cultivation system is another important factor that needs to be considered to achieve a high production rate. The different ways of cultivation of microalgae, that is, open and closed systems, are mainly optimized with respect to mixing, circulation of air, optimal light, harvesting efficiency, lowering risk of contamination, removal of unwanted gas, and possibility of integrating different industrial processes (wastewater treatment and CO_2 sequestering). Open systems like raceway ponds integrated with wastewater and flue gas utilization are the most economical ways of obtaining biomass of high value. Use of hybrid cultivation (i.e., combination of open ponds and photobioreactors) is another strategy to optimize algal biomass yield at the cultivation stage.

There are different ways to harvest microalgae biomass from cultivation media such as flocculation, sedimentation, and centrifugation. However, there is no universal method that can be efficiently applied in all processes. Designing a harvesting method depends on cell size, density, cell surface properties, and the end-use application. Using a combination of processes could be one approach to improve the harvesting efficiency and reduce the cost of the process. A two-stage harvesting process comprising thickening and dewatering is also an effective way to improve harvesting efficiencies. Bioflocculation or chemical flocculation followed by gravity sedimentation or centrifugation is reported to be the most cost-effective harvesting technique specifically for biofuels applications. For producing high-value products where the addition of flocculant can result in contamination, membrane separation or electric-based separation could be better alternatives.

Extraction is an important step in deciding the product yield and quality. Process intensification technologies such as ultrasound and hydrodynamic cavitation have been found to assist the extraction of desired products from the biomass. Use of supercritical fluids at mild extraction conditions and use of pulsed electric field technology using green solvents have been found to be effective compared to conventional solvent extraction. Ultrasound and hydrodynamic cavitation have also been found to assist extraction in an economical way.

In addition to the PI approach for general microalgae processes, there is a need to develop product-specific PI approaches. A PI approach that uses a strong heterogeneous catalyst and ultrasonic irradiation is found to enhance the transesterification process in biodiesel production.

Recently, more sustainable options have been attempted for microalgae production. These include the use of wastewater (specifically after secondary treatment) as a source of nutrients and flue gas emissions from the industry as a source of CO_2. This has the potential to solve environmental problems of wastewater pollution and flue gas emission. This is gaining more interest as the concept of a "microalgal bio-refinery" wherein the facility is expected to integrate production of biomass and its conversion to fuels, power, and chemicals so that each component of the microalgae is extracted, processed, and valorized.

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CHAPTER 28

Microalgal biorefineries

Alice Ferreira, Luisa Gouveia

LNEG, National Laboratory of Energy and Geology I.P., Bioenergy and Biorefinery Unit, Lisbon, Portugal

Chapter outline

28.1 Introduction 771
28.2 Biorefinery concept 772
28.3 Microalgae-based biorefinery 773
28.3.1 Wastewater treatment 774
28.3.2 Carbon dioxide mitigation 774
28.3.3 Biofuel generation 775
28.3.4 Valuable products obtained from microalgae 777
28.3.5 Microalgae-based bioplastics 781
28.3.6 Microalgae-based biofertilizers 781
28.4 Examples of microalgae-based biorefinery 782
28.4.1 *Nannochloropsis* sp. biorefinery 782
28.4.2 *Anabaena* sp. biorefinery 783
28.4.3 *Chlorella vulgaris* biorefineries 784
28.4.4 *Chlorella protothecoides* biorefinery 786
28.4.5 *Chlamydomonas reinhardtii* biorefinery 786
28.4.6 *Dunaliella salina* biorefinery 787
28.4.7 *Dunaliella tertiolecta* biorefinery 787
28.4.8 *Arthrospira* (*Spirulina*) biorefinery 788
28.4.9 *Spirogyra* sp. biorefinery 788
28.4.10 *Scenedesmus obliquus* biorefinery 789
28.4.11 *Tetraselmis* sp. biorefinery 790
28.5 Conclusions 791
Acknowledgments 791
References 791

28.1 Introduction

The search for renewable fuels has gained attention due to the higher energy demand imposed by the ever-increasing global population. In this context, microalgae are now accepted as a significant alternative source for renewable fuels. In addition to the microalgal lipids that could

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potentially be converted into biodiesel, the microalgal carbohydrates could be used in fermentation processes (to generate bioethanol and/or biohydrogen). Microalgae contain, in addition to lipids and carbohydrates, many other valuable components, including polyunsaturated fatty acids, antioxidants, and pigments. These compounds could widen the market opportunities of microalgae products and open up further possibilities of coupling production of microalgae for biofuels and high-value compounds (Chew et al., 2017).

Microalgae are grown in open ponds or closed systems that involve mixing and concentrating processes. Moreover, the downstream processing, which includes harvesting, drying, cell rupture, compounds extraction, and conversion technologies, can be challenging. Altogether, the microalgae production for biofuels is economically unfeasible. One path to drive down the cost of biofuels is to reduce the cost of biomass production (i.e., cultivation/harvesting). However, recent techno-economic analysis work has demonstrated that reducing the costs to a level that would enable biofuel economic viability is extremely challenging (Laurens et al., 2017). Thus, another way for turning economic and energy balances more favorable is to derive multiple products in a single cycle (biorefinery concept) (Bhalamurugan et al., 2018), and to use effluents as a nutrient source for cultivation.

As more promising bioproducts are developed and evaluated, a higher value can be added to the microalgal biomass, thereby lowering the pressure on increasing the productivity to achieve rigorous cost targets. This versatility and huge potential of tiny microalgae could support a microalgae-based biorefinery and bioeconomy, opening up vast opportunities in the global algae business (Laurens et al., 2017; Gouveia, 2014). The microalgae could play an important role in the response to the worldwide biofuel demand, together with the production of high-value-added products and assisting in some other environmental issues such as water stream bioremediation, and carbon dioxide mitigation (Gouveia, 2014).

28.2 Biorefinery concept

A biorefinery is a facility (or network of facilities) that integrates biomass conversion processes and equipment to produce transportation biofuels, energy, and high-value products from biomass. The concept of a biorefinery is similar to that of a traditional petroleum refinery, in which biomass is converted into multiple marketable chemicals, fuels, and products (Chew et al., 2017).

A biorefinery chain includes the pretreatment and separation of biomass components and the subsequent conversion to generate a spectrum of different intermediates and products. By producing multiple products, a biorefinery can take advantage of the differences in biomass components and intermediates, maximizing the value of the biomass feedstock and preventing resource loss and environmental impacts (Singh and Gu, 2010; Zhu, 2015).

Biorefineries are found in multiple sectors at an industrial scale, which allows the concentration of various products processing (Chew et al., 2017).

28.3 Microalgae-based biorefinery

Microalgae play a major role in the production of biofuels and bio-based chemicals, making them a promising alternative to many natural components and sources. Microalgae high-value products can be enhanced under stressed environmental conditions and be used as feedstock for different products. Extracted microalgal lipids can be employed as potential feedstock for biodiesel production, while carbohydrates can be used as a carbon source in fermentation industries to replace conventional carbohydrate sources such as simple sugars or lignocellulosic biomass. Moreover, some microalgae contain long-chain polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA), that can act as health food supplements, as well as proteins and pigments that exhibit properties desired by the food, feed, and pharmaceutical industries to treat certain diseases (Chew et al., 2017).

The advantage of using microalgae is the rapid growth rate and high photosynthetic efficiencies with small amounts of water, nutrients, and atmospheric CO_2 in comparison to terrestrial plants. It does not create the competition for land and food crops, since they can grow on degraded land and marginal areas (Khoo et al., 2011). Another virtue of microalgae is their ability to grow on industrial wastewaters by using their excess nutrients, while simultaneously promoting a more sustainable wastewater treatment. Furthermore, they can sequester the excess CO_2, not only from the atmosphere, but also from anthropogenic flue gases from pollutant industries, such as cement plants and thermoelectric stations, contributing immensely for the reduction of greenhouse gases (GHG) emissions (Cheah et al., 2015). Hence, microalgae have been considered as a sustainable feedstock for the biorefinery industries of the future, and some microalgae-based biorefineries have already been developed, as it will be shown in Section 28.4.

Nevertheless, several challenges still need to be tackled during the development of microalgae-based biorefinery technologies. The most challenging problems include high investment and operation costs, difficulty in controlling the culture conditions, contamination of bacteria or undesired algae, and unstable light supply and temperature, among others. Several strategies have been proposed to solve these issues. The selection of the most adequate microalgal strains in terms of target product, tolerance, and adaptation capacity to environmental conditions is very important for stable and sustainable microalgae cultivation. In addition, identifying the most advantageous culture conditions and operation design is critical for improving the productivity of microalgae and derived products. Finally, a high-efficiency and low-cost downstream processing (harvesting, drying, cell rupture, extraction, conversion) should be developed. Appropriate treatment of the wastes produced from microalgae systems as well as recycling of water used during microalgae cultivation processes are also critical issues. Finally,

life-cycle analysis, energy balance, and cost assessment should also be performed to justify the economic feasibility and environmental impacts (Yen et al., 2013).

28.3.1 Wastewater treatment

The combination of wastewater treatment with microalgal cultivation was first highlighted in the 1950s by Oswald and Gotaas (1957). Since then, algal-bacterial systems have arisen as a promising platform to support a sustainable and low-cost wastewater treatment due to the ability of microalgae to grow in nutrient-rich environments together with the accumulation of nutrients from wastewaters, and the need of reducing microalgae production costs (Ferreira et al., 2018; Posadas et al., 2018).

Microalgal-bacterial processes provide an effective treatment for replacing conventional secondary and/or tertiary treatment, with lower associated costs and environmental impacts. They can play a dual role of bioremediation of wastewaters due to their potential for cost-free oxygenation and simultaneous nutrient removal, while producing valuable biomass with concomitant CO_2 sequestration. Furthermore, this microalgae-based remediation allows nutrient recycling into a valuable biomass that can be further processed for different applications, without secondary pollution (Ferreira et al., 2018; Rawat et al., 2011). This strategy represents a double benefit for both parts, since microalgae provides the cleaning of wastewaters, while offering a source of water and nutrients that is readily available and at a lower cost (Cuellar-Bermudez et al., 2017).

Microalgal-based wastewater treatment is achieved through photosynthesis, by which microalgae supply O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO_2 released from bacterial respiration. Therefore, this avoids the use of intensive mechanical aeration, reducing operation costs and minimizing pollutant volatilization (Muñoz and Guieysse, 2006).

A wide range of microalgae such as *Chlorella*, *Scenedesmus*, *Phormidium*, *Botryococcus*, *Chlamydomonas*, and *Spirulina* have already been used for treating different wastewaters with promising results (Ferreira et al., 2018; Gao et al., 2018; Kong et al., 2010; Martínez et al., 2000; Mata et al., 2013; Posadas et al., 2015, 2014; Wang et al., 2010). These studies have shown that microalgae provide an effective wastewater treatment, while avoiding the use of fresh water and nutrients.

28.3.2 Carbon dioxide mitigation

The fixation of CO_2 performed by photosynthetic organisms on Earth has contributed significantly to the global carbon cycle. The CO_2 produced from natural or human activities can be consumed by plants and algae, converting it into biomass and other metabolic products

through photosynthesis and the Calvin cycle. Moreover, the CO_2 fixation is accompanied by production of microalgae biomass, which could be converted to a variety of biofuels, pigments, cosmetics, nutritious food, and animal feed, representing additional benefits from the microalgae CO_2 fixation process. Since microalgae-based CO_2 fixation is much faster and more efficient (around 10–50 times higher) than that of terrestrial plants, it has thus been considered to have the potential to serve as a commercially feasible process for mitigation of CO_2 emissions (Ho et al., 2011). Most microalgae can fix the dissolved inorganic carbon and CO_2 in the gaseous effluents to form chemical energy through photosynthesis. Their ability to tolerate high CO_2 contents allows them to capture CO_2 efficiently from streams such as flue gases and flaring gases (CO_2 content of 5%–15%) (Hsueh et al., 2007) and are dependent of the microalga species.

28.3.3 Biofuel generation

Microalgae represent a promising alternative based on inherent advantages such as rapid growth rate, high lipid yields, high CO_2 uptake rate, lower land use, lower water consumption, daily harvesting, etc. However, microalgae production remains economically unsustainable. The possibility of coupling wastewater treatment, using nutrients from waste streams (e.g., WWs and/or CO_2 flue gas emissions), with microalgae cultivation is crucial to provide a positive energy return (Lundquist et al., 2010; Pittman et al., 2011). Furthermore, it can bring additional benefits to the reduction of environmental impact and disposal problems (Mata et al., 2013). Efforts have been made in order to advance the commercial feasibility of microalgae-derived biofuels, focusing on the improvement of processing steps, from the production of feedstock to fuel conversion processes (Quinn and Davis, 2015).

The conversion technologies for microalgal biomass can be divided into four categories: thermochemical conversion, biochemical conversion, transesterification, and photosynthetic microbial fuel cell. The main factors affecting the choice of conversion process are the quantity and type of biomass feedstock, economic considerations, specification of projects, and the end form of the desired product (Brennan and Owende, 2010).

28.3.3.1 Thermochemical conversion

Thermochemical conversion consists of the thermal decomposition of organic materials in biomass to extract fuel products (Brennan and Owende, 2010). This includes the processes of gasification, thermal liquefaction, pyrolysis, and direct combustion. These conversion techniques are a promising pathway to separate the different microalgal compounds due to their small footprint, shorter processing times, feedstock flexibility, efficient nutrient recovery, and no fugitive gas emissions (Ferreira et al., 2015). Furthermore, the high temperatures eliminate possible pathogens and bioactive compounds, leaving only minor residues (Razzak et al.,

2013). Gasification is the chemical process where carbonaceous materials are converted to synthesis gas (syngas) at high temperatures (800–1000°C). Syngas is a mixture of CO, H_2, CO_2, N, and traces of CH_4. It can be used to make a wide range of fuels and chemical intermediates or it can be directly burnt to be used as a fuel for gas engines. For thermal liquefaction, the algal biomass will undergo liquefaction, at a low temperature (300–500°C) and high pressure (5–20 MPa), to decompose the biomass into smaller molecules with higher energy density. On the other hand, pyrolysis depicts the thermal degradation of biomass in an oxygen-free atmosphere under 350–700°C. This process has potential for large-scale production and can generate biofuels with medium-low calorific power (bio-oil, biochar, biogas) (Brennan and Owende, 2010). The pyrolysis gases usually contain CO, CO_2, light hydrocarbons (C1–C4), and H_2. Regarding the biochar, this presents a high content of C, some H, and a minimum of O. Biochar can be used in various ways such as a soil amendment, energy carrier, adsorbents, and catalyst support. Finally, bio-oil is a complex mixture of oxygenated compounds, water (15–40 wt%) and some fine char particles (Fermoso et al., 2018). In a direct combustion, biomass is burnt in the presence of air, producing carbon dioxide, water, and heat. Energy is generated through the combustion of biomass and higher efficiencies can be achieved with the co-combustion techniques in coal-fired power plants (Brennan and Owende, 2010).

28.3.3.2 Biochemical conversion

The biochemical conversion illustrates the biological processing of biomass into biofuels for energy production. Examples of biochemical conversion processes include anaerobic digestion, alcoholic fermentation, and photobiological hydrogen production. Anaerobic digestion involves the conversion of organic wastes into biogas, which is composed mainly of CH_4 (55%–75%) and CO_2 (25%–45%). The biogas produced from algal biomass was found to contain high energy value and the energy recovery is comparable to that of the extraction from cell lipids. Due to the rising cost of energy, the anaerobic digestion of biomass is becoming attractive as an alternative for fuel production (Brennan and Owende, 2010; Suganya et al., 2016). As for alcoholic fermentation, biomass materials that contain sugars, starch, or cellulose are converted into ethanol through the action of yeasts (Brennan and Owende, 2010). Biological hydrogen (bioH_2) can be produced mainly by two routes: photobiologically—biophotolysis of water using green algae and cyanobacteria and photo-decomposition of organic compounds by photosynthetic bacteria (Das and Veziroglu, 2008)—and by bacterial fermentative processes such as dark fermentation. The photobiological hydrogen production occurs due to the split of the water into hydrogen ions and oxygen, through the algae. Firstly, the algae are grown photosynthetically in normal conditions, and then cultured by inducing anaerobic conditions to stimulate hydrogen production. Secondly, the simultaneous production of photosynthetic hydrogen and oxygen gas will take place and these gases will be spatially separated (Chew et al., 2017). Dark fermentation is an indirect technology in which several genera of bacteria (namely *Clostridium* and *Enterobacter*) can use the carbohydrates, proteins, and lipids as substrates to produce H_2, CO_2, and organic acids, through the acidogenic pathway.

28.3.3.3 Transesterification

Transesterification is the reaction of triglycerides with alcohol (usually methanol) in the presence of a catalyst to produce fatty acid chains (biodiesel) and glycerol. Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) derived from a renewable lipid feedstock such as algal oil. Microalgal biodiesel is renewable, biodegradable, nontoxic, and produces fewer emissions when compared to petroleum diesel (Brennan and Owende, 2010).

28.3.3.4 Photosynthetic microbial fuel cell

Microbial fuel cells are bio-electrochemical devices that have the capacity to generate electricity from the biodegradation of organic matter under anaerobic conditions. The integration of microalgal photosynthesis with microbial fuel cells has shown potential in the production of an oxygen-rich environment and the removal of CO_2 (Uggetti and Puigagut, 2016). The photosynthetic microbial fuel cell consists of an anode and a cathode separated by a proton exchange membrane. The bacteria in the anode oxidize the organic compounds, producing electrons, which are transferred to the cathode electrode through an external circuit, producing electricity. The benefit of this system is that bacteria in the anode can also treat biodegradable wastes. In addition, microalgae in the cathode can fixate CO_2, nitrogen, and phosphorus while simultaneously producing a biomass rich in value compounds which could be used in food, feed, nutraceuticals, and supplements. The whole system allows the effluent treatment, production of microalgae valuable biomass, and production of bioelectricity, which is very useful especially in remote areas (Gouveia et al., 2014).

28.3.4 Valuable products obtained from microalgae

Microalgae are excellent sources of nutritional compounds due to their high content of antioxidants and pigments (carotenoids such as fucoxanthin, lutein, beta-carotene, cantaxanthin and/or astaxanthin, and phycobilliproteins) and the presence of long-chain PUFAs (e.g., EPA and DHA) and proteins (essential amino acids methionine, threonine, and tryptophan) (Gouveia, 2014).

Moreover, microalgae have also been screened for new pharmaceutical compounds with biological activity, such as antibiotics, antiviral, anticancer, enzyme inhibitory agents, and other therapeutic applications. They have been reported potentially to prevent or reduce the impact of several lifestyle-related diseases (Ebrahimi-Mameghani et al., 2014; Shibata et al., 2007, 2003; Shibata and Sansawa, 2006) with antimicrobial (antibacterial, antifungal, antiprotozoal), antiviral (including anti-HIV), and antidiabetes functions; they also have cytotoxic, antibiotic, and antitumor properties as well as bio-modulatory effects such as immunosuppressive and antiinflammatory roles (Lauritano et al., 2016; Burja et al., 2001; Singh et al., 2005). Furthermore, algae are believed to have a positive effect on the reduction of cardio-circulatory and coronary diseases, atherosclerosis, gastric ulcers, wounds, constipation,

anemia, hypertension, obesity, and diabetes (Go et al., 2016; Nuño et al., 2013; Yamaguchi, 1996; Yook et al., 2015).

28.3.4.1 Lipids

Microalgal lipids usually account for approximately 30%–50% of their total weight (Chew et al., 2017). Some microalgae can accumulate a high percentage of lipids depending on the environmental conditions in which they are grown. Stress conditions, such as nitrogen starvation, high temperature, pH shift, and high concentration of salts, are required to enhance lipid productivity (Kwak et al., 2016). The higher lipid productivity when compared to other lipid-based energy crops makes microalgae attractive as a raw material for biodiesel, health food supplements, and cosmetic applications (Yeh and Chang, 2012).

28.3.4.2 Proteins

Proteins account for the major constituents of microalgae, comprising 50%–70% of the total composition. Moreover, microalgae present a favorable amino acid profile, especially in essential amino acids (e.g., lysine, valine, isoleucine, leucine, and branched chain amino acids) that are adequate for human's requirements according to WHO/FAO/UNU recommendations, with minor deficiencies among the sulfur containing amino acids methionine and cysteine (Becker, 2007). Proteins are, therefore, one of the most important products of microalgae biorefineries and can be used for human and animal nutrition (Chew et al., 2017).

28.3.4.3 Carbohydrates

Microalgae can have a high carbohydrate content which can be easily stored due to its relatively high photo conversion efficiency. Algal carbohydrates are mainly composed of glucose, starch, cellulose, and various kinds of polysaccharides. Among these, glucose and starch can be used for bioethanol and biohydrogen production (Batista et al., 2014; Ferreira et al., 2012; John et al., 2011; Karemore and Sen, 2016; Miranda et al., 2012), while polysaccharides have biological functions as storage, protection, and structural molecules. Microalgal polysaccharides have the capacity to modulate the immune system and inflammatory reactions, being a promising source of biologically active molecules, such as cosmetic additives, food ingredients, and natural therapeutic agents (Chew et al., 2017).

28.3.4.4 Pigments

Microalgal pigments can be divided into three basic classes: carotenoids (carotenes and xanthophylls), chlorophylls, and phycobiliproteins. Chlorophylls and carotenoids are generally fat-soluble molecules, whereas phycobiliproteins are water-soluble. These pigments have been used as precursors of vitamins in both food and animal feed (Marques et al., 2011b), additives and coloring agents in food applications, biomaterials, and in the cosmetic and pharmaceutical industries (Chew et al., 2017).

28.3.4.4.1 Carotenoids

Carotenoids are fat-soluble pigments that are accessory pigments in plants. The most common algal carotenoids are lutein, astaxanthin, β-carotene, zeaxanthin, and lycopene. The microalgae carotenoids have been associated and claimed to reduce the risk of: (1) certain cancers (Gerster, 1993; Lupulescu, 1994; Tanaka et al., 2012; Willett, 1994); (2) cardiovascular diseases (Giordano et al., 2012; Kohlmeier and Hastings, 1995); and (3) macular degeneration and cataract formation (Snodderly, 1995; Weikel and Chiu, 2012). They may also possibly have an effect on the immune system and may influence chronic diseases (Meydani et al., 1995; Park et al., 2010). The global carotenoids market was valued at US$1577 million in 2017 and is projected to reach US$2098 million by 2025, registering a CAGR of 3.6% from 2018 to 2025 (Allied Market Research, 2019).

Most of the lutein produced commercially is extracted from the petals of the marigold flower. However, microalgae are gaining in importance due to higher lutein productivities. Furthermore, microalgae require a smaller land area and less labor when compared to marigold cultivars (Fernández-Sevilla et al., 2010). The amount of lutein produced by microalgae can vary depending on the environmental conditions, namely temperature, pH, light intensity, salinity, and nitrogen amount (Guedes et al., 2011). The most common microalgae for producing lutein include *Muriellopsis* sp., *Scenedesmus almeriensis*, *Chlorella protothecoides*, *Chlorella zofingiensis*, *Chlorococcum citriforme*, and *Neospongiococcus gelatinosum* (Fernández-Sevilla et al., 2010). Regarding market price, the cost of lutein extracted from the microalga *S. almeriensis* is approximately 2.5 US$/g lutein (Molina et al., 2005; Sánchez et al., 2008).

Zeaxanthin is generally a yellow-colored carotenoid mainly used in pharmaceutical, cosmetic, and food industry applications (Sajilata et al., 2008). *S. almeriensis* and *Nannochloropsis oculate* are the most commonly used microalgae for zeaxanthin production (Granado-Lorencio et al., 2009; Guillerme et al., 2017). The market value for zeaxanthin produced by *S. almeriensis* is around 10 US$/g zeaxanthin (Granado-Lorencio et al., 2009; The Insight Refinery, 2016).

Astaxanthin is a carotenoid from the xanthophyll family that acts as a potent antioxidant, having strong antiaging, sun-proofing, antiinflammatory and immune system-boosting effects, and skin protection from ultraviolet radiation. Certain microalgae like *Haematococcus pluvialis* (Panis and Carreon, 2016) and *C. zofingiensis* (Guedes et al., 2011) have already been successfully used for producing commercial astaxanthin. For example, for astaxanthin obtained from *H. pluvialis*, the market value is approximately 1.8 US$/g astaxanthin (Cuellar-Bermudez et al., 2015; Panis and Carreon, 2016; Shah et al., 2016).

β-Carotene has been used as a coloring agent, an antioxidant, and a vitamin-A supplement. It also possesses antiaging and anticancer properties (Pisal and Lele, 2005). The most commonly used microalgae for the production of β-carotene are *Dunaliella salina*, *S. almeriensis*, and *Dunaliella bardawil* (Guedes et al., 2011; Pisal and Lele, 2005). For instance, β-carotene from *Dunaliella* sp. has an approximate market value of 0.3–0.7 US$/g β-carotene (Markou and Nerantzis, 2013).

Lycopene is considered to be one of the most influential antioxidant and an effective sunscreen agent. It is also known to possess anticarcinogenic and antiatherogenic properties, reducing the risk of chronic diseases like cancer and cardiovascular diseases (Agarwal and Rao, 2000; Mourelle et al., 2017). An *in vivo* study showed that algal lycopene obtained from *Chlorella marina* exhibited a high antioxidant and antiinflammatory effect in high cholesterol-fed rats (Renju et al., 2014).

28.3.4.4.2 Chlorophylls

Chlorophylls are lipid-soluble pigments with low polarity (Chew et al., 2017). One or more types of chlorophyll are present in microalgae, but the main types are chlorophylls *a*, *b*, and *c*. Due to the structural differences, chlorophyll *a* has blue/green pigment with maximum absorbance from 660 to 665 nm and chlorophyll *b* has green/yellow pigment with maximum absorbance from 642 to 652 nm (Begum et al., 2016).

Chlorophyll is an essential compound, used not only as an additive in pharmaceutical but also in cosmetic products. Chlorophyll *a* has been extensively used as a coloring agent because of its stability. Green microalgae have the highest chlorophyll content among all algae, and it is already commercialized from *Chlorella* species (Chew et al., 2017). On the other hand, *Spirulina platensis* has only chlorophyll *a*, being used as a natural color in food, cosmetic, and pharmaceutical products (Begum et al., 2016). Moreover, chlorophyll derivatives can exhibit health-promoting activities, such as wound healing and antiinflammatory properties (Ferruzzi and Blakeslee, 2007). Additionally, Balder et al. (2006) suggested that the consumption of chlorophyll was associated to a decrease in the risk of colorectal cancer.

28.3.4.4.3 Phycobiliproteins

Phycobiliproteins are the major photosynthetic accessory pigments in cyanobacteria and red algae. These include phycocyanin, allophycocyanin, phycoerythrin, and phycoerythrocyanin (Sekar and Chandramohan, 2008). Phycobiliproteins are used not only commercially as natural dyes and fluorescent agents, but also in pharmaceutical (antioxidant, anti-allergic, antiinflammatory, neuroprotective and hepatoprotective agents) and cosmetic industries (perfumes and eye makeup powders). The major sources of phycobiliproteins are *Arthrospira* (*Spirulina*) sp., *Arthrospira platensis*, and *Aphanizomenon flos-aquae* (de Jesus Raposo et al., 2013; Odjadjare et al., 2017).

28.3.4.5 Polyunsaturated fatty acids (PUFAs)

PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are widely recognized as essential compounds in human health. The increasing demands for PUFAs has motivated the replacing of fish oil for microalgae as source of DHA and EPA, playing an increasing role in the food industry due to the depletion of marine resources and off-flavors (Wang et al., 2015). DHA-rich algal oil is usually obtained from microalgae such as

Schizochytrium, *Ulkenia*, *Isochrysis galbana*, *Chlorella pyrenoidosa*, *Chlorella ellipsoidea*, and *Crypthecodinium* (Matos et al., 2017; Winwood, 2013).

28.3.5 Microalgae-based bioplastics

Plastics and their by-products are littering our cities, oceans, and water streams, and contributing to health problems in humans and animals. These polymers take many years to decompose because they are hydrophobic and do not undergo the action of microorganisms. These issues have been greatly aggravated due to the economic growth from developed and developing countries and the increases in population. Hence, it is absolutely mandatory to reduce the amount of discarded plastics and create biodegradable ones, combining practicality with economy issues. The most common polymers used in the formulation of edible films are proteins (gelatin, casein, wheat gluten, and zein), polysaccharides (starch and chitosan), and lipids (waxes), which are used alone or combined. These biopolymers are highly biodegradable and decompose easily into inorganic CO_2 and water (Santacruz et al., 2015). The use of vegetable raw materials could be a very favorable alternative, being the microalgae one of the most demanded feedstocks. Some studies already exist on microalgae-based bioplastics (Morales-Jímenez et al., 2020; Rocha et al., 2020; Abdo and Ali et al., 2019; Kato, 2019; Mathiot et al., 2019; Zeller et al., 2013).

28.3.6 Microalgae-based biofertilizers

The continuous use of arable land for cultivation has led to loss of essential nutrients, such as nitrogen and phosphorus, in the soil. Thus, fertilizers play a vital role in improving agriculture to achieve maximum yields. The intensive use of chemical fertilizers and pesticides in agriculture practices has led to a overdependency on synthetic agrochemicals, which are not only finite resources and toxic, but are also rising in price (Chakhalyan et al., 2008).

Biofertilizers are cost-effective, eco-friendly, and renewable resources, that play a major role in the controlled mineralization and fertilization processes (Kawalekar, 2013). Biological fertilizers contain living or latent microorganisms or natural compounds derived from organisms such as algae, bacteria, and fungi, which can help in improving soil fertility and stimulating plant growth (Abdel-Raouf et al., 2012). Hence, the use of biofertilizers-based microalgae would provide a possible solution. In addition, preliminary results using microalgae biomass grown in effluents strongly suggest an important biostimulant capacity, which could have a significant impact on plant growth and seed germination indexes when applied to soil with minimal pretreatment (Navarro-Lopez et al., 2020). Special attention should be given to algal biomass and/or microalgal extracts (Michalak et al., 2017, 2016; Navarro-Lopez et al., 2020). Microalgae biomass is also known to act as a pesticide, protecting plants from diseases, insects, and abiotic stress (e.g., high salinity, drought, and frost), and is thus an alternative to chemical pesticides (Khan et al., 2019).

Most cyanobacteria are capable of fixing atmospheric nitrogen and can be effectively used as biofertilizers (Bhalamurugan et al., 2018). Some studies have already been developed using microalgae as biofertilizer, achieving promising results in seed germination, plant growth, production of flowers, increase in pigments, and soil fertility (Agwa et al., 2017; Dineshkumar et al., 2017; Faheed and Fattah, 2008; Garcia-Gonzalez and Sommerfeld, 2016; Renuka et al., 2016; Song et al., 2005). These studies suggest that microalgae are an efficient, economical, and safe biofertilizer to substitute for chemical fertilizers in enhancing plant growth, and have no detrimental effect on the plant.

28.4 Examples of microalgae-based biorefinery

28.4.1 Nannochloropsis *sp. biorefinery*

Nobre et al. (2013) developed a biorefinery surrounding *Nannochloropsis* sp. microalga with the extraction of value-added compounds such as carotenoids and fatty acids (namely EPA) for food and feed purposes, as well as lipids for biodiesel production. The fractionated recovery of these compounds was done by supercritical fluid extraction using CO_2 and ethanol as an entrainer. After the extraction process, the biomass leftovers were used as substrate for *Enterobacter aerogenes*, in a dark fermentation process, to produce bioH_2 (Fig. 28.1). The maximum bioH_2 yield was 60.6 mL H_2/g alga (Nobre et al., 2013).

Ferreira et al. (2013) did a Life Cycle Inventory (LCI) of the whole process (microalgae cultivation, dewatering, milling, extraction, and H_2 production), evaluating the energy consumption, CO_2 emissions, and economic factors. The authors showed and analyzed five possible pathways and two biorefineries (Fig. 28.2).

The analysis of pathways 1, 2, and 5 considers a system boundary that includes the *Nannochloropsis* sp. microalgal culture and the final product output (fatty acids, pigments, or bioH_2, respectively). For the remaining pathways, 3 and 4, the bioH_2 production from the leftover biomass from Soxhlet extraction (SE) and supercritical fluid extraction (SFE), respectively, was evaluated. The authors concluded that the oil production pathway by SE (3) achieved the lowest energy consumption (176–244 MJ/MJ_{prod}) and CO_2 emissions (13–15 kg CO_2/MJ_{prod}). However, the biorefinery considering the production of oil, pigments, and H_2 via SFE was the most economically viable.

From the net energy balance and the CO_2 emission analysis, Biorefinery 1 (biodiesel SE + bioH_2) presented the best results. Biorefinery 2 (biodiesel SFE + bioH_2) showed results in the same range of those in Biorefinery 1. However, the use of SFE produced high-value pigments in addition to the fact that it is a clean technology which does not use toxic organic solvents.

Therefore, Biorefinery 2 was the best in terms of energy, CO_2 emissions, and cost (Ferreira et al., 2013).

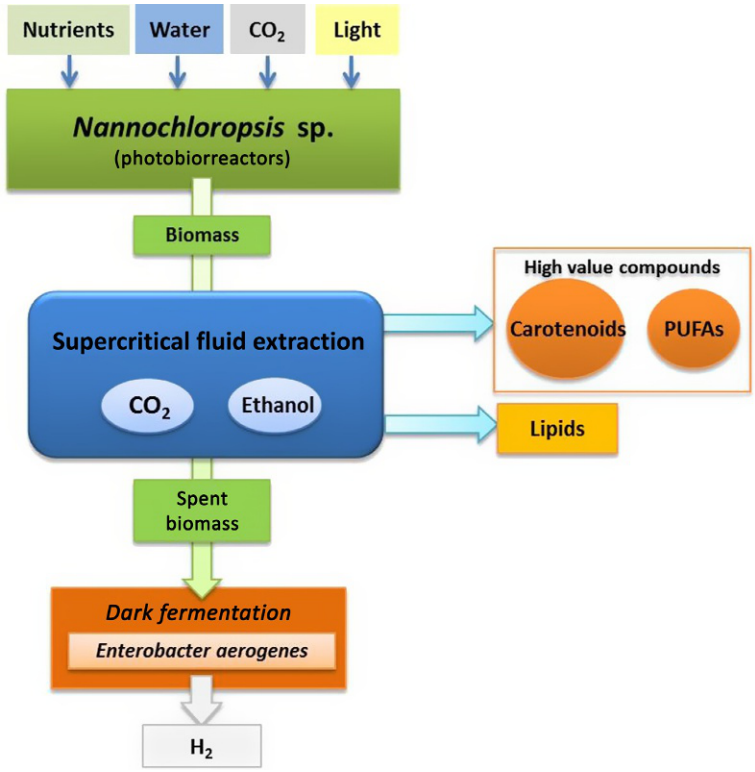

Fig. 28.1

Nannochloropsis sp. biorefinery. *Adapted from Nobre, B.P., Villalobos, F., Barragán, B.E., Oliveira, A.C., Batista, A.P., Marques, P.A.S.S., Mendes, R.L., Sovová, H., Palavra, A.F., Gouveia, L., 2013. A biorefinery from Nannochloropsis sp. microalga—extraction of oils and pigments. Production of biohydrogen from the leftover biomass. Bioresour. Technol. 135, 128–136. https://doi.org/10.1016/j.biortech.2012.11.084.*

28.4.2 Anabaena *sp. biorefinery*

The experimental biohydrogen production by photoautotrophic cyanobacterium *Anabaena* sp. was studied by Marques et al. (2011a). Hydrogen production from the *Anabaena* biomass leftovers was also achieved by fermentation through the *E. aerogenes* bacteria and was reported by Ferreira et al. (2012) (Fig. 28.3).

Different culture conditions and gas atmospheres were tested in order to maximize the autotrophic bioH_2 yield versus the energy consumption and CO_2 emissions. The authors stated that the best conditions included an Air+CO_2 +20% N_2 gas atmosphere and medium light intensity (384 W) (Ferreira et al., 2012). The yielded H_2 could be increased using the biomass leftovers through a fermentative process; however, this would mean higher energy consumption as well as an increase in CO_2 emissions.

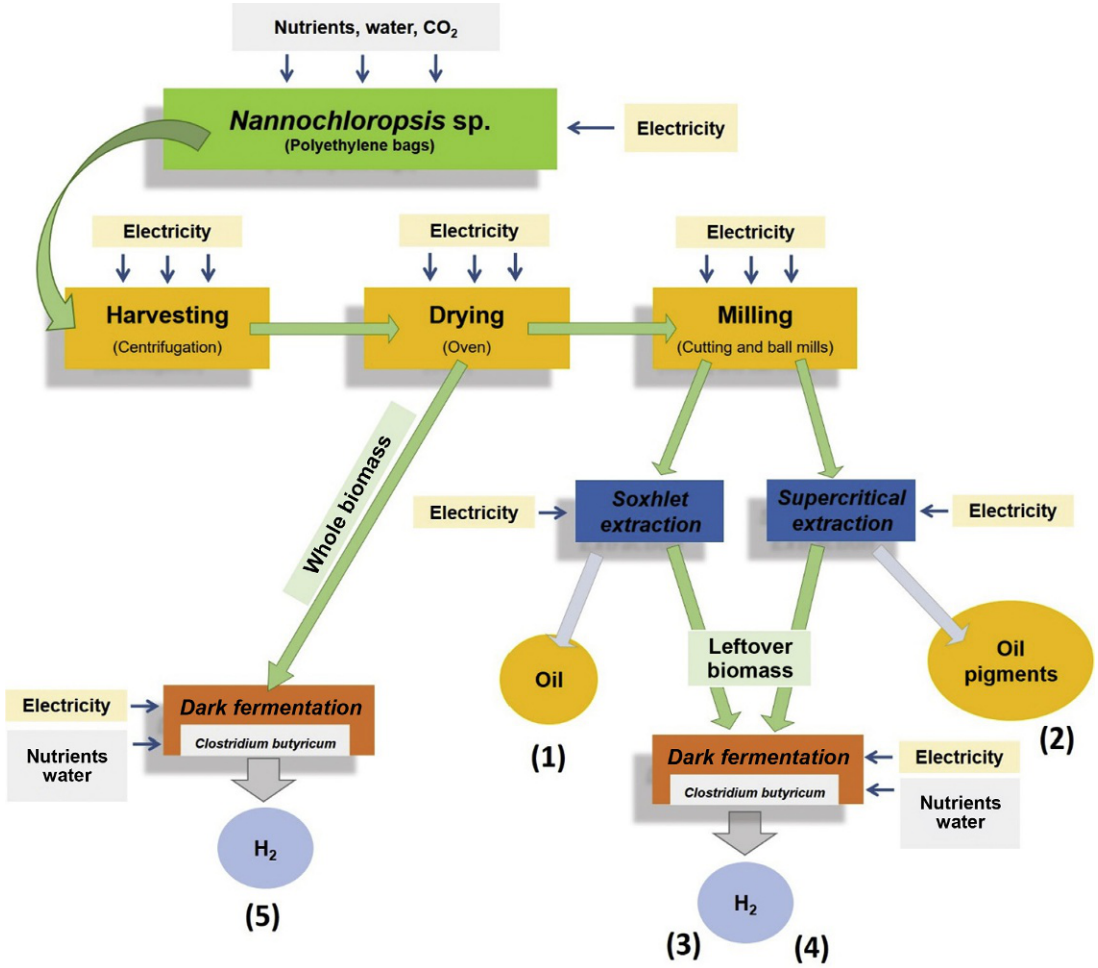

Fig. 28.2

Nannochloropsis sp. possible biorefineries: pathways 1 and 3 represent Biorefinery 1, pathways 2 and 4 are Biorefinery 2, and pathway 5 is the direct bioH_2 production. *Adapted from Ferreira, A.F., Ribeiro, L.A., Batista, A.P., Marques, P.A.S.S., Nobre, B.P., Palavra, A.M.F., da Silva, P.P., Gouveia, L., Silva, C., 2013. A biorefinery from nannochloropsis sp. microalga—energy and CO_2 emission and economic analyses. Bioresour. Technol. 138, 235–244. https://doi.org/10.1016/J.BIORTECH.2013.03.168).*

28.4.3 Chlorella vulgaris *biorefineries*

Chlorella vulgaris is one of the most intensively researched microalgae. Therefore, a lot of work has been done concerning biorefinery from this microalga.

Collet et al. (2011) worked on a biorefinery using *C. vulgaris* with lipid extraction followed by methane production from the remaining biomass. The authors developed a Life Cycle Assessment (LCA) and demonstrated that the microalgal methane is the worst case, when compared to microalgal biodiesel and diesel, in terms of abiotic depletion, ionizing radiation,

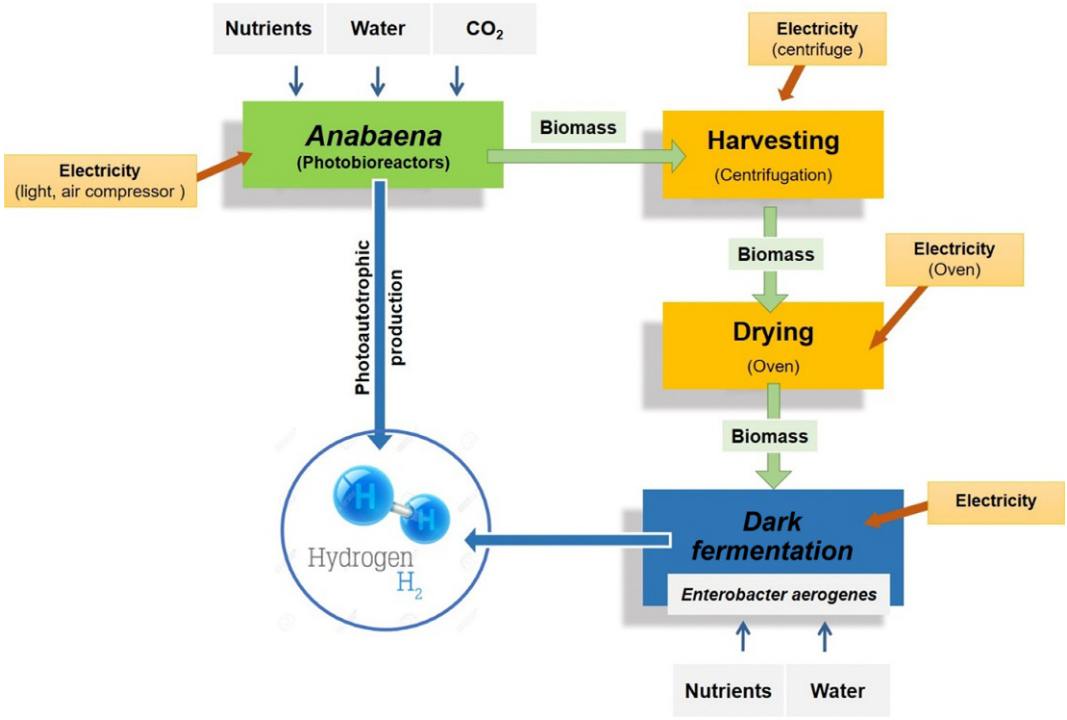

Fig. 28.3

Anabaena sp. biorefinery: production of biohydrogen through two pathways (autotrophically and by dark fermentation with *Enterobacter aerogenes*). *Adapted from Ferreira, A.F., Marques, A.C., Batista, A.P.B., Marques, P.A.S.S., Gouveia, L., Silva, C.M., 2012. Biological hydrogen production by Anabaena sp.—yield, energy and CO_2 analysis including fermentative biomass recovery. Int. J. Hydrogen Energy 37, 179–190. https://doi.org/10.1016/j.ijhydene.2011.09.056).*

human toxicity, and possible global warming. These negative results are mainly due to a strong demand for electricity. For the land use category, algal biodiesel also had a lesser impact than algal methane. However, algal methane is a much better option regarding acidification and eutrophication.

Another work by Ehimen et al. (2011) consider the simultaneous production of biodiesel and methane in a biorefinery concept. The authors obtained biodiesel from a direct transesterification process on the *Chlorella* biomass, and methane through anaerobic digestion of the biomass residues. The maximum methane concentration obtained was 69% (*v*/v), with a specific yield of 0.308 m^3 CH_4/kg VS, at 40°C and a C/N mass ratio of 8.53. The biodiesel yield was not reported (Ehimen et al., 2011).

Gouveia et al. (2014) studied the simultaneous production of bioelectricity and added-value pigments with wastewater treatment. Fig. 28.4 represents the photosynthetic algal microbial fuel cell (PAMFC), where *C. vulgaris* is present in the cathode compartment and a bacterial consortium in the anode compartment. The authors proved that the light intensity increases the

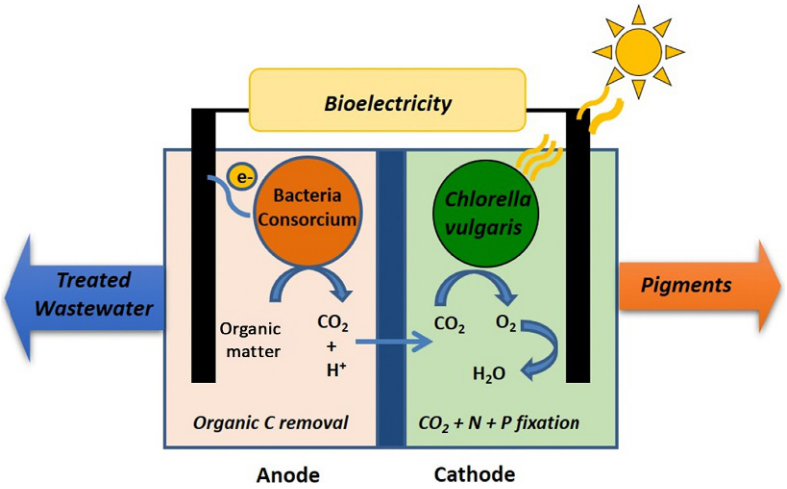

Fig. 28.4

C. vulgaris biorefinery: photosynthetic algal microbial fuel cell (Gouveia et al., 2014).

PAMFC power and increases the carotenogenesis process in the cathode compartment. The maximum power produced was 62.7 mW/m^2 with a light intensity of 96 μE/(m^2 s).

Another example of a *C. vulgaris* biorefinery is a bioethanol-biodiesel-microbial fuel cell as reported by Powell and Hill (2009). This fuel cell consisted of an integration of *C. vulgaris* (in the cathode) that captures the CO_2 emitted by yeast fermenters (in the anode). The study demonstrated the possibility of generating electrical power and oil for biodiesel, in a bioethanol production facility. After oil extraction, the remaining biomass could be used for animal feed supplementation (Powell and Hill, 2009).

28.4.4 Chlorella protothecoides *biorefinery*

The biorefinery developed by Campenni' et al. (2013) consisted in the extraction of lipids and carotenoids from *Chlorella protothecoides* grown autotrophically and with nitrogen deprivation and the addition of a 20 g/L NaCl solution. The leftover biomass could be used for hydrogen or bioethanol production, as the residue still contains sugar (Fig. 28.5).

The total carotenoid content was 0.8% (*w*/*w*), which includes canthaxanthin (23.3%), echinenone (14.7%), free astaxanthin (7.1%), and lutein/zeaxanthin (4.1%), which can be used for food applications. Moreover, the total lipid content reached 43.4% (*w*/*w*), with a favorable fatty acid composition that complies with the biodiesel EN 14214 quality specifications (European Standard EN 14214, 2004) and can be used for the biodiesel industry.

28.4.5 Chlamydomonas reinhardtii *biorefinery*

Mussgnug et al. (2010) studied the production of biohydrogen from *Chlamydomonas reinhardtii* followed by biogas (methane) production by anaerobic fermentation of the leftover biomass.

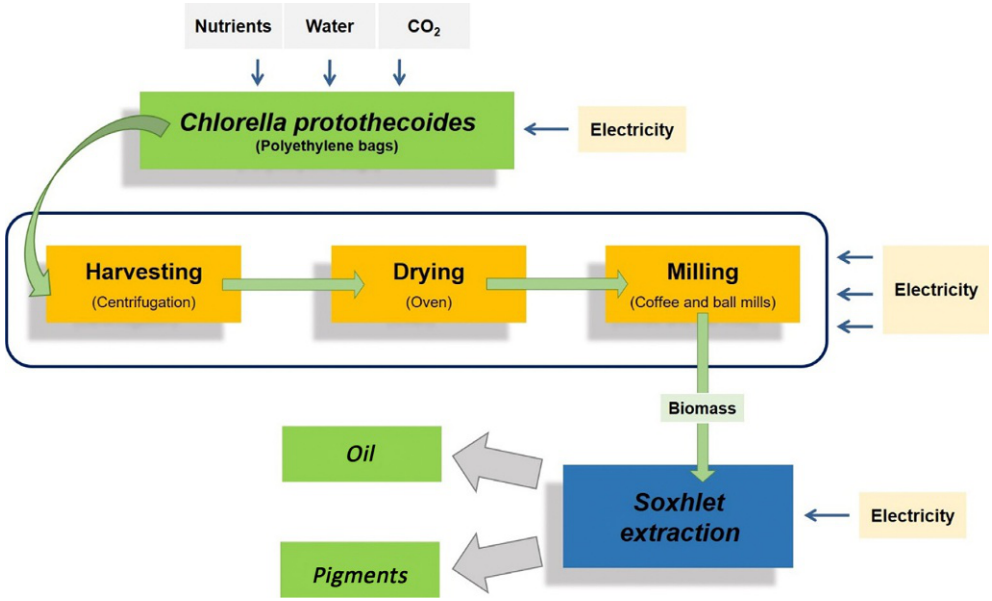

Fig. 28.5

Chlorella protothecoides biorefinery. *Adapted from Campenni' L., Nobre B.P., Santos C.A., Oliveira A.C., Aires-Barros M.R., Palavra A.M.F. and Gouveia L., Carotenoid and lipid production by the autotrophic microalga Chlorella protothecoides under nutritional, salinity, and luminosity stress conditions, Appl. Microbiol. Biotechnol.* ***97****, 2013, 1383–1393, https://doi.org/10.1007/s00253-012-4570-6.*

The authors verified that using the biomass after hydrogen production instead of the fresh biomass increased the biogas production around 120%. They concluded that these results were due to the storage compounds with high fermentative potential, such as starch and lipids, which are the key to microalgae-based integrated processes for value-added applications (Mussgnug et al., 2010).

28.4.6 Dunaliella salina *biorefinery*

Sialve et al. (2009) showed the production of methane from *Dunaliella salina* after oil extraction for making biodiesel. For shorter hydraulic retention time (HRT, 18 days), the authors achieved a much higher yield (up to 50%), compared to the values reported by Collet et al. (2011) using *C. vulgaris*.

28.4.7 Dunaliella tertiolecta *biorefinery*

Lee et al. (2013) investigated the integration of chemoenzymatic saccharification and bioethanol fermentation after lipid extraction of *Dunaliella tertiolecta* biomass for biodiesel production. The bioethanol production achieved a yield of 0.14 g ethanol/g residual biomass and 0.44 g ethanol/g glucose. According to the authors, this strategy could improve the economic feasibility of a microalgae-based integrated process.

28.4.8 Arthrospira (Spirulina) biorefinery

Olguín (2012) studied a biorefinery with the double purpose of producing oleaginous microalgae grown in wastewater and *Arthrospira* grown in seawater supplemented with anaerobic effluents from animal waste for the production of biofuels (biogas, biodiesel, and biohydrogen) and high-value products (PUFAs, phycocyanin, and fish feed). This study highlighted that the biorefinery strategy offers new opportunities for cost-effective and competitive production of biofuels along with nonfuel applications.

28.4.9 Spirogyra sp. biorefinery

Pacheco et al. (2015) developed a biorefinery from the sugar-rich microalga *Spirogyra* sp. for the production of bioH_2 and pigments (Fig. 28.6). The authors carried out an economic and Life Cycle Analysis of the whole process and concluded that the sugar content of the microalgae must be increased in order to achieve higher bioH_2 yields.

The potential energy production and food-grade protein and pigments revenue per cubic meter of microalga culture per year was estimated as 7.4 MJ, US $412, and US $15, respectively, thereby contributing to the cost efficiency and sustainability of the whole bioconversion

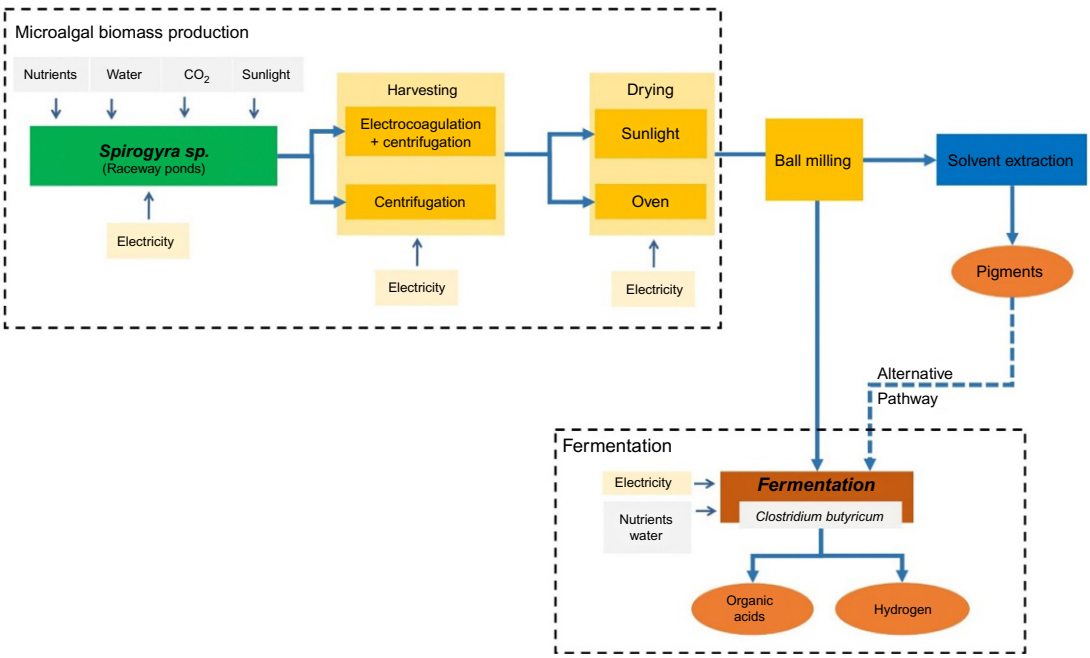

Fig. 28.6

Spirogyra sp. biorefinery. *Adapted from Pacheco, R., Ferreira, A.F., Pinto, T., Nobre, B.P., Loureiro, D., Moura, P., Gouveia, L., Silva, C.M., 2015. The production of pigments & hydrogen through a Spirogyra sp. biorefinery. Energ. Conver. Manage. 89, 789–797. https://doi.org/10.1016/J.ENCONMAN.2014.10.040).*

process (Pinto et al., 2018). Moreover, the use of alternative methods for harvesting and dewatering as well as pigment extraction is crucial to increase the economic viability of the process. Electrocoagulation and solar drying were used in this study and were able to reduce the energy requirements by 90% (Pacheco et al., 2015).

Overall, the major energy consumers and CO_2 emitters of the process were the centrifugation of the microalgal biomass and heating for the fermentation. Pigment production thus becomes necessary to improve the economic benefits of the biorefinery. Nonetheless, it is essential to reduce the extraction energy requirements.

28.4.10 Scenedesmus obliquus *biorefinery*

Ferreira et al. (2019) used microalga *S. obliquus* to treat wastewater from the brewery industry successfully. Furthermore, they used the obtained biomass for different applications, including biofuel production (bioH_2 and pyrolytic bio-oil), subcritical water extraction (SWE) of bioactive compounds (e.g., phenols, flavonoids), and biofertilizers/biostimulants (Fig. 28.7).

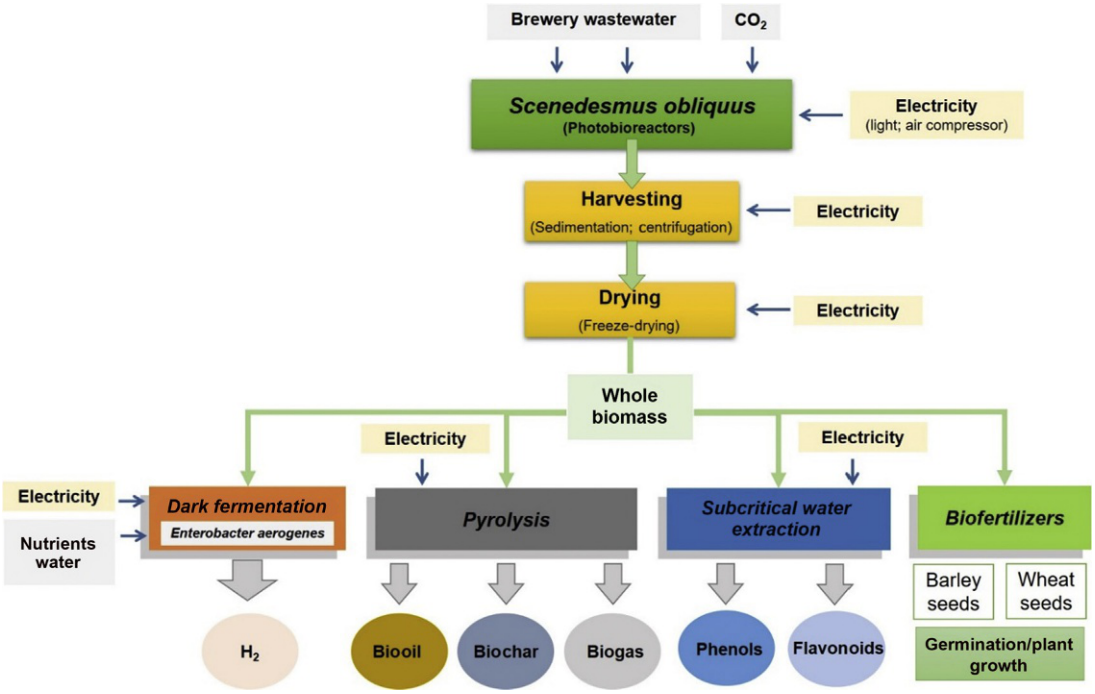

Fig. 28.7

Scenedesmus obliquus biorefinery. *Based on Ferreira, A., Ribeiro, B., Tavares, M.L.A., Vladic, J., Vidovic, S., Cvetkovic, D., Melkonyan, L., Avetisova, G., Goginyan, V., Gouveia, L., 2019. Scenedesmus obliquus microalga-based biorefinery—from brewery effluent to bioactive compounds, biofuels and biofertilizers—aiming a circular bioeconomy. Biofuels Bioprod. Biorefin. 13, 1169–1186).*

The authors achieved high removal efficiencies, obtaining clean waters that meet the standards imposed by Portuguese law (Decree-Law No 236/98, 1998).

SWE of the microalgal biomass allowed recovery yields of 1.016 mg GAE (gallic acid equivalent)/mL for phenols and 0.167 mg CE (catechin equivalent)/mL for flavonoids at 200°C. Furthermore, the high temperatures had a sterilizing effect on extracts, which could be beneficial for future food and feed applications.

The wastewater-grown *S. obliquus* biomass was also upgraded into biofuel production, achieving a yield of 67.1 mL H_2/g VS for bioH_2 production, and 64% for bio-oil and 30% for biochar produced from the pyrolysis process (Ferreira et al., 2017, 2019).

Lastly, the potential of *S. obliquus* biomass was evaluated in barley and wheat seeds, and the authors verified that this microalga has an enhancing capacity for plant germination and growth. Moreover, this capacity was further increased when the microalga was grown in brewery wastewater, which is a double benefit for the viability of a biorefinery approach.

28.4.11 Tetraselmis *sp. biorefinery*

Pereira (2019) used *Tetraselmis* sp. CTP4, which is a very robust and tolerant microalga strain, to produce a biorefinery that included biofuel and added-value streams.

To minimize harvesting and drying costs, the microalgal biomass was first extracted with ethanol and the crude ethanolic extract was fractionated using a liquid–liquid triphasic system (LTPS). The authors noted the presence of added-value molecules with antioxidant and metal chelating properties, namely phospholipids and carotenoids. The nonpolar and water-soluble phases, as well as the leftover biomass from the ethanolic extraction were upgraded into different biofuel applications, namely biodiesel, bioethanol, and biogas, respectively.

The authors obtained a biodiesel composed mainly of palmitic and oleic acid esters, with low amounts of polyunsaturated fatty acid esters. They achieved a bioethanol yield of 0.46 g ethanol/g fermentable sugar through yeast fermentation after enzymatic hydrolysis. Lastly, the anaerobic digestion of the residual biomass with or without glycerol supplementation resulted in biomethane yields of 64% and 83%, respectively (Pereira, 2019).

Furthermore, the biochemical composition of the spent biomass also showed to be adequate for food and feed applications (Pereira et al., 2019). Pereira et al. (2020) incorporated the defatted biomass of *Tetraselmis* sp. CTP4 into the feed of juvenile gilthead seabream (*Sparus aurata*), obtaining a similar growth to feed enriched with soybean meal. This means that the defatted microalgal biomass could potentially replace the use of soybean meal in aquaculture feeds, contributing to fulfill the protein demands for EU animal feed market.

Overall, this innovative biorefinery based on *Tetraselmis* sp. CTP4 allowed an effective separation of valuable compounds present in wet microalgal biomass, with the potential for an effective scale-up extraction process.

28.5 Conclusions

The microalgal business is still very new, but it is currently accepted as a doorway to a multibillion industry, since microalgae are an ecologically safe feedstock for biofuels and products with high commercial value. Furthermore, microalgae have the ability to convert any type of wastewater into a low environmental impact effluent, which in turn could serve as a biofertilizer for plants by improving the fertility of the soil and/or to made bioplastic for a cleaner environment. Thus, the sector needs to be developed further to respond to the constant demand for eco-friendly innovations to meet human needs regarding food, water, and energy.

The implementation of a biorefinery platform for microalgae production is therefore crucial to make exploitation of microalgae cheaper and competitive and support a microalgae-based bioeconomy. A microalgae-based biorefinery should integrate several processes, taking advantage of the various products synthesized by the microalgae for different industries, such as food, feed, energy, agricultural, pharmaceutical, cosmetic, and chemical. Furthermore, this should be done with minimal environmental impact by recycling the nutrients and water (wastewater bioremediation), and by mitigating the CO_2 from the flue gases.

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CHAPTER 29

The bioeconomy of microalgae-based processes and products

Mauro Vigani

Countryside and Community Research Institute, University of Gloucestershire, Cheltenham, United Kingdom

Chapter outline

29.1 Introduction 799
29.2 Algae bioeconomy and sustainability 800
29.3 Algae products and circularity 803
29.4 Supply chain characteristics 807
29.5 Algae production and trade 808
29.6 Conclusions 819
References 820

29.1 Introduction

Algae and seaweeds have been exploited for thousands of years, mainly by coastal communities, as a source of human food, cattle feed, fertilizers, and construction material, but it is only during the last century that the large-scale exploitation of this resource has been developed (Milledge, 2011).

Recently, algae and seaweed products and biomass have been at the center of greater interest by researchers, the industry, and governments for their potential contribution to sustainable growth. Algae are nowadays "seen as important resources in the bioeconomy context for their relevant role to the conservation of marine ecosystems and their value for a variety of commercial applications that reduce the pressures on land-based products or nonrenewable resources" (EMODnet, 2018).

National and international institutions, such as the United States (US), the European Commission (EC), or the Organisation for Economic Co-operation and Development (OECD), issued official strategies for the development of the bioeconomy, in which algae and seaweeds are often included. These strategies attempt to address and contribute to a number of

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the United Nations' Sustainable Development Goals (SDGs). Indeed, the 2030 Agenda for Sustainable Development often cites the bioeconomy as a means to achieve food security and nutrition, and the use of natural resources, in a way that ensures sustainable economic, social, and environmental development (FAO, 2018). In this context, algae and seaweeds can contribute directly to the SDGs concerning: economic growth and eradication of poverty (SDG 1); food security and nutrition (SDG 2); water and sanitation (SDG 6); affordable and clean energy (SDG 7); sustainable consumption and production (SDG 12); and oceans, seas, and marine resources (SDG 14).

Despite the promising utilization of algae and the overall growth of the sector, currently several challenges need to be addressed in order for the algae sector to express its potentials fully for the bioeconomy and the SDGs. These challenges include technology development for improving the commercial viability of algae products and biomass, and accurate economic and market data to support industrial and institutional planning. Moreover, targeted policies should be developed considering that algae and seaweeds biomass must be managed sustainably in a context of climate change and anthropogenic pressure affecting marine ecosystems (Camia et al., 2018).

The reminder of this chapter addresses these issues by: discussing the application of the concepts of bioeconomy, blue economy, and circular economy to algae and seaweeds; presenting the complex portfolio of applications of algae and seaweeds across industrial sectors; describing the characteristics of the algae supply chains; and analyzing global macroeconomic data on production and trade of algae and seaweeds.

29.2 Algae bioeconomy and sustainability

The fast growth rate of the global population and the constrained amount of natural resources for supporting the livelihood of a larger number of individuals is leading governments and researchers to propose a shift from a resource-intensive type of economy to a more sustainable and resource efficient one. Researchers, governments, and international organizations often refer to this new type of approach to economic activities as a "bioeconomy" or "bio-based economy." The ultimate objective of the bioeconomy is to replace fossil-based product with bio-based products, which would reduce environmental problems and global warming (Didem Özçimen et al., 2018).

The bioeconomy is described by the EC as a type of economy that "[…] encompasses the production of renewable biological resources and the conversion of these resources and waste streams into value-added products, such as food, feed, bio-based products and bioenergy" (EC, 2012). Therefore, the bioeconomy concerns a number of economic sectors (e.g., agriculture, forestry, fisheries, food, bio-based chemicals and materials, and bioenergy), including macro- and microalgae.

When considering macro- and microalgae, a more specific concept can be used: the "blue bioeconomy." This is defined by the OECD (2016) as encompassing: "[…] ocean-based industries, (such as shipping, fishing, offshore wind and marine biotechnology), but also the natural assets and ecosystem services that the ocean provides (fish, shipping lanes, CO_2 absorption and the like)." The World Bank adds to this definition some sociological aspects, specifying that "the blue economy concept seeks to promote economic growth, social inclusion, and the preservation or improvement of livelihoods while at the same time ensuring environmental sustainability of the oceans and coastal areas" (Vierros and De Fontaubert, 2017).

The development of the bioeconomy is built on two main premises (ENRD, 2018): (i) an optimal exploitation of biomass wastes and residues through the extraction of materials and energy; and (ii) improvement of the biomass potential by using more productive species, biotechnological advances, and innovative extraction and processing technologies. The main constraint for shifting toward bio-based industrial products is to guarantee a stable and sufficient supply of biomass that does not compete with the production of food and feed, and that is price-competitive (Ronzon et al., 2017). In this sense, the benefit of algae is that their production does not require fertile soils or arable land and they can be grown on marginal lands, without impacting the production of food and feed (Trentacoste et al., 2015). However, price-competitiveness is still an issue.

The two concepts of bioeconomy and blue economy are not sufficient to describe the potential economic and sustainability opportunities of macro- and microalgae. First of all, although most algae are produced or harvested from the seas and oceans, they can also be produced and used in inland waters, and therefore the definition of blue economy is too narrow. Second, the bioeconomy is about not only using biomass instead of other nonrenewable resources, but also delivering efficient resource use, sustainable growth, and mitigation and adaptation to climate change (ENRD, 2018). For this reason, an additional concept needs to be introduced, which is the "circular economy." According to the EC (EC, 2018a), "in a circular economy, the value of products and materials is maintained for as long as possible. Waste and resource use are minimized, and when a product reaches the end of its life, it is used again to create further value." In this context, the OECD highlights three core elements of circular systems: (i) they focus on closing resource loops rather than on linear economic systems; (ii) they aim at reducing material flows, with some degree of material circularity; and (iii) they increase the efficient use of natural resources, materials, and products, in all economic activities and not only those that have a high material use (McCarthy et al., 2018). In other words, "a circular economy entails markets that give incentives to reusing products, rather than scrapping them and then extracting new resources. In such an economy, all forms of waste […] are returned to the economy or used more efficiently" (UNCTAD, 2018).

The bioeconomy and the circular economy are relevant for regional, industrial, waste, environmental, climate change, and research policies, which are all crucial to address growing global demand of goods and the decline of fossil resources (Ronzon et al., 2017). In order to achieve the objective of such policies, about 50 countries (or groups of countries) across Africa, the Americas, Europe, Asia, and the Pacific have set out initiatives and strategies to develop the bioeconomy at national or regional level. Most of the strategies focus on research and innovation for the bioeconomy, for example, in South Africa, Japan, and Malaysia (ENRD, 2018). The US national bioeconomy strategy was initially drafted by the Office of Science and Technology Policy and the Executive Office of the President, with the participation of different federal agencies, and mainly concerns the production of renewable energy (Trentacoste et al., 2015). Within the US bioeconomy strategy, the "National Bioeconomy Blueprint" outlines the core technologies for the bioeconomy, namely genetic engineering, DNA sequencing, and automated high-throughput manipulations of biomolecules. The 2008 Farm Bill initiated the development of a variety of programs focused on R&D, such as the Bioenergy Program for Advanced Biofuels, the Rural Energy for America Program, and the Biomass Research and Development Initiative. In addition, the Biomass Crop Assistance Program provides financial support to farmers to promote farming of bioenergy crops (Trentacoste et al., 2015).

The EU's Bioeconomy Strategy (EC, 2018b) is probably the most articulated, going beyond research and innovation. It is part of the EU's decarbonization strategy and consists of a high-level document outlining the EC's priorities for economic activities based on renewable biological resources from land and sea, reducing fossil fuel dependency, and improving the economic and environmental sustainability of primary production and processing industry, while underlining sustainability, circularity, and multidisciplinarity. As renewable biological resources, algae are included in the EU's Bioeconomy Strategy, in particular in the Blue Growth Strategy that supports the sustainable growth in the marine and maritime environment (Camia et al., 2018).

Despite the bulk of the biomass coming from agriculture, forestry, and fisheries, algae have a special role with strong potential for the development of the bioeconomy. Algae are a renewable resource highly efficient in converting solar energy into biomass. Their production does not require freshwater or pesticides; on the contrary, it reduces coastal eutrophication and improves water quality by absorbing excessive amounts of nutrients and emitting oxygen. If cultivated at a large scale, algae's uptake and storage of CO_2 can contribute to mitigate climate change and ocean acidification (Ronzon et al., 2017). From an ecosystem point of view, algae farms provide a shelter to fish, allowing algae to be combined with aquaculture production through integrated multitrophic systems, obtaining larger fish stocks, higher algae growth rates, and cleaner waters (Ronzon et al., 2017).

29.3 Algae products and circularity

The actual and potential contribution to the bioeconomy and circular economy becomes apparent by going through the large variety of products and sectors linked to both macro- and microalgae. Algae uses include products for human consumption, animal feed, agriculture, energy, pharmaceuticals, cosmetics, raw materials for the chemicals industry, and bioremediation. This is summarized in Fig. 29.1, which depicts not only the variety of sectors linked to macro and microalgae, but also the contribution to environmental sustainability through bioremediation of soils and waters and the circularity through the exploitation of algae biomass residuals after the primary use.

The largest amount of algae products supplies the food and feed markets. Nonprocessed or dry algae are part of traditional food in Asian countries, such as mozuku, kombu, and wakame. Seaweed meals are important for aquaculture and, in general, algae are a source of protein for livestock and pet feed instead of conventional protein sources such as soybean and fish meal.

Important extracts are used as food and feed additives, ingredients, and colorants. The most common are carrageenan, alginate, and agar, which have gelling, thickening, and stabilizing properties, and are used in soups, dairy products, fruit preserves, ice cream, and other desserts, and as clarifying agents in brewing. Recently, they have been successfully used as a vegetarian substitute for gelatin.

The variety of high-value compounds extracted from algae and used as nutraceuticals is remarkable. Given their high commercial value, these compounds are often used as supplements in infant formulas and dietary products. Carotenoids (α-, β-carotene; astaxanthin; lutein; zeaxanthin; canthaxanthin; fucoxanthin; phytoene; phytofluene; violaxanthin; antheraxanthin; echinenone; cryptoxanthin) are probably the most important high-value extracts and are used as additives, nutritional supplements, and colorants. In addition to carotenoids, the following compounds are extracted from both macro- and microalgae:

- polyunsaturated fatty acids (PUFAs): eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid, oleic acid, lauric acid;
- vitamins: B, 12, E;
- phycobiliproteins: phycocyanin, phycoerythrin, allophycocyanin;
- chlorophyll;
- polysaccharides: sulfated polysaccharides, nostoflan;
- sterols: brassicasterol, sitosterol, stigmasterol; and
- phenolic and volatile compounds: β-cyclocitral; α-, β-Ionone; neophytadiene; phytol; pentadecane; heptadecane.

The second most important utilization of algae after food and feed is the production of energy. Algae biomass is processed through anaerobic digestion for the production of transport fuel in

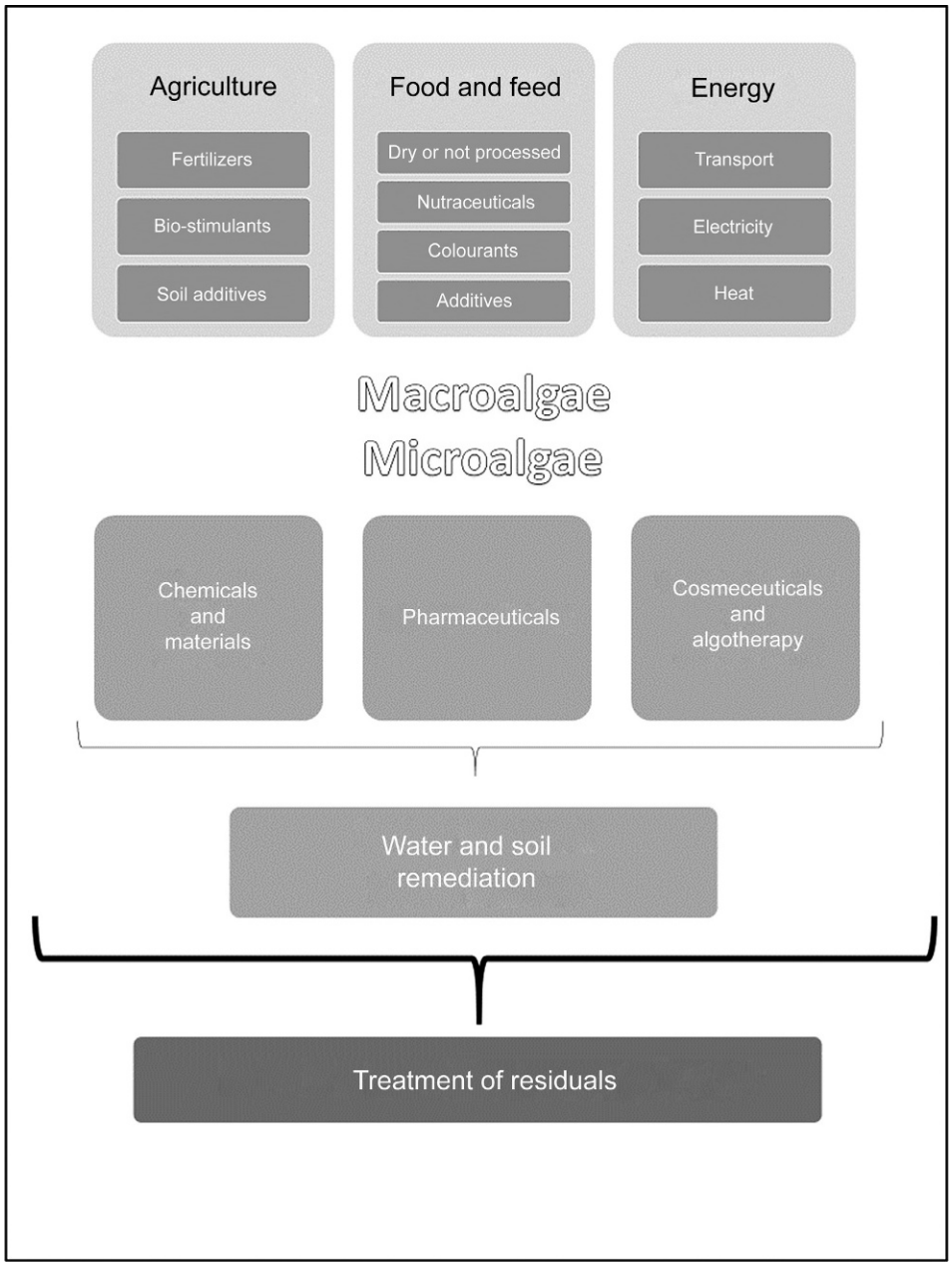

Fig. 29.1
Macro and microalgae products.

modified engines and through thermochemical conversion (e.g., gasification, liquefaction, pyrolysis) to obtain liquid fuels. The components that can be extracted are several: alcohols, biodiesel, kerosene, lipids, hydrocarbons, and carbohydrates. In addition, the bulk biomass can be used for the production of electricity and heat through combustion.

The agricultural sector is the one that can benefit the most from future potential algae applications, especially for fertilizers, pesticides, crop stimulants, and soil additives, although currently commercial products are limited. This potential is linked to the increasing public concern regarding the use of synthetic chemical herbicides, pesticides, and fertilizers, the growing demand for organic and conservation agriculture products, and the policy support for sustainable agricultural practices (EUMOFA, 2018).

Raw biomass of both macro and microalgae can be applied to agricultural land as slow-releasing fertilizers and for improving the soil organic matter and water retention capacity (Barsanti and Gualtieri, 2018). Cyanobacteria and eukaryotic green microalgae enhance the mineralization and mobilization of soil nutrients and induce crops' production of bioactive compounds, such as polysaccharides, growth hormones, and antimicrobial compounds. In particular, crops inoculation with nitrogen-fixing cyanobacteria can reduce the use of chemical nitrate fertilizer (Renuka et al., 2018). However, it should be noted that because agriculture requires large amounts of fertilizations, the amount of algal biomass that needs to be produced should increase dramatically (Renuka et al., 2018).

Microalgae are also potential biopesticides through eliciting metabolic processes for plant defense enzymes, which lead to enhanced plant immunity to pathogens. Similarly, microalgae are potential crop bio-stimulants inducing the production of plants' growth hormones and β-glucans (Renuka et al., 2018).

Other important compounds are extracted from macroalgae and used in several industrial sectors. Alginate and agar are used in the textile and paper industries as substrates for printing and sizing, and they are considered safer and easier to decompose than other synthetic substrates. Alginate is also used as a flux binder in the production of welding rods. Ethanol is an essential solvent in pharmaceuticals, toiletries and cosmetics, detergents and household cleaners, coatings and inks, and processing solvents. Lipids are used for the production of adhesives, phenols for anticorrosive coating, and acrylic styrene for inks.

Chlorophylls and thylakoid isoprenoids from microalgae are used for the production of paints pigments, while from both macro- and microalgae it is possible to obtain a variety of bioplastics (hybrid plastics; plastics derived from cellulose of lipid extracted algal biomass (Khanra et al., 2018)) or compounds for the production of bioplastics: lactic acid and poly lactic acid; bio polyethylene; ethylene monomer for the production of polyethylene.

Cosmeceutical compounds from algae include photo-protective molecules such as carotenoids, terpenes, tocopherols, and pyrenoine, but also surfactants for hair care and glycolipids and

phospholipids. Alginate is a thickener and moisture and color retainer used to produce lipsticks. Algotherapy (seaweed baths) has a niche market that is expected to grow (EUMOFA, 2018).

The pharmaceutical industry makes use of alginate and agar from macroalgae. The former is added to tablets to facilitate the decomposition and release of the active ingredients and as a protector of the stomach mucosa, while agar is commonly used as a substrate for the culture of microbacterial and as a laxative and an appetite suppressant. Another compound extracted from macroalgae useful in the pharmaceutical industry are phlorotannins which have antidiabetic, anticancer, antioxidation, antibacterial, radioprotective, and anti-HIV properties. From microalgae it is possible to obtain phycobiliprotein for clinical or research immunology, sterols for cholesterol lowering and antiinflammatory products, poliketides which have antibacterial and antifungal properties, and other substances such as PUFAs, terpenoids, glycolipids, and phospholipids (Barsanti and Gualtieri, 2018).

Apart from being a primary source of products and high-value compounds, algae are useful organisms for the remediation of soils and waters from environmental pollutants due to industrial and civil activities. Macroalgae are particularly useful for the remediation of fish farm waste in Integrated Multi-Trophic Aquaculture (IMTA), while microalgae provide more diversified opportunities. Some microalgae facilitate the degradation of oil and petroleum residuals and can enhance the development of heterotrophic microorganisms degrading oil. Blue-green microalgae contribute to the desalinization of soils by trapping excesses of sodium (Renuka et al., 2018). High Rate Algal Ponds (HRAPs) and biofilms are used for the treatment of municipal wastewater, allowing the retrieval of nutrients such as nitrate and phosphate currently not recovered from sewage treatment works (STWs). This increases resource efficiency in a circular way, turning environmental pollutants that can cause water eutrophication into inputs for algal biomass production at lower costs. Similarly, microalgae can be used for the remediation of wastewaters from meat, fish, and potatoes processing.

The final step in the circularity of algae bioeconomy is represented by the opportunities of further exploiting algae biomass residuals after their primary use for the production of products and high-value compounds. First of all, residual biomass can be recycled back as nutrients into the microalgae cultivation system. Second, the transesterification process of microalgal lipids to biodiesel produces glycerol as a by-product that can be converted to polymers, solvents, or polyesters, or used to generate electricity directly in biofuel cells. Residual lipids in the defatted biomass resulting from biodiesel extraction can be reused as a supplement for animal feed, while the defatted biomass can be used either as a source of pigments, as bioactive compounds and proteins, or to produce biogas through anaerobic digestion. Furthermore, the digestate resulting from biogas production can be used as a soil fertilizer and additive (Barsanti and Gualtieri, 2018). Other residual biomass can be exploited for its content of phlorotannins and subsequently further used for: anaerobic digestion; production of biochar; pyrolytic conversion for biodiesel; and multitrophic aquaculture systems.

29.4 Supply chain characteristics

The production of both macro- and microalgae and the extraction of high-value compounds is integrated in a highly vertically coordinated supply chain, where producers have direct contractual arrangements with buyers (e.g., seaweed producers directly supply producers of alginates and other marine hydrocolloids, under contract) (EUMOFA, 2018). Moreover, the algal biomass is circularly exploited across different stages of the supply chain in order to improve the commercial viability of the products.

Large-scale macroalgae production can contribute significantly to jobs creation, but requires a close dialog and cooperation between different stakeholders in the supply chain, in particular between the industry, the research community, the regulatory authorities, and consumers (Ronzon et al., 2017). However, this depends on the production systems and on the final products, which vary significantly across countries. For example, while in Asia macroalgae production is mainly based on cultivation, in Europe it is generally based on manual or mechanical harvesting by boat, which is subject to regulatory restrictions. Seaweed harvesting is environmentally sensitive and therefore regulated with licenses, harvesting authorizations, quotas by harvesting zone or by boat, harvesting size, and rotation systems (EUMOFA, 2018). In particular, kelp is the object of several preservation programs, which can restrict the mechanical harvesting or create protected areas. In some cases, kelp harvesting has been prohibited—for example, in the Spanish Basque country due to the implementation of a Natura 2000 marine area (EUMOFA, 2018).

The level of integration within the supply chain depends also on the overall competitiveness and relative negotiating power between suppliers and buyers. The lower competitiveness of EU producers with respect to Asian ones can put them in an unfavorable position within the supply chain, making producers more vulnerable to unfair trade practices. In order to improve the position of the EU producers within the supply chain, some suggest developing and adapting the Asian model to the EU's characteristics. Even though this might improve the overall competitiveness of the EU, it would be difficult for EU producers to achieve the same level of productivity and quality to penetrate highly regulated markets with high standards such as the Japanese one, competing against producers in the Philippines, Indonesia, Tanzania, and Zanzibar (EUMOFA, 2018).

Producers of microalgae have an even stronger integration within the supply chain, especially the nutritional and energy supply chains, with close links with buyers or even carrying out contract work (EUMOFA, 2018). This is because the extraction of high-value-added compounds from microalgae is technology-demanding and requires high investment costs, therefore buyers have an interest to provide producers with technologies or services to obtain products at the quality level needed (EUMOFA, 2018). However, despite the large variety of different high-value compounds that can be extracted from microalgae, currently only PUFAs

and carotenoids are economically viable thanks to their high market value; other compounds are produced in quantities too small to compete with alternative products (Barsanti and Gualtieri, 2018). This is the case, for example, of algal biofuel, which has costs of production that are significantly higher than those for other biofuels or fossil fuel, because of the heavy energy demand (electricity and heat) and material consumption for the algae biomass production (Jez et al., 2017).

In order to reduce production costs of algal biofuels, two main strategies can be identified: (1) to combine the biofuel production with other secondary products, such as biopolimers (Khanra et al., 2018); and (2) to use renewable energy production technologies, such as photovoltaics and biogas self-production from microalgae cake (Jez et al., 2017). In other words, the most cost-competitive way to produce microalgae products is through biorefineries.

Integrated biorefineries are currently under development to reduce macro- and microalgae production costs by directly exploiting the circular use of inputs and outputs. In a biorefinery, inputs derive as much as possible from waste streams, nutrients are recycled, and energy and capital costs are minimized. The output biomass is exploited into a variety of commercial products through reuse of residuals and, in order to guarantee market access, the level of integration within and the control over the supply chain are maximized. Moreover, any opportunity of diversification is explored, including provision of ecosystem services (Schlarb-Ridley and Parker, 2013).

For example, the production of algal biofertilizers is input-intensive and therefore can be achieved using nonpotable water sources and cheap sources of algal nutrients, combined with extraction of high value added by products (Renuka et al., 2018). As an alternative, integration with fish farming can represent a source of inputs, while providing valuable ecosystem services. Finally, capital costs can be reduced in the long term by co-developing algal farms with offshore wind farms (Schlarb-Ridley and Parker, 2013).

29.5 Algae production and trade

Analyzing the algae potential to contribute to the global bioeconomy and circular economy is constrained by a systematic lack of detailed economic data. A few sources are publicly available to researchers and practitioners looking for a thorough understanding of production and trade patterns of algae and their products. However, a few useful sources are currently available online for preliminary analyses.

The most comprehensive source of data is without doubt the FishStatJ (Software for Fishery and Aquaculture Statistical Time Series)[a] developed by the UN FAO which provides time series data for the period 1950–2017 on production quantities and values (both for sea harvests

[a] Available at http://www.fao.org/fishery/statistics/software/fishstatj/en (last accessed June 2019).

and aquaculture), and imports and exports quantities and values. This information is available at national, regional, or global levels. However, it should be noted that FishStatJ is a resource mainly for fish and fisheries products, therefore data on algae is much more limited, especially for what concerns microalgae.

A second important source of algae production data is EUROSTAT. Two main limitations can be mentioned for this data source. First of all, because of official agreements between the EU and the FAO on fisheries data exchange, data contained in the EUROSTAT databases are virtually the same as the data in FishStatJ. Second, given that EUROSTAT is a service for the EU and its citizens, data concerns exclusively the 28 EU member states, plus Norway and Iceland.

Another data source for EU countries is the recently developed free database European Marine Observation and Data Network[b] (EMODnet), which currently contains data on the location of EU producers of both macroalgae and microalgae, specifying production methods.

Regarding trade data, an alternative source with respect FishStatJ is the UN Comtrade database through its WITS service (World Integrated Trade Solutions),[c] which provides trade and protection data for an extensive range of commodities between 222 countries since 1962. Although this represents an important resource for global commodities, when it comes to algae the level of detail is quite aggregated, providing the following classifications: (i) Standard International Trade Classification, Revision 4 (SITC) Revision 4 "29297—Seaweeds & other algae"; (ii) World Customs Organization Harmonized System (HS) 2017 "121221—Seaweeds and other algae; fit for human consumption, fresh, chilled, frozen or dried, whether or not ground"; and (iii) UN's Central Product Classification (CPC) "0493—Seaweeds and other algae, fresh or dried, whether or not ground." These classifications are not sufficiently detailed to understand the uses and destination of algae and their products. In contrast, the FishStatJ data are classified also according to the International Standard Statistical Classification of Aquatic Animals and Plants (ISSCAAP), which distinguishes between brown, green, and red algae (by harvest and aquaculture) and therefore enables users to trace back each algae's main uses and products.

For the reasons outlined above, in what follows we focus on FAO FishStatJ data to obtain insights on global algae production and trade patterns, and to derive potential future developments and opportunities at national levels.

From 1950 to 2016, the total blue economy (all marine and aquaculture products, both fish and algae), increased from 20 mio tonnes to 200 mio tonnes, a ninefold increase. In the same period, algae production rose from 0.56 mio tonnes to 32.7 mio tonnes, about a 60-fold increase.

[b] Available at http://www.emodnet-humanactivities.eu (last accessed June 2019).

[c] Available at https://wits.worldbank.org/WITS/WITS/Restricted/Login.aspx (last accessed June 2019).

While the algae production in 1950 was just 2.8% of the total blue economy, in 2016 it rose to 16.5%. This demonstrates a significant growth in use and consumption of algae at the global level. In particular, given the growing importance of marine resources for food and nutrition security, it suggests that algae are playing an increasing and substantial role in achieving the Agenda 2030 for SDGs.

The growing importance of algae in the overall blue economy is linked to a transformation in the production systems and processes. Fig. 29.2 shows the changing quantity of algae produced from 1950 to 2016 by harvesting and aquaculture.

From Fig. 29.2, it appears clear that the raise in algae production is due to aquaculture rather than harvest. The latter remained almost constant since 1950, rising from 0.5 mio tonnes to 1.4 mio tonnes with an average annual growth rate of 1.55%, while aquaculture production grew at an average annual growth rate 10.5%, increasing from 0.0.35 mio tonnes to 32 mio tonnes of algae. Therefore, significant contribution of algae to the blue economy is linked to the development of new production and processing technologies. In this respect, new inland production systems such as open and closed ponds, as well as genetic improvement of algae strands, contributed significantly to increase the productivity and the content concentration of valuable compounds for human and animal nutrition and for green chemicals.

Among the different types of algae, red and brown algae production is the one with the highest growth since 1950 (Fig. 29.3). Currently, red algae represent about 55% of global production and brown algae 41%, while green algae represents only 0.11%.

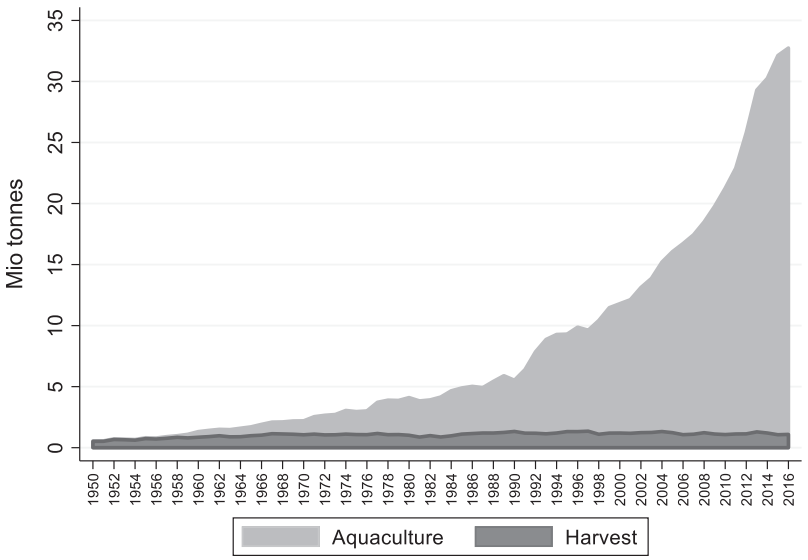

Fig. 29.2
Evolution of algae production 1950–2016 by aquaculture and harvest (author's elaboration on FAO data).

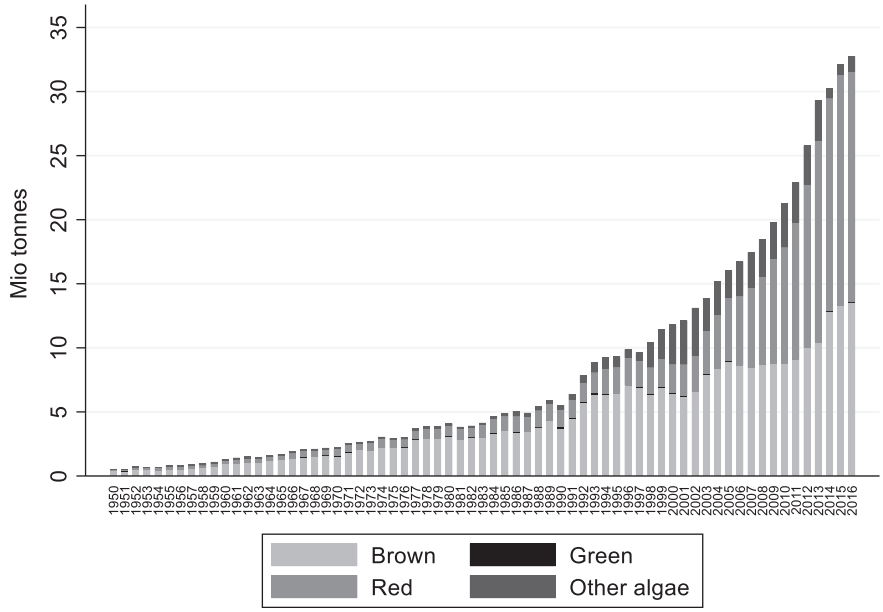

Fig. 29.3
Evolution of production 1950–2016 by type of algae (author's elaboration on FAO data).

The global production of red algae had an impressive boom since 1950, from 0.1 mio tonnes almost exclusively harvested from the seas, to 18 mio tonnes in 2016, 98.9% of it from modern aquaculture.

The main product extracted from red algae (*Rhodophyta*) is probably agar, obtained from the species *Gelidium*, *Gigartina*, and *Gracilaria*. Red algae of the genus *Pyropia* are produced and used in Japan as nori, which is a key ingredient and element of Japanese cuisine. However, from red algae a variety of compounds for pharmaceutics and cosmetic purposes are extracted.

Concerning brown algae, until the end of the 1950s their production was almost exclusively through harvesting from the marine ecosystem and consisted of about 0.4–0.6 mio tonnes. From the 1960s, aquaculture production started and kept growing nutil the most recent years with an average annual growth rate of 11.1%. In 2016, brown algae production from aquaculture reached 12.9 mio tonnes, while brown algae harvesting was still 0.59 mio tonnes.

Commercialized brown algae belongs mainly to the class *Phaeophyceae*, which includes a variety of kelps like the giant kelp (*Macrocystis pyrifera*), bull kelp (*Nereocystis*), Chilean kelp (*Lessonia*, *Durvillaea*, and *Macrocystis*), North European kelp (*Laminaria hyperborean*), as well as mozuku (*Cladosiphon okamuranus*), kombu (*Saccharina japonica*), and wakame (*Undaria pinnatifida*). While mozuku, kombu, and wakame are almost exclusively used as food

for human consumption, other kelps have multiple uses. Kelps are one of the major sources of alginate. From kelp ashes, products such as soap, glass, and sodium carbonate can be obtained. Most recent and innovative uses of kelps are for renewable energy, thanks to its high growth rate and decay which make methane and sugars (for ethanol production) yielding quite efficient, avoiding competition with crops for food security such as corn ethanol and avoiding consumption of fresh water for irrigation.

As explained above, green algae global production quantity is rather small in comparison to red and brown algae. Moreover, global production of green algae is quite volatile, as illustrated in Fig. 29.4. During the 1950s, green algae production was between 0.006 and 0.032 mio tonnes; it peaked in 1998 at 0.1 mio tonnes and in the most recent years has been about 0.03–0.05 mio tonnes. Harvesting from the seas is much more important for green algae than for red and brown algae.

Despite these much lower production volumes, the group of green algae contains species of microalgae particularly important for the production of high-value compounds for nutrition and green chemistry. In particular, *Dunaliella salina*, *Haematococcus pluvialis*, and *Chlorella*, apart from being used dried as valuable food supplements, are also important sources of β-carotene, astaxanthin, cantaxanthin, and lutein. The extraction of these carotenoids from green algae is generally more expensive than the alternative of synthetic synthesis, but the microalgae-based molecules have better chemical conformation with respect to the synthetic isomers that make them preferable for high-value and sensitive products such as infant formula, fish pigment enhancers, and dietary supplements (Mauro Vigani et al., 2015).

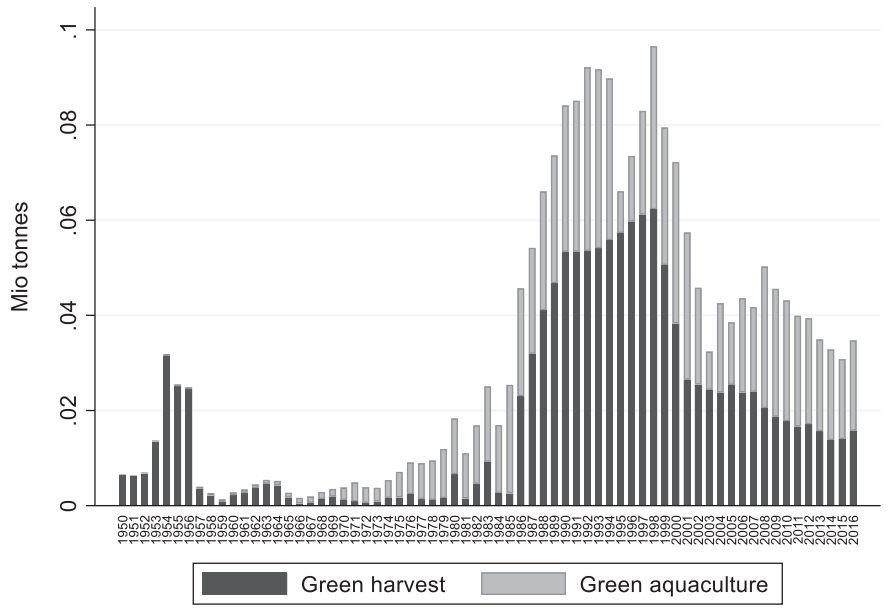

Fig. 29.4
Global production of green algae by harvest and aquaculture (author's elaboration on FAO data).

The fourth group contains a miscellaneous variety of algae, among which two are worth mentioning. The first are algae of the genus *Zostera*, which over the centuries have been used as food and also as material for construction for thatching roofs and as filling for mattresses and cushions, but more recently interest has grown regarding the production of renewable energy. The second are microalgae of the genus *Spirulina*, which are commonly sold and used dried as dietary supplements to improve the content of vitamins and proteins in food; however, they can also be a source for the extraction of the pigment phycocyanin. More recently, *Spirulina* has been used in the cosmetic industry for personal care and antiaging skin products.

Because microalgae are excluded from the main production and trade databases, it is difficult to identify detailed free sources of microalgae data. According to recent figures from the European Algae Biomass Association, the global number of companies producing microalgae (some of them among other products) in 2016 was above 2000, with a total biomass production exceeding 40,000 tonnes/year. These companies employ more than 30,000 workers globally and the total turnover of the sector in 2015 exceeded 2.6 billion €/year.

Global production of algae overtime changed not only in terms of quantities, but also in global geographical patterns. The moving from marine harvesting production methods in the 1950s to aquaculture methods in the 1980s and 2000s, as well as changing consumer attitudes toward algae and the new uses of algae for green chemicals and renewable energy, contributed to transform the geography of global algae production. This is evident from both Tables 29.1 and 29.2.

Looking at Table 29.1, it is evident that the 20 main producers of algae significantly changed from 1950, to 1985, and to 2016. Production quantities grew in all countries, but in some much more than in others. As a result, countries like Japan, the USA, France, and the UK keep moving down in the ranking, while others such as Spain, Morocco, and Portugal moved to 28th, 25th, and 29th position, respectively. In contrast, China had a constant average growth since the 1950s of about 9% every year, which makes this country the main global producer since the 1980s. Moreover, it is interesting to note that countries entering the top 20 in 2016 such as Malaysia, Zanzibar, India, and Madagascar all border the Indian Ocean, suggesting an increasing importance of this global region for the production of algae.

Changing geographical patterns of global production occurred also among red, brown, and green algae (Table 29.2). Red algae production was progressively concentrated in fewer Asian countries, of which Indonesia is by far the main producer, with an average annual growth rate of about 24.6% since 1985.

However, it should be noted that a few countries are developing the production of red algae, becoming part of the top 10 global producers (Malaysia, Zanzibar, Madagascar, and Peru). Brown algae global production is dominated by China, which produces about 84% of the total global production of brown algae. In contrast, countries that were important producers of brown algae until the mid-1980s, such as the USA and Russia, have progressively reduced their volumes of production, suggesting a process of global delocalization.

Table 29.1: Top 20 producing countries in 1950, 1985, and 2016 (thousands of tonnes of all algae types, author's elaboration on FAO data).

N.	1950		1985			2016		
1	Japan	145.40	China	1907.12	↑	China	16,700.00	=
2	USSR	142.59	North Korea	721.00	↑	Indonesia	11,100.00	↑
3	USA	101.58	Japan	707.48	↓	Philippines	1404.78	↑
4	France	35.00	South Korea	443.84	↑	South Korea	1360.76	=
5	UK	30.00	Philippines	184.41	↑	North Korea	553.00	↓
6	Spain	16.00	Chile	182.41	↑	Japan	470.91	↓
7	South Korea	16.00	Norway	133.32	↑	Chile	344.57	↓
8	Morocco	10.30	USSR	122.16	↓	Malaysia	205.99	+
9	Portugal	10.00	Indonesia	121.19	↑	Norway	169.47	↓
10	Iceland	10.00	USA	81.24	↓	Zanzibar	111.14	+
11	Mexico	8.00	France	70.74	↓	France	55.54	=
12	South Africa	8.00	Mexico	39.64	↓	Ireland	29.55	↑
13	Norway	5.30	Ireland	30.20	+	India	22.58	+
14	Canada	5.14	Canada	27.42	=	Madagascar	18.22	+
15	Chile	5.00	South Africa	22.10	↓	Iceland	17.99	↑
16	Indonesia	1.00	Iceland	15.15	+	Russia	15.21	+
17	Italy	1.00	Australia	13.63	+	Peru	14.83	+
18	Argentina	1.00	Argentina	12.91	=	Mexico	13.14	↓
19	North Korea	0.62	UK	12.76	↓	Canada	12.73	↓
20	Philippines	0.59	Taiwan	11.54	+	South Africa	12.38	↓

Table 29.2: Top 10 producing countries of red, brown, and green algae in 1950, 1985, and 2016 (thousands of tonnes, author's elaboration on FAO data).

N.	1950		1985			2016		
				Red				
1	Japan	30.00	Japan	361.81	=	Indonesia	11,100.00	↑
2	USSR	17.08	Philippines	173.13	+	China	4230.29	↑
3	Spain	15.00	Chile	146.00	↑	Philippines	1404.20	↓
4	Morocco	10.00	Indonesia	121.19	↑	South Korea	409.77	↑
5	Portugal	10.00	South Korea	114.78	↑	Japan	300.68	↓
6	Canada	5.10	China	102.06	+	Malaysia	205.99	+
7	Chile	5.00	USSR	25.53	↓	Zanzibar	111.14	+
8	South Korea	1.00	Canada	22.61	↓	Chile	99.09	↓
9	Argentina	1.00	North Korea	21.00	+	Madagascar	18.22	+
10	Italy	1.00	Argentina	12.61	↓	Peru	14.82	+
	Indonesia	1.00						
	South Africa	1.00						
				Brown				
1	USSR	124.20	China	1715.95	+	China	11,400.00	=
2	USA	100.60	North Korea	700.00	+	South Korea	931.09	↑
3	Japan	80.00	Japan	306.21	=	North Korea	550.00	↓
4	France	30.00	South Korea	299.93	+	Chile	245.43	↑
5	UK	30.00	Norway	133.32	↑	Norway	132.96	=
6	Iceland	10.00	USA	81.24	↓	Japan	132.84	↓
7	Mexico	8.00	France	67.57	↓	France	55.04	=
8	South Africa	7.00	Chile	36.03	+	Ireland	29.45	+
9	Norway	5.30	USSR	33.78	↓	Iceland	17.99	+
10	South Korea	4.00	Mexico	31.38	↓	USA	0.01	↓
				Green				
1	Japan	5.40	South Korea	12.91	↑	South Korea	15.88	=
2	South Korea	1.00	Philippines	11.28	↑	India	12.35	+
3	Philippines	0.19	Japan	1.04	↓	China	3.74	+
4			Fiji	0.15	+	New Zealand	1.07	+
5			Argentina	0.01	+	Italy	0.80	+
6						Philippines	0.59	↓
7						Fiji	0.14	↓
8						Taiwan	0.11	+
9						Spain	0.03	+
10						Chile	0.01	+

Regarding green algae, it is worth noting how their production has become increasingly diversified. In 1950, only three countries were producing green algae (Japan, South Korea, and the Philippines), becoming five in 1985 with the addition of Fiji and Argentina, and in 2016 a total of 13 countries were producing green algae, of which only the top 10 are displayed in Table 29.2. The fact that countries like Italy, Fiji, Taiwan, and Spain, which are not competitive producers of red and brown algae, are (relatively) large producers of green algae suggests that green algae production requires a certain degree of specialization, probably due to the niche nature of its market.

Since 1976, international trade in algae and their products significantly increased, in terms of both export and imports. Fig. 29.5 shows global export and import trends in the period 1976–2016 and predicted fitted values with 95% confidence interval (CI) using simple ordinary least squares (OLS) regression. As one can see, both imports and exports increased steadily inthe 40-year period. According to the regressions' coefficients, the value of the global algaeexports increased by 25 mio US $ each year of the period, while imports increased by 15.5 mio US $ each year. In the period 1990–2008, annual growth rate of global algae exports wasabout 4.402%, which, according to World Trade Organization data (World Trade Organization, 2009), is about the same growth rate of the global exports of main food commodities in the same period (4.446%).

In 2016, the main exporting countries of algae and their products were South Korea (200.8 mio US $), Chile (146.9 mio US $), China (144.1 mio US $), and Indonesia (132.9 mio US $), which are all among the top 10 producers (see Table 29.2). In the 1970s, the main exporter was France, followed by a few other European countries (Portugal, Germany, the UK, Ireland, Italy, and the Netherlands). Therefore, trade data also confirm a structural change in the global geographical patterns of algae production. Currently the main importers of algae and their products are Asian countries (Japan, China, Thailand, and Taiwan) and the USA. While Asian countries have traditionally been importers of algae and their products, it is worth noting that in 1976, the USA was importing barely any algae.

The growth in global exports since 1976 was driven mainly by red and brown algae, with an average annual growth rate of about 4 mio US $ and 3.6 mio US $, respectively. In the most recent years, the main exporters of both red and brown algae were, once again, South Korea, China, and Taiwan. Exports of green algae currently account for about 0.13% and they mostly come from South Korea.

In contrast, global imports are driven by brown algae with an average annual growth rate of about 3.9 mio US $ since 1976, while global imports of red algae remain almost stable. The main importers of brown algae are Japan, Taiwan, South Korea, and China, suggesting a strong intra-industry trade.

In terms of comparative advantage, this has been calculated in Table 29.3 using the Revealed Comparative Advantage indicator developed by Balassa (1965).

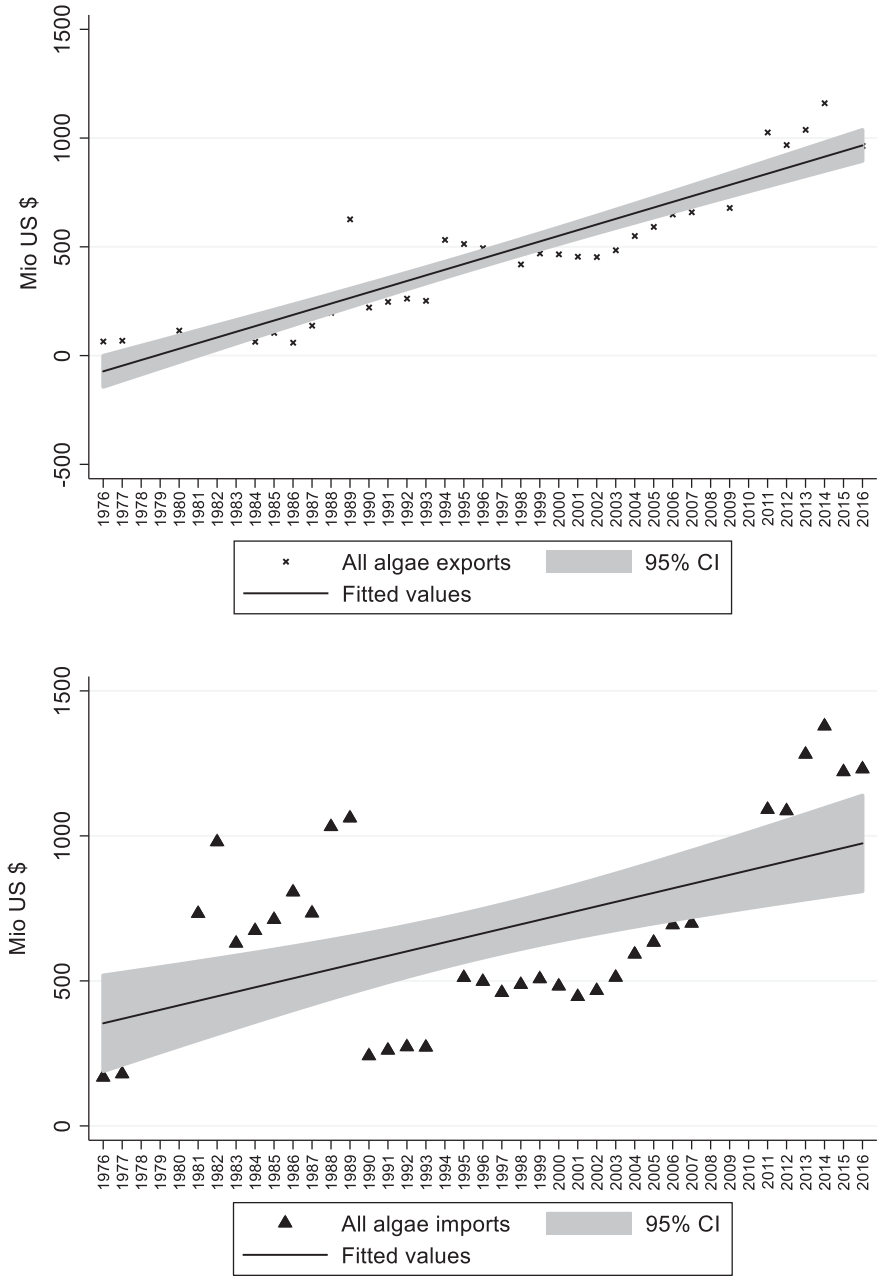

Fig. 29.5
Global export and import trends 1976–2016 of all algae types (author's elaboration on FAO data).

Table 29.3: Revealed comparative advantage in 1976, 1995, and 2016 (author's elaboration on FAO data).

N.	1976		1995			2016		
All algae								
1	France	31.43	South Korea	8.42	+	Cayman	148.04	+
2	Portugal	8.42	Philippines	7.29	+	Saint Lucia	56.94	+
3	Japan	1.78	Chile	3.76	+	Israel	24.30	+
4	Taiwan	0.97	Japan	3.63	↓	South Korea	15.90	↓
5	Ireland	0.69	Kiribati	3.35	+	Turkmenistan	13.46	+
6	Germany	0.35	Ireland	3.13	↓	Niger	11.36	+
7	UK	0.26	China	3.02	+	Brunei	8.43	+
8	Italy	0.23	Switzerland	2.63	+	Ireland	5.51	↓
9			Morocco	2.43	+	Tanzania	5.42	+
10			Italy	1.58	↓	Indonesia	4.91	↑
11			Portugal	1.44	↓	Switzerland	4.73	↓
12			Taiwan	1.32	↓	Chile	4.11	↓
13			Spain	1.22	+	Kuwait	3.67	+
14			Madagascar	1.18	+	Austria	3.01	+
15			Indonesia	1.15	+	Italy	2.97	↓
16			Singapore	0.70	+	Morocco	2.52	↓
17			USA	0.60	+	Laos	2.03	+
18			Germany	0.50	↓	Lebanon	1.82	+
19			Czechia	0.46	+	Philippines	1.72	↓
20			France	0.44	↓	Spain	1.62	↓
Red								
1	Japan	6.76	Japan	9.97	=	South Korea	2.898	↑
2			South Korea	1.73	+	Indonesia	1.68	+
3						China	1.094	+
4						Taiwan	0.239	+
Brown								
1			South Korea	3.63		South Korea	3.622	=
2						China	1.625	+
3						Taiwan	0.436	+
4						New Zealand	0.018	+
Green								
1			South Korea	3.63		South Korea	4.802	=

According to Balassa's indicator, a given country has a comparative advantage (RCA > 1) in a given product when the product's share in national exports is higher than the product's share in the world exports. In contrast, if the RCA < 1, the country reveals a comparative disadvantage in that product. Patterns of comparative advantage changed from 1976 to 1995 and then to 2016. At the beginning of the period, only three countries had a comparative advantage in algae, two of which were European: France and Portugal. In the 1990s, the highest comparative advantages are found in the traditional Asian countries producing and consuming algae and their products. The comparative advantage of the two European countries decreased, and France moved to a comparative disadvantage. In the most recent years, strong changes in the comparative advantages occurred. The fact that countries such as the Cayman and Saint Lucia islands currently have the highest comparative advantage is due to the fact that their exports of fisheries and aquaculture products were almost exclusively composed of algae, but quantities are extremely low. However, it is interesting to note that countries such as Israel currently have a relatively large comparative advantage. This is most probably due to changes in production technologies developed by Israel and the fact that modern inland open ponds are particularly used in this country. South Korea has a comparative advantage in producing all algae type and in particular of red algae.

It should also be noted that levels of comparative advantage can be influenced by specific government policies and interventions that can affect trade flows. However, governments tend to support directly the less competitive sectors rather than the competitive ones, therefore the figures in Table 29.3 are likely to reflect the real comparative advantage based on the sectors' competitiveness rather than on policy support. For example, the most developed and integrated policy supporting the bioeconomy and circular economy is currently in the EU, but few EU countries show a comparative advantage in 2016, namely Ireland, Italy, and Spain.

29.6 Conclusions

Global algal production has significantly increased in the last 50 years, and it changed in production methods and geographical patterns with a progressive concentration from Western to Asian countries. This suggests that algae are playing an increasing role in achieving the Agenda 2030 SDGs in some parts of the globe, and that significant contribution of algae to the bioeconomy is linked to the development of new production and processing technologies, passing from harvesting to aquaculture.

Despite the progress achieved by the sector, various important challenges and constraints need to be addressed in order to transform the current potentialities into actual products and processes for the bioeconomy.

The first challenge is to develop an accurate market information system that provides more detailed and reliable data on algae supply and demand, and on biomass types and sources. More

accurate national statistics would support the development and implementation of useful policies for algal bioeconomy, and would improve consumer awareness and understanding of algae products. In order to improve the availability of data on microalgae, collaboration and support from the algae industry sector are essential.

A multitude of companies are interested in using algal biomass, but they are held back by the high price of algae and by the shortage of supply due to limited current production capacity. Therefore, a second important challenge consists of progressing in technological innovation at different levels of the supply chain to produce sufficient algal biomass feedstocks and products at reduced production costs to increase competitiveness. Innovation and technologies are necessary to identify new high-yielding algal species or strains, to improve the methods for storage of surplus algal biomass, and to increase the efficiency of biorefineries. Moreover, the knowledge transfer between research and industry should be strengthened.

Finally, it is important to develop policy frameworks supporting the development of supply chains and marketing of new products through consistent bioeconomy policies. Such policies should consider the sustainable exploitation of algae resources, taking into account the pressure on the marine environment and ecosystem.

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CHAPTER 30

Life cycle assessment of microalgae-based processes and products

Lucas Reijnders

IBED, University of Amsterdam, Amsterdam, The Netherlands

Chapter outline

30.1 Introduction 823

30.2 What is life cycle assessment (LCA) and in which respects can LCA be useful? 823

30.3 Application of peer-reviewed life cycle assessment to microalgae-based products and processes 827

30.3.1 Biofuels 828

30.3.2 Applications in the food and feed sector 833

30.3.3 Other applications 835

30.4 Conclusions 836

References 836

30.1 Introduction

Life cycle assessment (LCA), also called life cycle impact assessment or life cycle environmental impact assessment, is widely used to assess the environmental impacts, or environmental burden, of products from cradle to grave. It can be applied to current and future products and processes. Section 30.2 of this chapter deals with the questions: what is life cycle assessment (LCA) and in which respects can LCA be useful? Section 30.3 considers the application of peer-reviewed life cycle assessment to microalgae-based processes and products, including processes and products based on cyanobacteria. Section 30.4 presents the conclusions of this chapter.

30.2 What is life cycle assessment (LCA) and in which respects can LCA be useful?

Environmental life cycle assessment (LCA) has been developed to establish environmental impacts of products from cradle to grave. Full life cycle assessments are divided into four stages:

- goal and scope definition;
- inventory analysis;

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- impact assessment; and
- interpretation.

In the goal and scope definition stage, the object, aim, and scope of life cycle assessment are determined. The product is commonly defined in terms of a functional unit (e.g., 1 kg of n-3 fatty acids or 1 MJ of microalgal biodiesel). In addition, in this stage system boundaries are defined. The full life cycle may be considered, but also parts thereof, e.g., cradle to factory gate, or, in case of biofuels, cradle to pump. As to the determination of aim, it might, for instance, be established that the LCA should be comparative; e.g., comparing fish-based and microalgal n-3 fatty acids. Life cycle assessment may also serve to identify parts of the life cycle with relatively large environmental impacts, which in turn may be the basis for efforts aimed at the reduction of such impacts by applying alternative technologies. For instance, LCA methodology has been applied to the process step of harvesting of microalgae for the production of bio-oil or n-3 fatty acids. Harvesting by conventional centrifugation is very energy-intensive (Mu et al., 2017; Barr and Landis, 2018). In view thereof, e.g., harvesting by membrane filtration has been suggested as an alternative to conventional centrifugation (Barr and Landis, 2018).

In the case of full microalgal product life cycles, a variety of matters regarding system boundaries should be decided. It should, for instance, be established whether the production of water for microalgal production and wastewater treatment associated with the production of microalgal products should be included or excluded. As to road transport involved in such life cycles, it should be decided whether only energy use for transport and emissions should be included or also, and to what extent, road building and the production of transport vehicles. Differences in system boundaries may lead to differences in outcomes of LCAs.

Related to the establishment of system boundaries is the valuation of avoided processes, inputs, and outputs, including nonproduct outputs such as emissions and "wastes." Such valuations should be carefully considered. For instance, in a life cycle assessment of microalgal biofuel, Handler et al. (2013) used a scenario in which the effluent from a wastewater treatment plant was used as an input. This was given a credit as the need for wastewater treatment was considered as an avoided process. However, microalgal biofuel production systems are currently not as good as state-of-the-art wastewater treatment facilities in Western industrialized countries (Reijnders, 2019). In addition, (co-)products, such as microalgal meal, may be negatively impacted by wastewater-based microalgae cultivation systems (e.g., Hess and Quinn, 2018). Or, to take another example, in several LCAs, flue gas containing CO_2 derived from fossil fuel combustion is assumed to be an input in the cultivation of microalgae (Reijnders, 2017). This input can be valued at zero (because flue gas CO_2 can be considered to be a waste), as a negative emission (as CO_2 is sequestered by algae), or as a positive emission

(as such CO_2 is apparently valuable) (Reijnders, 2017). The emission of the greenhouse gas CO_2 originating in the combustion of biofuels and biofuel production residues is furthermore often valued at zero, because it is assumed that this emission will be sequestered by the generation of new biomass. However, in practice the generation of new biomass in part serves the sequestration of CO_2 emitted by the combustion of fossil fuels. Moreover, there is no guarantee that the sequestration in biomass of CO_2 emitted by the combustion of biofuels will be indefinite (Reijnders, 2019). Differences in assumptions about the valuation of avoided processes, inputs, and outputs can lead to differences in the outcomes of life cycle assessments. Also, in the goal and scope definition stage, the matter of allocation is to be considered for processes that have more than one output. For instance, in biodiesel production from microalgae, biodiesel, glycerol, and microalgal meal may be marketable cooutputs, whereas there are also production residues. How should the environmental burden be distributed over, or allocated to, these outputs? Allocation can be done on the basis of monetary units (e.g., prices), on the basis of physical units (e.g., mass, energy content), or on the basis of substitution. In the latter case, the environmental impacts of a similar output are used. There is no agreement about the best way to allocate environmental impacts to outputs. Different ways to allocate the environmental burden can lead to different outcomes of life cycle assessments (e.g., Reijnders, 2017).

In the inventory analysis stage, the necessary data for all environmentally relevant avoided processes, processes, inputs, and outputs, including nonproduct outputs, involved in the product life cycle are gathered and a set of quantified environmental interventions (see Box 30.1) per functional unit (e.g., x kg phosphate emitted into fresh surface water per kg n-3 fatty acids) is generated.

In the impact assessment stage, data gathered at the inventory stage are "converted" into environmental impacts. This conversion includes characterization: the calculation of impact category indicator results (e.g., Hauschild et al., 2013). A range of environmental impacts may be considered in impact assessment (see Box 30.1).

In the characterization step of impact assessment, there is aggregation on the basis of a common unit of an impact category indicator. For instance, regarding impacts on climate, emissions of all greenhouse gases (CO_2, CH_4, N_2O, and a number of halogenated hydrocarbons) should be included (Reijnders, 2015, 2017). Furthermore, land use and land use change affecting ecosystem carbon stocks, which impact greenhouse gas concentrations in the atmosphere, should be included. Changes in such carbon stocks can be associated with the establishment of microalgae-based production facilities or the cultivation of crops supplying inputs to heterotrophic microalgal processes (Reijnders, 2015; Arita et al., 2016). The emission of all greenhouse gases and the effect of changing ecosystem carbon stocks are aggregated on the basis of CO_2 equivalents—the common unit of the impact category

BOX 30.1 Interventions potentially leading to environmental impacts that may be considered in life cycle assessment of microalgae-based production (Reijnders, 2015; Colotta et al., 2016).

Interventions potentially leading to environmental impacts that may be considered in life cycle assessment

Abiotic resource consumption leading to resource depletion (e.g., due to consumption of fossil fuels and freshwater)
Land use (change) affecting ecosystems and carbon stocks
Water consumption affecting ecosystems
Greenhouse gas emissions that affect climate (e.g., linked to combustion and the conversion of N-compounds into N_2O)
Other interventions that may affect climate (e.g., due to change in albedo or evaporation of water)
Emissions leading to acidification of surface water and/or soils (e.g., related to transport and N-inputs)
Nutrient emissions leading to eutrophication (e.g., related to emissions of microalgae-based production into surface water)
Emissions leading to photo-oxidant formation (e.g., related to transport)
Emissions that are hazardous to human health (e.g., heavy metal emissions related to inputs of wastewater or the use of synthetic phosphates)
Emissions that may lead to ecotoxicity (e.g., related to inputs of waste water or the use of synthetic phosphates)
Emissions that may lead to nuisance (e.g., of noise, odor) (e.g., related to transport or waste water inputs)
Emissions of waste heat that may affect ecosystems (e.g., related to generation needed for production)

indicator (Reijnders, 2015, 2017). CO_2 equivalents are established assuming a specific timeframe for the impact, e.g., 100 years (Hauschild et al., 2013). There are, furthermore, characterizations that aggregate many of the environmental impacts covered by Box 30.1, leading to estimates of overall single scores for environmental impacts (cf. e.g., Smetana et al., 2015, 2017; Taelman et al., 2015).

In LCA practice, only one or a few of the interventions indicated in Box 30.1 may be considered, for instance, greenhouse gas emissions, fossil fuel consumption, and/or nutrient emissions. Life cycle assessment also may serve the calculation of the energetic return on energy investment in biofuels or EROI. The EROI is the quotient between the energy generated by combustion of biofuels and the primary energy input in the biofuel life cycle, or a part thereof, such as cradle to factory gate. The primary energy input in the biofuel life cycle should include the embodied energy (cumulative cradle-to-cultivation-facility energy consumption) of material inputs and of biofuel production facilities. In practice, LCAs regarding microalgal

fuels often estimate EROIs. It has been argued that the cradle-to-factory-gate EROI should be 5–8 to qualify microalgal fuels for large-scale production and usage (Chisti, 2013; Reijnders, 2018).

For many of the interventions presented in Box 30.1, the actual environmental impact is specific to location and the temporal character of the intervention. Conventional life cycle assessments have no time and place specificity for interventions; however, life cycle impact assessment can be adapted to include place and time specificity by combination with other tools (e.g., Hellweg et al., 2005; Rehr et al., 2010).

In the interpretation stage, the validity of the outcomes of previous stages is evaluated. Uncertainty analyses considering uncertainties in data and modeling belong to this stage. So does sensitivity analysis regarding the choices made in establishing system boundaries, valuation of avoided processes, inputs and outputs, and allocation methodology. Also in this stage, conclusions may be drawn, e.g., as to priorities for reducing the environmental burden of products.

In practice, not all life cycle assessments fully comprise all four stages. There are many examples of life cycle assessments without uncertainty and sensitivity analyses.

30.3 Application of peer-reviewed life cycle assessment to microalgae-based products and processes

Arthrospira platensis, *Chlorella vulgaris*, *Dunaliella salina*, *Isochrysis galbana*, *Nostoc sphaeroides*, *Spirulina maxima*, and *Spirulina platensis* are commercially produced for application in feed and/or food (Chen et al., 2019). *C. vulgaris* is also commercially cultivated for the production of the food ingredients astaxanthin, beta-carotene, canthaxanthin, and chlorophyll, and *D. salina* for the production of beta-carotene (Chen et al., 2019). *Haematococcus pluvialis* is used to produce astaxanthin and canthaxanthin (Chen et al., 2019). *Cryptothecondium cohnii*, *Schizochytrium*, *Thraustochytrium*, and *Ulkenia* are commercially cultivated for the production of the food ingredient docosahexaenoic acid (Chen et al., 2019).

Many other products derived from microalgae, including cyanobacteria, have been proposed, including proteins, lipids, n-3 and n-6 fatty acids, sterols, microalgal oil, hydrocarbons, vitamins, polysaccharides, phycobiliproteins, zeaxanthin, lutein, phycocyanin, phycoerythrin, antioxidants, extracts and oil for application in cosmetics, and a variety of biofuels (such as biodiesel, ethanol, methanol, bio-crude, biogas, and hydrogen) (Chew et al., 2017; Kothari et al., 2017; Papadaki et al., 2017; Smetana et al., 2017; Soni et al., 2017; Enamala et al., 2018; Hu et al., 2018; Kahn et al., 2018; Chaudry et al., 2019; Chen et al., 2019). Cyanobacteria have been proposed as a platform for the production of a variety of chemicals, including mannitol,

erythrol, 1,3-butanediol, acetone, isobutyraldehyde, isobutanol, butanol, ethylene, 2-methyl-butanol, terpenes such as isoprene and limonene, and for the production of polymers such as poly 3-hydroxybutyrate (Khanra et al., 2018; Van den Berg et al., 2019; Chen et al., 2019). Several microalgae, including cyanobacteria, have been proposed as fertilizers (Chen et al., 2019). Microalgae-based processes have been suggested for wastewater treatment (e.g., Maga, 2017; Quiroz-Arita et al., 2019). Only a part of these microalgae-based products and processes have been made the object of LCA studies. Available life cycle assessments will be discussed next.

30.3.1 Biofuels

In practice, a major focus has been on the life cycle assessment of microalgal biofuels. In those assessments, often a limited number of the interventions outlined in Section 30.2 are considered. Fossil fuel consumption, EROI (energetic return on energy investment), and greenhouse gas emissions are often considered, other interventions presented in Box 30.1 much less (Colotta et al., 2016). The studies in this field mainly regard biodiesel (methanol- or ethanol esters of microalgal oil-based fatty acids) derived from freshwater autotrophic microalgae produced in a variety of ways, including biorefinery-based production. These studies have been reviewed by Slade and Bauen (2013), Reijnders (2017), Tu et al. (2017), Ketzer et al. (2018), and Reijnders (2018). Biodiesel is obtained by extracting oil (lipids) from dried autotrophic microalgae followed by transesterification with methanol or ethanol. Chang et al. (2015), Orfield et al. (2015), and Siqueira et al. (2018) assessed biodiesel from microalgae heterotrophically cultivated in fermenters. Jet fuel derived from autotrophic microalgae has also been the object of LCA studies. Other fuels that have been considered in LCA studies are biogas (containing methane), obtained by anaerobic conversion of autotrophic microalgae, hydrogen produced in a photobioreactor, hydrocarbons generated by *Botryococcus braunii*, biocrude (obtained by thermochemical treatment of autotrophic microalgae), and fuels derived from biocrude. From these studies, the following conclusions can be drawn:

- None of the LCA studies dealing with microalgal fuels regards commercial production. This, in combination with variable assumptions as to system boundaries, allocation, and the valuation of avoided processes, inputs and outputs, and about what commercial production would look like, leads to widely diverging results (Slade and Bauen, 2013; Tu et al., 2017; Reijnders, 2017, 2018).
- Estimated cradle-to-factory-gate EROIs are below the factor 5–8 that, as pointed out in Section 30.2, should be met to qualify for large-scale application.
- Adequate uncertainty and sensitivity analyses of LCA outcomes are often lacking.

30.3.1.1 Biodiesel

The cultivation stage of autotrophic microalgae (e.g., Ketzer et al., 2018; Togarcheti et al., 2017) and the combined stages of harvesting, drying, and extraction of such microalgae (e.g., De Boer et al., 2012; Tu et al., 2017) have emerged as important determinants of life cycle environmental burdens of biodiesel production. In view of the former, energy-efficient open raceway ponds have been preferred over bioreactors (e.g., Ketzer et al., 2018). In view of the latter, e.g., the use of membrane filtration, flocculation, ultrasonic technology, and (belt) filter presses (instead of centrifugation for harvesting), solar drying and ultrasonic extraction have been proposed as ways to reduce environmental impacts (e.g., Udom et al., 2013; Soni et al., 2017; Barr and Landis, 2018; Tu et al., 2018; Shi et al., 2019). A study using a relatively wide range of the interventions outlined in Table 1, regarding the operation of a pilot-scale raceway pond and two photobioreactors for the cultivation of autotrophic microalgae in the Netherlands, concluded that energy consumption for temperature control was the main contributor to the overall environmental burden of operating microalgae cultivation facilities (Pérez-López et al., 2017). Choosing appropriate weather conditions (e.g., relatively warm temperatures and low rainfall for raceway ponds) may limit the environmental burden of cultivation (Pérez-López et al., 2017).

Greenhouse gas emissions for autotrophic microalgal biodiesel life cycles and cradle-to-factory-gate and cradle-to-pump parts of such life cycles estimated in peer-reviewed studies range from −95.7 g CO_{2eq} to +534 g CO_{2eq} MJ^{-1} biodiesel, whereas the cradle-to-grave greenhouse gas emission for conventional fossil diesel is estimated to be 79–96 g CO_{2eq} MJ^{-1} (Reijnders, 2017; Tu et al., 2017; Wu et al., 2018). A negative greenhouse gas emission for microalgal diesel is linked to the use of energy-credits and regards studies in which carbon originating in fossil fuel-derived CO_2 is still sequestrated in the biofuel. During the full life cycle, such carbon will be released. Most full peer-reviewed LCA studies suggest that the (full) life cycle greenhouse gas emission of freshwater autotrophic microalgae-derived biodiesel will exceed the life cycle greenhouse gas emission of fossil biodiesel (Tu et al., 2017). Whether microalgal diesel in commercial practice will have higher or lower life cycle greenhouse gas emissions than fossil diesel is as yet uncertain.

Chang et al. (2015) estimated a similar life cycle CO_2 emission for fossil diesel and biodiesel derived from heterotrophically cultivated thraustochytrids. Orfield et al. (2015) suggested a lower life cycle CO_2 emission for heterotrophic microalgal diesel than for fossil diesel. Siqueira et al. (2018) estimated a lower CO_2 emission for biodiesel derived from heterotrophically grown *Phormidium autumnale* than for fossil biodiesel. However, in all three studies, the large CO_2 emission linked to heterotrophic growth (Yang et al., 2000; Lowry et al., 2015) and the changes in ecosystem carbon stocks linked to the production of agriculture-based carbon sources used in heterotrophic cultivation were neglected.

Published EROIs for biodiesel derived from freshwater autotrophic microalgae range from <1 to <4.1. EROIs for biodiesel from microalgae grown in raceway ponds are often higher than EROIs for biodiesel from microalgae grown in bioreactors, with hybrid production systems using both bioreactors and raceway ponds (e.g., Adesanya et al., 2014) having an intermediary position (Chisti, 2013; Ketzer et al., 2018; Reijnders, 2018). Estimated EROIs for biodiesel derived from autotrophic microalgae grown in bioreactors tend to be <1 (Ruiz et al., 2016; Ketzer et al., 2018; Reijnders, 2018).

Chang et al. (2015) estimated EROIs for biodiesel derived from heterotrophically cultivated thraustochytrids ranging from 0.5 to 1.35, dependent on the carbon source used. The EROIs for heterotrophic microalgal biodiesel estimated by Orfield et al. (2015) varied from 0.6 to 1.6 and did not include the embodied energy of nonenergy inputs. The EROIs regarding various scenarios for producing biodiesel with sucrose as carbon source by *Phormidium autumnale*, in which the embodied energy in nonenergy inputs such as sucrose, water, and nutrients was not included, were estimated at <2 (Siqueira et al., 2018).

Estimates of the life cycle consumption of freshwater for microalgal biodiesel vary, but suggest a life cycle fresh water consumption for microalgal biodiesel that is larger than the life cycle freshwater consumption of fossil diesel (Batan et al., 2013; Orfield et al., 2015). The actual life cycle consumption for future commercial production of biodiesel based on freshwater microalgae is uncertain and strongly dependent on the recycling rate of freshwater used in biodiesel production (Reijnders, 2019). The life cycle emission of N- and P compounds to water is likely to be larger for microalgal biodiesel than for fossil diesel (Reijnders, 2019). When agriculture-based carbon sources are applied, land use associated with heterotrophic cultivation of microalgae is likely to be larger than for autotrophic cultivation (Orfield et al., 2015).

30.3.1.2 Hydrocarbons generated by B. braunii

Chaudry et al. (2019) published a cradle-to-hydrocarbon-refinery LCA for extracellular hydrocarbons generated by *B. braunii* in a process that does not destruct (dewatered) *B. braunii* cells. There is no commercial application of this technology, so assumptions were made as to what a commercial process would look like. With such assumptions, Chaudry et al. (2019) estimated an EROI of 1.04. In view of credits applied for carbon sequestration and product displacement, a negative greenhouse gas emission was estimated for cradle-to-hydrocarbon-refinery hydrocarbons (Chaudry et al., 2019). Taking account of the emission of greenhouse gases on combustion and the energy input needed to refine *B. braunii* hydrocarbons, the full life cycle greenhouse gas emission can be estimated to exceed the corresponding full life cycle greenhouse gas emission for fossil diesel.

30.3.1.3 Microalgal fuels obtained by hydrothermal liquefaction

The energy input in the part of the life cycle including harvesting, drying, and oil extraction is an important determinant of the life cycle environmental burden of biodiesel derived from autotrophically grown microalgae (De Boer et al., 2012; Tu et al., 2017). For this reason, thermochemical treatments of such microalgae, especially hydrothermal liquefaction technology, have been suggested as a basis for microalgal biofuel (biocrude) production (e.g., De Boer et al., 2012; Patel et al., 2016a; Mu et al., 2017; Mathimani and Mallick, 2019). In hydrothermal liquefaction, all kinds of organic substances in microalgae serve biofuel production, not only the lipid fraction (Biller and Ross, 2011).

Hydrothermal liquefaction generates, apart from biocrude, mixtures of gases, watery solutions, and (semi-)solids (char, tar) (Barreiro et al., 2013; Mathimani and Mallick, 2019). To be suitable as a liquid fuel (e.g., for cars) biocrude needs refining (Barreiro et al., 2013; Mathimani and Mallick, 2019). It has been suggested to refine biocrude in refineries for fossil crude oil. A problem with this suggestion is the high N content of biocrude (usually 5%–7%) (Barreiro et al., 2013), which is at variance with fossil crude oil. When the N content is not sharply reduced in refining, the fuel output of refining will give rise to high NO_x emissions (Barreiro et al., 2013).

Preferentially, the cooutputs of biocrude have to be (or be converted into) useful products. The watery output from hydrothermal liquefaction, which contains plant nutrients, may be recycled to microalgal production facilities (Barreiro et al., 2013; Patel et al., 2016b). As the watery output of hydrothermal liquefaction also contains high concentrations of organic substances, it may serve the production of heterotrophic microalgae (Barreiro et al., 2013). The gaseous output of hydrothermal liquefaction contains substantial amounts of CO_2, H_2, and low-molecular weight hydrocarbons (Barreiro et al., 2013). Applications for energy generation and hydrogenation may be considered. The (semi)solid output of hydrothermal liquefaction may be subjected to further thermochemical treatment, which might leave ashes as a residue.

There is no large-scale commercial application of hydrothermal liquefaction technology to microalgae, and the optimal conditions for commercial application of this technology remain to be established (Patel et al., 2018; Mathimani and Mallick, 2019). There may furthermore be a case for (energy-intensive) treatment before hydrothermal liquefaction (e.g., by flash hydrolysis, mechanical cell disruption, microwave treatment, or pulsed electric field) (Barreiro et al., 2013; Biller et al., 2013; Bessette et al., 2018).

Whether a commercial hydrothermal liquefaction process for microalgae will emerge is uncertain. The substantial development effort directed at hydrothermal liquefaction of terrestrial biomass has failed to generate a commercial process (e.g., Barreiro et al., 2013). Available LCAs for hydrothermal liquefaction of microalgae cast doubt on the assumption that hydrothermal liquefaction leads to a reduced energy input in biofuel production, if compared with

biodiesel production (Liu et al., 2013; Connelly et al., 2015). Thus, it cannot be established with certainty whether commercial hydrothermal liquefaction technology can indeed reduce the life cycle greenhouse gas emissions of freshwater microalgal fuel, if compared with commercial freshwater microalgal diesel production.

Liu et al. estimated that the pilot-scale EROI for autotrophic microalgae-derived gasoline produced using hydrothermal liquefaction is about 1, and might increase to 2.5–3 on further development. This is well below the value of 5–8 for the cradle-to-factory-gate EROI suggested as a criterion for large-scale application (Chisti, 2013; Reijnders, 2018).

30.3.1.4 Microalgal fuels produced by other high-temperature thermochemical processes

The generation of microalgal fuels produced by other high-temperature thermochemical processes has not progressed beyond the laboratory stage (Xu et al., 2011; Khoo et al., 2013; Yang et al., 2019). Xu et al. (2011) and Khoo et al. (2013) have studied thermochemical biofuel production by gasification and pyrolysis of autotrophically grown microalgae or microalgal biomass remaining after lipid extraction. Most of the product in the processes studied by Xu et al. (2011) and Khoo et al. (2013) is char. Xu et al. (2011) and Khoo et al. (2013) suggest EROIs <2 for bio-oil and biogas. They estimated an EROI of 2.27 and a greenhouse gas emission that would be much higher than for fossil fuel. Bio-oil generated by pyrolysis may have a nitrogen content of 5.2%–16.3% and an O/C ratio of 0.23 (Yang et al., 2019), which would necessitate further refining before being used as a liquid fuel in transport, for example.

30.3.1.5 Biojet fuel

Several peer-reviewed LCA studies have dealt with biojet fuel derived from autotrophic microalgae (Augusdinata et al., 2011; Lokesh et al., 2012; Cox et al., 2014; Fortier et al., 2014; Bessette et al., 2018). Lokesh et al. (2012) studied hydrotreatment of microalgal diesel, leading to decarboxylation, decarbonylation, deoxygenation, hydrocracking, and isomerization. Cox et al. (2014) considered hydrocracking of microalgal oil. Augusdinata et al. (2011) studied a process gasifying microalgal lipid and using the Fischer-Tropsch synthesis to generate biojet fuel. Fortier et al. (2014) and Bessette et al. (2018) studied refining of biocrude obtained by hydrothermal liquefaction of autotrophic microalgae to generate biojet fuel. Lokesh et al. (2012) estimated an EROI of 1.135, Cox et al. suggested an EROI of about 0.8–1, dependent on type of allocation, and Bessette et al. (2018) proposed an EROI of 1.05, all much below 5–8, suggested for widespread application of the technology.

All the studies mentioned here suggest a climate benefit of the biojet fuel considered. However, in view of the absence of commercial biojet fuel production and the uncertainties about the climate impacts of biodiesel and biocrude produced by hydrothermal liquefaction outlined above, it is uncertain whether a climate benefit will materialize in commercial biojet fuel production practice.

30.3.1.6 Biogas

A way to circumvent the energy intensive harvesting, drying, and oil extraction stages in biofuel production is the conversion of (wet) microalgae into biogas containing methane by anaerobic conversion (e.g., Milledge et al., 2019). The availability of microalgal organic substances to anaerobic conversion is partial (Milledge and Heaven, 2017; Xiao et al., 2019). Hydrothermal pretreatment, which is energy-intensive, can enhance methane production from microalgal biomass. Following a hydrothermal pretreatment process studied by Xiao et al. (2019), methane production increased by 57%. Anaerobic conversion of microalgae may be combined with the anaerobic conversion of wastewater (Tasca et al., 2019).

Available life cycle assessments of anaerobic conversion of microalgal biomass without pretreatment do not show a clear advantage over autotrophic microalgal biodiesel production. Collet et al. (2011) estimated similar life cycle fossil fuel-derived CO_2 emissions for natural gas and biogas derived from the anaerobic conversion of cultivated autotrophic microalgae. Fugitive emissions of CH_4 and N_2O from the biogas lifecycle were not included in the study of Collet et al. (2011), whereas these emissions might make a substantial contribution to the life cycle greenhouse gas emissions (Frank et al., 2012; Reijnders, 2017). ter Veld (2012) estimated an EROI of 3.2 for autotrophic microalgal biogas production, while not including treatment of wastewater from cultivation and valuing at zero the embodied energy in production facilities and the embodied energy for water and CO_2 used in cultivation. Milledge and Heaven (2017) excluded wastewater treatment and the embodied energy of nonenergy materials and production facilities, and estimated an EROI of 2.4–3.4 for autotrophic microalgal biogas generation. Maga (2017) estimated and EROI of 1.91 for biogas generated from autotrophic microalgae grown in municipal wastewater. Maga (2017) used data from a pilot plant, but the estimated EROI of 1.91 may be overly optimistic as a large energy credit was included for the full substitution of municipal wastewater treatment.

30.3.1.7 Hydrogen

Rosner and Wagner (2012) applied life cycle assessment to H_2 production by *Chlamydomonas reinhardtii* in a photobioreactor, considering energy use, climate change, eutrophication, and acidification. They concluded that the H_2 production rates should be increased by a factor of about 100 to become competitive with H_2 production technologies such as biomass gasification and wind-based and photovoltaic electrolysis.

30.3.2 Applications in the food and feed sector

30.3.2.1 Feed for aquaculture

Taelman et al. (2013) performed an LCA using cumulative exergy extraction from the natural environment (not included in Box 30.1) as intervention. This intervention might be considered indicative for the overall environmental burden (Finnveden et al., 2016).

Taelman et al. (2015) suggested that microalgal biomass-based feed for carnivorous fish might lead to a lower exergy extraction from the natural environment than conventional fish meal.

30.3.2.2 Proteins

The application of microalgal proteins in food and feed has been the object of life cycle assessment using a relatively wide range of the interventions outlined in Box 30.1 (Smetana et al., 2017; Ye et al., 2018). Taelman et al. (2015) studied microalgal protein meal using cumulative exergy extraction from the natural environment as an intervention. From the studies of Smetana et al. (2017) and Ye et al. (2018), it appears that when protein mass is used in the functional unit, microalgal protein is linked with a larger life cycle burden than proteins derived from terrestrial crops such as soybean and maize. Taelman et al. (2015) estimated that soybean meal would be associated with a lower cumulative exergy extraction from the natural environment than microalgal meal. (Potential) differences in nutritional value of the proteins obtained from different sources were not considered in the studies discussed here. Smetana et al. (2017) found higher environmental burdens for protein concentrate from autotrophic and heterotrophic cultivation of cyanobacteria than for protein in pork and beef. Smetana et al. (2017) suggested that a mixture of soybean protein concentrate and protein concentrate derived from heterotrophically cultivated *C. vulgaris* using high moisture extrusion may be associated with a lower environmental burden than protein in pork and beef. Using hydrolyzed food waste was also suggested as a way to reduce the environmental impacts of cyanobacterial protein concentrate (Smetana et al., 2017). The assumption underlying this suggestion is that the environmental burden of food waste can be valued at zero, which is a debatable assumption, as in this context, food waste is a useful input.

30.3.2.3 Cyanobacterial hydrolysate

Laboratory-based meat production using cyanobacterial hydrolysate was subjected to a cradle-to-plate LCA including most of the interventions mentioned in Box 30.1 and compared with chicken meat and terrestrial plant-based meat substitutes (Smetana et al., 2015). Laboratory-based meat production had a larger estimated overall environmental burden than chicken meat and a much larger environmental burden than gluten-based and soybean meal-based meat alternatives. Due to the absence of commercial production, data used by Smetana et al. (2015) for estimating the environmental impacts of laboratory-based meat production were much more uncertain than the data used for chicken meat and terrestrial-plant-based meat substitutes.

30.3.2.4 N-3 fatty acids

Barr and Landis (2018) compared n-3 fatty acids extracted from fish with n-3 fatty acids coextracted from autotrophic microalgae in a biorefinery with protein for application in feed as a coproduct. Four of the interventions outlined in Box 30.1 were considered. The study was based on assumptions about future n-3 fatty acid production from microalgae. It was concluded

that when a low-energy harvesting methodology is to be used, the cradle-to-factory-gate environmental burdens of microalgae-based n-3 fatty acids and fish-based n-3 fatty acids might be similar.

30.3.2.5 Phycocyanin

Papadaki et al. (2017) studied various recovery methods for phycocyanin from the cyanobacterium *Spirulina platensis*, using nine of the interventions outlined in Box 30.1. It was concluded that, per kg phycocyanin, the ultrasound-assisted extraction of dried cyanobacteria in the buffer had the lowest cradle-to-factory-gate environmental impacts.

30.3.3 Other applications

30.3.3.1 Fertilizer

De Souza et al. (2019) compared microalgae as a nitrogen fertilizer (using empirical data from a small pilot plant processing wastewater in Brazil) and the synthetic fertilizer urea. It was suggested that microalgal fertilizer had a better life cycle performance in terms of terrestrial acidification and freshwater ecotoxicity but a much worse performance regarding climate change and freshwater eutrophication. The use of photovoltaic power and a higher N-content of wastewater were suggested as ways to improve the environmental performance of the microalgal fertilizer. It might be argued that there is in this study a negative bias regarding microalgal fertilizer by not considering the presence of P in microalgal fertilizer and the small scale of the pilot plant used for empirical data. On the other hand, there may be a positive bias as to microalgal fertilizer because the equivalence of urea and microalgal fertilizer was not demonstrated by De Souza et al. (2019).

30.3.3.2 Extracted microalgal residue for polylactic acid (PLA) blends

Bussa et al. (2019) compared polylactic acid (PLA) with a polylactic acid blend containing extract from microalgal residue remaining after the production of lipoprotein. The residue was assumed to contain lipids, polysaccharides, and proteins. Equality regarding the functional performance of PLA and PLA blend was not demonstrated. Uncertainty following from the absence of commercial production was pointed out by Bussa et al. (2019). It was concluded that blended PLA might do better than PLA regarding, e.g., land use and terrestrial ecotoxicity but might do worse regarding, e.g., energy use and the contribution to global warming (Bussa et al., 2019).

30.3.3.3 Wastewater treatment

Quiroz-Arita et al. (2019) assessed the integration of cyanobacterial nutrient removal from sludge concentrate in a municipal wastewater treatment plant. A specified nitrogen removal rate was used as a functional unit. Anaerobic codigestion of cyanobacteria and sludge was included in the integrated wastewater treatment process. The fates of the digestate originating in

anaerobic treatment and the substances present therein were not considered in this study. In the absence of data from a pilot-scale or commercial application of this technology, assumptions were made about the modeling of the integration. On the basis of this, Quiroz-Arita et al. (2019) suggested that energy use and greenhouse gas emissions might be reduced by 8% and 17%, respectively.

30.4 Conclusions

The LCAs discussed in this chapter are characterized by assumptions about what microalgae-based processes would look like in the future. This leads to relatively large uncertainties regarding the outcomes of such LCAs. Nevertheless, several conclusions can be drawn from these studies. In processes that require extraction of dry algae, the combined harvesting, drying, and extraction steps are associated with relatively large environmental burdens. In addition, the cultivation stage has been identified as linked to relatively large environmental impacts. Microalgal biomass-based feed for carnivorous fish might lead to a lower exergy extraction from the natural environment than conventional fish meal. Proteins derived from cyanobacteria tend to have larger estimated life cycle environmental impacts than proteins derived from cultivated terrestrial plants such as maize and soybean. Whether commercial microalgal fuels will have a climate benefit compared with fossil fuels appears to be uncertain. The energetic return on energy invested (EROI) of all microalgal fuels for which peer-reviewed LCAs are available does not meet the criterion of 5–8, which would allow for widespread usage.

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CHAPTER 31

Exergy analysis applied to microalgae-based processes and products

Karina A. Ojeda[a], Eduardo Sánchez-Tuirán[a], Jose Gonzalez-Diaz[a], Manuel Gomez-Ochoa[a], Vyacheslav Kafarov[b]

[a]Process Design and Biomass Utilization Research Group (IDAB), University of Cartagena, Chemical Engineering Program, Cartagena, Colombia [b]Center for the Sustainable Development of Industry and Energy, Industrial University of Santander, School of Chemical Engineering, Bucaramanga, Colombia

Chapter outline

31.1 Introduction 842
- 31.1.1 Benefits of algae biofuel 842
- 31.1.2 Basic algae biology 842

31.2 Cultivation and harvesting technologies 843
- 31.2.1 Cultivation systems 843
- 31.2.2 Harvesting technologies 844

31.3 Extraction technologies 844

31.4 Current state of microalgae-feedstock biorefineries 845
- 31.4.1 Products from algae 845

31.5 Exergy analysis in microalgae-feedstock biorefineries 846
- 31.5.1 Main concepts of exergy 846
- 31.5.2 Exergy formulation 846
- 31.5.3 Exergy applied to biomass processes 847
- 31.5.4 Exergy analysis in a microalgae-feedstock biorefinery 848

31.6 Case studies 848
- 31.6.1 Case 1: Biodiesel production from *Chlorella vulgaris* microalgae through acid esterification and basic transesterification using methanol 849
- 31.6.2 Case 2: Biodiesel production from *C. vulgaris* microalgae through a pretreatment with $ZnCl_2$ and basic transesterification with ethanol 853

References 857

Further reading 859

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31.1 Introduction

31.1.1 Benefits of algae biofuel

Microalgae are renewable feedstocks for biofuels and chemicals (Yang et al., 2019). Due to their high oil content and environmentally friendly nature, they have been increasingly considered as an option to respond to global climate change (Yang et al., 2019; Zheng et al., 2019). Among their advantages over traditional biomass-to-biofuels platforms, it has been found that different water sources can be used to cultivate microalgae. They are also capable of using the CO_2 in the air as a carbon source to grow and reproduce (Chen et al., 2015). In addition, they can grow in low-quality agricultural land (Sovová and Stateva, 2019). These are some of the reasons why microalgae is a promising raw material for biorefinery-based-platforms.

31.1.2 Basic algae biology

The biomolecular composition of a microalga cell includes protein, carbohydrate, lipid, and nucleic acid. The internal composition of a microalga cell also includes protein, carbohydrate, lipid, and nucleic acid. Lipids can be extracted and transformed into biodiesel, and the carbohydrate components can be fermented into bioethanol (Sudibyo et al., 2017). Table 31.1 lists some useful microalgae species for biodiesel production and their lipid content under normal cultivation conditions.

Table 31.1: Lipid content of some microalgae species.

Microalgae	Lipid content (% dry wt)	Microalgae	Lipid content (% wt)
Achnanthes sp.	44.5	*Anabaena cylindrica*	4–7
Amphora sp.	21	*Chlamydomonas reinhardtii*	6
Ankistrodesmus sp.	29–40	*Chlorella vulgaris*	49–52
Botryococcus braunii	25–75	*Chlorella pyrenoidosa*	38
Chlorella sp.	28–32	*Chlorella sorokiniana*	22–24
Chlorococcum sp.	19	*Dunaliella bioculata*	8
Cylindrotheca sp.	16–37	*Dunaliella salina*	6–25
Dunaliella salina	6–25	*Nannochloropsis* sp.	30
Ellipsodium sp.	27	*Nannochloropsis granulata*	28.5
Hormidium sp.	38	*Nannochloropsis oculata*	45
Isochrysis sp.	25–33	*Neochloris oleoabundans*	35–54
Monoraphidium sp.	20	*Porphyridium cruentum*	9–14
Nannochloris sp.	20–35	*Prymnesium parvum*	22–38
Nitzschia sp.	45–47	*Scenedesmus dimorphus*	10
Phaeodactylum sp.	20–30	*Scenedesmus obliquus*	30–50
Scenedesmus sp.	20–21	*Scenedesmus quadricauda*	1.9
Schizochytrium sp.	50–77	*Tetraselmis* sp.	20–50

Modified from Han, B., Goh, H., Chyuan, H., Yee, M., Chen, W., Ling, K., 2019. Sustainability of direct biodiesel synthesis from microalgae biomass: a critical review. Renew. Sustain. Energy Rev. J. 107, 59–74; Sati, H., Mitra, M., Mishra, S., Baredar, P., 2019. Microalgal lipid extraction strategies for biodiesel production: a review. Algal Res. 38, 101413.

The lipids content in microalgae can reach up to 75% of their biomass (Lara and Graciano, 2019). Thus, several microalgae species are identified as potential biodiesel feedstock due to their high lipid accumulation abilities (Han et al., 2019). Therefore, research topics have been focused on cultivation, harvesting, and transformation technologies to increase microalgae productivity.

31.2 Cultivation and harvesting technologies

Microalgae can grow in different environments, such as fresh-, sea-, or wastewater (Lozano-Garcia et al., n.d.). Their cultivation is associated with the usage of nitrogen and phosphorus nutrients. Wastewater can be used as a source of these two components. In consequence, algae cultivation can help in the purification of water (Lim et al., 2017).

31.2.1 Cultivation systems

There are two main types of cultivation systems: open and closed. The choice of which system to use should be based on factors such as source of nutrients and investment cost (Wayne et al., 2018).

Open systems can be divided into unstirred, race track-type, and circular pond. Unstirred pounds are placed in natural water systems. Therefore, this type of system is low cost in terms of construction and operation. Its main drawback is that the CO_2 present in the air dissolves in the water and this generates a reduction in the biomass performance. The race track-type pond is common in commercial cultivation of microalgae. The mixing in this system is generated by paddle wheels, which causes better microalgae biomass yield. Finally, circular ponds are designed considering the basics of wastewater treatment ponds. These ponds have a disadvantage to control the temperature of the system. However, the mixing in these open ponds is more effective than in unstirred ponds (Wayne et al., 2018).

On the other hand, closed systems can be classified into vertical tube, horizontal tube, stirred tank, and flat panel photobioreactors. Vertical tube photobioreactors are divided into two types: bubbles and airlift column. Bubble column photobioreactors are characterized by a suitable mass transfer and high area-to-volume ratio. Airlift column photobioreactors appeared as a novel model of the bubble one, and are cost-effective to cultivate different types of microalgae. Horizontal photobioreactors are made up of several tubes arranged in several ways. On these, a large building surface area is required. Stirred tank photobioreactors are based on a fermentation tank concept. An agitator is utilized in these reactors to mix the culture medium and to guarantee optimal heat and mass transfer. Flat panel photobioreactors have low operation and installment costs, but some issues are associated to the culture temperature control (Wayne et al., 2018).

The main advantage of the open system is its simplicity and economy. However, this system, due to its design conditions, also presents some disadvantages associated to evaporation loss and pollution in the culture medium. On the other hand, the closed system addresses these disadvantages and controls contamination risk and reduces evaporation loss. However, this system requires a higher economic investment than the open system (Wayne et al., 2018; Klinthong et al., 2015).

31.2.2 Harvesting technologies

Harvesting is a step that represents an important impact in operational costs of a process based on microalgae as feedstock. Solid–liquid separation operations such as coagulation or flocculation sedimentation, flotation, and centrifugation have been utilized to harvest microalgae (Klinthong et al., 2015; Alkarawi et al., 2018). However, it is common to pair some of these techniques due to the low efficiency or high energy consumption of these separate operations. For example, coagulation and sedimentation are often combined (Alkarawi et al., 2018; Zhang et al., 2016).

31.3 Extraction technologies

Microalgae lipids have applications in several industries such as energy, pharmaceutical, and food. The final application requires an efficient and economical extraction process to operate properly (Lara and Graciano, 2019). According to Han et al. (2019), some factors must be considered for industrial lipid extraction: extraction efficiency, process duration, reactivity with lipids, capital and operational cost, process safety, waste generated, and solvent consumption. Lipid extraction starts with the microalgae cell wall disruption; the lipids can then be extracted in different ways (Lara and Graciano, 2019). Cell wall disruption is important to improve lipid extraction efficiency. It will allow direct access of solvent to the intracellular lipid, which increases the lipid recovery efficiency (Han et al., 2019). Cell disruption techniques are often classified into mechanical and nonmechanical. Some of these are shown in Table 31.2.

Lipids are separated by an extraction solvent (such as hexane, chloroform, acetone, ethanol, or methanol (Sati et al., 2019)) and converted to biodiesel by transesterification. Then, distillation, vacuum evaporation, or solid phase absorption techniques have to be applied to recover the solvent (Lara and Graciano, 2019). However, to optimize the extraction technologies and reduce the use of organic solvents, new research topics have been focused in apply green solvents, and simultaneous extraction and conversion lipids to biodiesel processes (Sati et al., 2019).

Table 31.2: Cell disruption techniques.

Mechanical	Nonmechanical
Conventional mechanical pressing	Conventional chemical solvents
Ultrasonic assistance	Acids
Microwave assistance	Nanoparticles
Electric pulse	Supercritical fluids
Cell homogenization	Ionic liquid
Sudden depressurization	Biological enzymes

Modified from Lara, M. and Graciano, G., 2019. Biomass recovery and lipid extraction processes for microalgae biofuels production: a review. Renew. Sustain. Energy Rev. 107, 87–107; Han, B., Goh, H., Chyuan, H., Yee, M., Chen, W., Ling, K., 2019. Sustainability of direct biodiesel synthesis from microalgae biomass: a critical review. Renew. Sustain. Energy Rev. J. 107, 59–74; Youn, S., Muk, J., Keun, Y., Oh, Y., 2017. Cell disruption and lipid extraction for microalgal biorefineries: a review. Bioresour. Technol. 244, 1317–1328.

31.4 Current state of microalgae-feedstock biorefineries

Current oil reserve levels demand the pursuit of new renewable sources of feedstock for biorefineries processes. In this context, a microalgae biorefinery potentially contributes through the replacement of conventional petroleum refineries (Mat et al., 2015; Brasil et al., 2017). Using this approach it is possible to phycoremediate wastewater and produce biodiesel in a sustainable way (Malla et al., 2018).

Diverse research related to the use of microalgae in biorefineries has been performed. For instance, synthetic natural gas from microalgae grown using CO_2 produced by a sugarcane biorefinery was assessed (Albarelli et al., 2018). A study about the optimal design of a microalgae biorefinery for producing polyhydroxybutyrate, astaxanthin, and biodiesel was carried out, and it was found that biodiesel production can be economically viable due to the incomes generated by the other products (García Prieto et al., 2017). The articulation of an algae biorefinery with palm oil mill to generate biogas through five technology alternatives was assessed, and the results showed that none of them was economically feasible (Nabila et al., 2018). The aforementioned researches have been focused in the microalgae valorization by means of high-value product, which demonstrates its potential in a biorefinery-based platform.

31.4.1 Products from algae

Products related to cosmetic, nutrition, pharmaceutical, and energy industries, such as fuels, pigments, vitamins, proteins, etc., are obtained from microalgae due to their composition based on proteins, lipids, and carbohydrates (Wayne et al., 2017).

Bioethanol and biodiesel are the main products obtained from microalgae related to the fuel industry. In recent years, bioethanol production from mixed microalgae culture has been

researched, and the effect of combined pretreatments on the sugar extraction from microalgae has also been analyzed (Shokrkar et al., 2017). Regarding biodiesel, its generation from microalgae using simultaneous extraction-reaction process was evaluated as well as the impact of some operation conditions such as temperature and reaction time on biodiesel yield (Shomal et al., 2019).

31.5 Exergy analysis in microalgae-feedstock biorefineries

31.5.1 Main concepts of exergy

Energy and exergy analysis are tools that can be applied to evaluate efficiency in a process (Gholami et al., 2018). However, energy balance does not offer information related to the energy degradation or irreversibilities of the system evaluated. This is why exergy analysis is important. This analysis can help to enhance the yield of the process because it identifies the lowest energy performance, irreversibilities, and environmental impacts of a specific pathway. Exergy is defined as "the maximum theoretical useful work that could be obtained from a system that interacts only with the environment if this has not reached the thermodynamic equilibrium" (Peralta-Ruiz et al., 2013).

31.5.2 Exergy formulation

In a material stream, the exergy content is the amount of work that could be obtained when the thermal, mechanical, and chemical equilibrium reaches a reference state by a sequence of reversible operations (Ojeda et al., 2011a). The energy and exergy balances for a stream process are defined as:

$$\text{Energy input} - \text{energy output} = \text{energy accumulation} \tag{31.1}$$

$$\text{Exergy input} - \text{exergy output} - \text{exergy consumption} = \text{exergy accumulation} \tag{31.2}$$

The exergy E of a system may be written as:

$$E = S(T - T_0) - V(p - p_0) + \sum n_i(\mu_i - \mu_{i0}) \tag{31.3}$$

where S represents entropy, V volume, n_i number of moles of substance i, T temperature, p pressure, and μ_i the chemical potential of substance i. The subscript o describes the state when thermodynamic equilibrium with the reference environment is established (Ojeda and Kafarov, 2009).

The exergy of a flow is written as:

$$E = H - H_0 - T_0(S - S_0) + \sum \mu_{i0}(n_i - n_{io}) \tag{31.4}$$

where H is the enthalpy. Energy and exergy efficiencies are defined as:

$$\eta = \frac{\text{energy in products}}{\text{total energy input}} \tag{31.5}$$

$$\eta_e = \frac{\text{exergy in products}}{\text{total exergy input}} \tag{31.6}$$

The standard exergy of many compounds is found in the literature. However, if not, the chemical exergy content of any pure substance can be calculated as:

$$B_{ch} = \Delta G_{Fo} + \sum N_i b_i \tag{31.7}$$

where ΔG_{Fo} is the standard Gibbs free energy of formation of the substance (J/kg), b_i the chemical exergy of the ith pure element of the substance (J/kg), and N_i the molar fraction of the ith pure element of the compound (J/kg).

For most chemical compounds, the Gibbs free energy of formation is available. However, if not, it may be calculated as:

$$\Delta G_{Fo} = A + BT \tag{31.8}$$

where T is temperature (K), ΔG_{Fo} (kcal), and A and B are functional groups contributions. Now, the chemical exergy of mixtures is defined as the sum of the chemical exergy of substances into the mixture plus the exergy loss due to the mixing process:

$$B = \sum N_i b_i + RT_o y_i \ln y_i \tag{31.9}$$

where b_i is the chemical exergy of the ith substance (J/kg), R is the gas law constant (J/kg K), T_o is the temperature (K), and y_i is the mole fraction of the ith substance. An overall exergy balance is possible by computing the exergy contents of all incoming and outgoing streams (Ojeda and Kafarov, 2009).

31.5.3 Exergy applied to biomass processes

The exergy concept allows calculation of the efficiency of a process by computing the efficiencies associated to each process step (Ojeda and Kafarov, 2009). Exergy analysis has been used to assess a variety of processes such as dimethyl ether (DME) production, milk processing, ammonia production, and crude oil distillation, among others (Bahadori and Nalband Oshnuie, 2019; Bühler et al., 2018; Flórez-orrego et al., 2019; Yan et al., 2019). In particular, the biomass-based process industry has been analyzed under this concept. Jaimes et al. (2010) analyzed a biodiesel production process in which the feedstock was palm oil. It was found that the largest exergy losses were generated due to the lack of heat integration in the process. Ojeda and Kafarov (2009) studied enzymatic hydrolysis in reactors

to obtain second-generation bioethanol from lignocellulosic biomass using an exergy analysis. A plug flow reactor with a packed enzyme was suggested for this stage of the process because this type of reactor showed better energy efficiency compared to a continuous stirred-tank reactor. Ojeda et al. (2011b) investigated three topologies to produce bioethanol from sugarcane bagasse. This study aimed to evaluate the performance of the sugarcane bagasse as feedstock for the production process. Considering the exergy analysis, the authors recommended developing improvements in pretreatment and simultaneous saccharification and fermentation steps as well as implementation of heat integration to increase the processes yield. Ojeda et al. (2011c) analyzed several biorefineries to produce second-generation bioethanol from sugarcane bagasse. Specifically, they evaluated the impact of process integration in the design of biorefineries to produce bioethanol from the mentioned feedstock. Exergy and life-cycle analysis was utilized to evaluate this impact. Ojeda et al. (2011a) developed an exergy analysis in several biorefineries using process integration. Bioethanol was obtained from acid pretreated biomass. Sequential hydrolysis and fermentation, simultaneous saccharification and fermentation, and simultaneous saccharification and co-fermentation were employed to design different study cases. Jaimes et al. (2012) analyzed two process alternatives to produce biodiesel from palm oil through an exergy analysis. The objective of this study was to calculate the irreversibility of each process step as well as its exergetic efficiencies.

31.5.4 Exergy analysis in a microalgae-feedstock biorefinery

Several studies related to exergy analysis of microalgae-feedstock biorefinery have been developed. Peralta-Ruiz et al. (2013) used it for screening three design alternatives for microalgae oil extraction in a large-scale process. Karimi (2017) used the concept of exergy efficiency in an ultrasound-assisted in situ transesterification of microalgae slurry to extract lipids and convert to biodiesel. Sanli (2019) developed energy, exergy, and sustainability analyses of a diesel engine fueled with diesel and microalgae biodiesel. Peralta-Ruiz et al. (2018) worked on a exergy analysis of biodiesel process from the microalgae biomass *Chlorella* sp. Restrepo-Serna et al. (2018) carried out energy, exergy, and economic and environmental analysis of a process for biodiesel production from microalgae oil that includes the cultivation, harvesting, and extraction stages.

31.6 Case studies

In this section, two biodiesel production processes from microalgae are evaluated using an exergetic approach. An exergy analysis is employed to determine the global exergy efficiency, exergetic emissions, and total irreversibilities associated to the biodiesel production. In addition, the steps of the processes are analyzed individually to calculate their contributions to the systems' efficiency, emissions, and irreversibilities.

31.6.1 Case 1: Biodiesel production from Chlorella vulgaris microalgae through acid esterification and basic transesterification using methanol

In order to develop the exergy analysis, the process was simulated using the commercial software Aspen Plus. The main operation conditions such as temperature and pressure were defined in the simulation as well as the physical exergy of the process streams. Chemical exergies were determined using the equations provided in Section 31.5.2. Component thermodynamic data were obtained using the NRTL method, and for decantation steps RK-Soave was considered. The feedstock stream considered for the process has a flow of 225 t/day of *C. vulgaris* microalgae oil (MAO), which has a 10 (wt%) of free fatty acids (FFA). A total of 199 t/day of biodiesel were produced from the aforementioned feedstock stream. Esterification (pretreatment) and transesterification steps with methanol were used in the process. The esterification and transesterification reactions were simulated in conversion reactors. The process considered the separation, biodiesel purification, and glycerol upgrading stages. This process was divided into several stages, as shown in Fig. 31.1.

During the esterification step, the FFAs in the MAO react with methanol in the molar ratio 60:1 (methanol:FFAs) at 60 C to produce biodiesel and water. This reaction takes place in a reactor (RX-01) using sulfuric acid as a catalyst. The reaction products are sent to a separation step where water and methanol that did not react are separated. Later, the esterified MAO is sent to transesterification with methanol and NaOH at 1% (wt) to produce biodiesel and glycerol. This transesterification reaction is performed at 60°C with a TG conversion of 0.97, and its product is sent to a decanter (DEC-02), where biodiesel is separated from glycerol.

A glycerol stream (120) that contains 81.9% of methanol transesterification is sent to a glycerol upgrading step in which it is separated from methanol with a purity of 78.6% (wt); the produced glycerol resulted in 23.0 t/day. On the other hand, the biodiesel stream generated by the decanter (DEC-02) goes into a tower (T-02) after reacting with H_2SO_4 in the reactor (RX-04). This column removes 98% of methanol present in the biodiesel stream. This biodiesel (stream 130), which is partially free of methanol, is sent to the washing step (TK-01), where it comes into contact with acidulated water (H_2SO_4 solution 5% (wt)). Later, the stream resulting from this washing is sent to a decanter (DEC-03) to separate partially the biodiesel from water and other impurities such as methanol, glycerol, and sulfuric acid. Finally, this biodiesel (stream 134) is sent to a tower (T-03), which produces a biodiesel-rich stream, 96.5 (wt%), with a flow of 199 t/day.

Taking into account the previous process description, information given by the software, and the equations of Section 31.5.2, the exergetic analysis was performed. During this analysis, both biodiesel and glycerol were considered as products of the process analyzed, and the results were expressed in terms of metric tonnes of biodiesel (BD) generated per day.

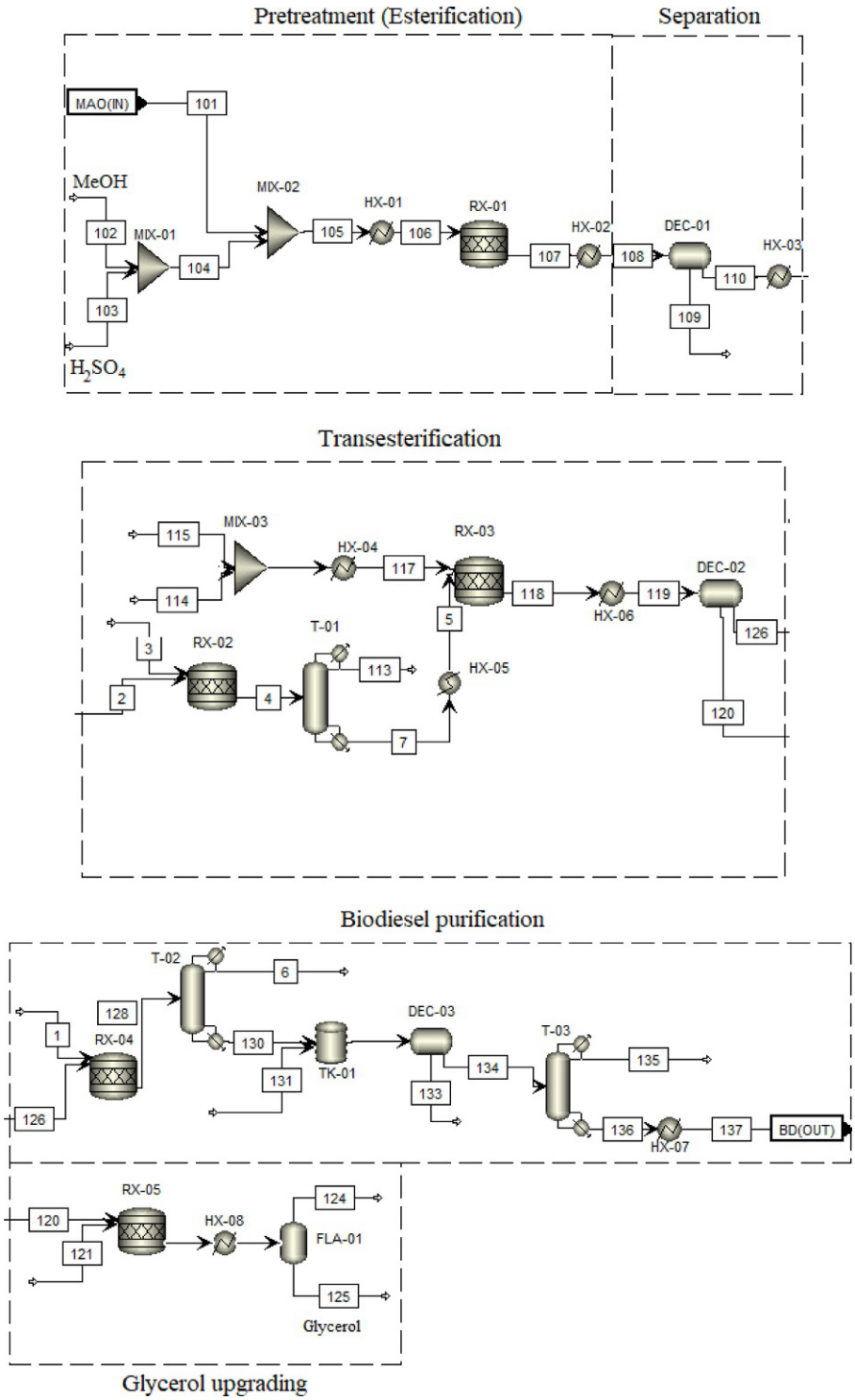

Fig. 31.1
Biodiesel production process (case 1).

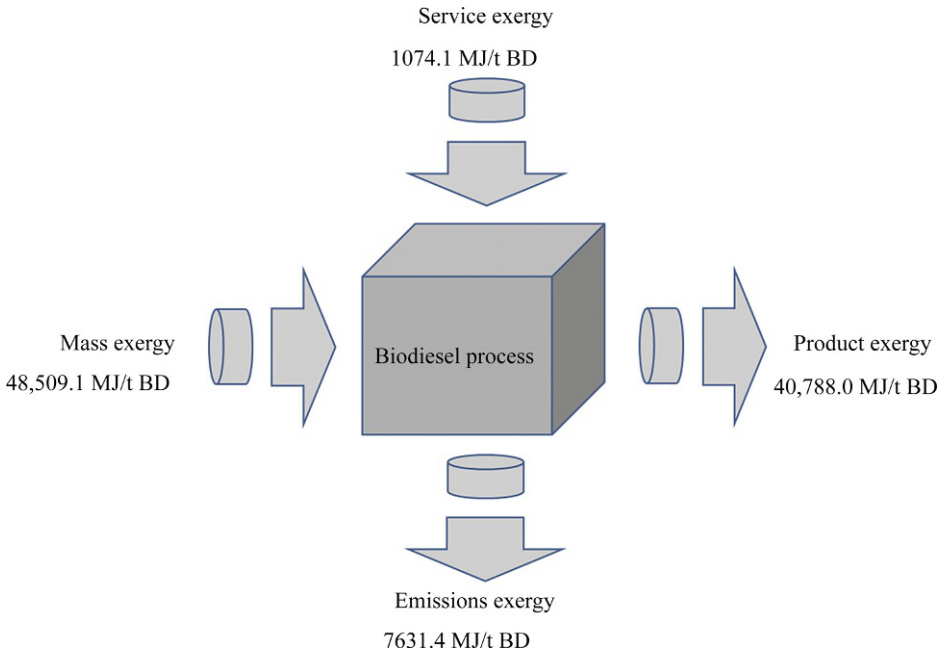

Fig. 31.2
Global exergy balance over the biodiesel process (case 1).

The results provided by the general exergy analysis over the system are given in Fig. 31.2. The exergy efficiency calculated for this process was 82.3%, which is higher than the reported by Peralta et al. (2010) for a similar process. This proves the potential that this process has if implemented. On the other hand, the emissions exergy, associated to the streams that are not considered as products, represents 15.7% of the mass exergy that enters to the process. Therefore, the implementation of some techniques related to process integration could improve the exergetic performance of this process.

This analysis also studied individually the five stages of the process: pretreatment (esterification), separation, transesterification, biodiesel purification, and glycerol upgrading. Fig. 31.3 presents the contribution of each stage to the process irreversibilities.

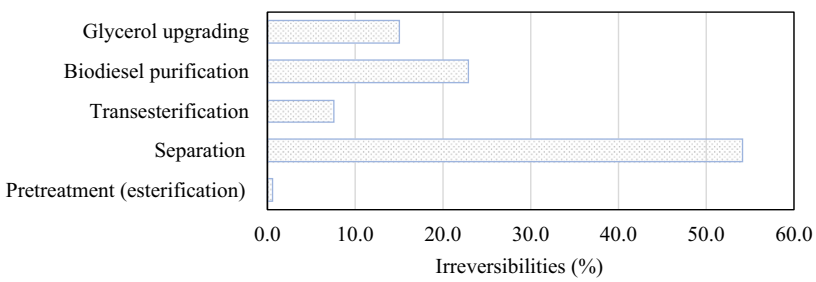

Fig. 31.3
Percentage of stage contribution to the process irreversibilities (case 1).

It was observed that the separation has the largest impact, since it causes more than 50% of the process irreversibilities. This can be explained by the fact that this step generates a stream considered as residual, which contains important amounts of biodiesel, water, and methanol that are being released to the atmosphere. Hence, it is suggested to apply mass integration methodologies to the process as a technique to recover these valuable products.

Fig. 31.4 shows the exergy efficiency of each process step (Peralta et al., 2010). The glycerol upgrading stage has a large exergy inlet compared to the exergy associated to the product generated in this unit. Therefore, process exergy efficiency is lower than other stages considered. Heat integration methodologies could help in the exergy inlet decrease since the heating and cooling utilities could decrease, and this would generate the same impact in the exergy inlet.

In this work, the exergy emissions of the process steps were also analyzed in Fig. 31.5. Exergy emissions are associated to the exergy of a stream that leaves a process unit and is not considered as a product stream.

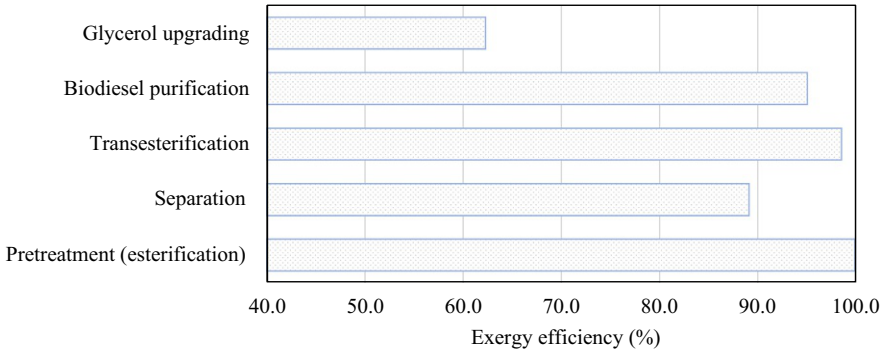

Fig. 31.4
Process steps exergy efficiency (case 1).

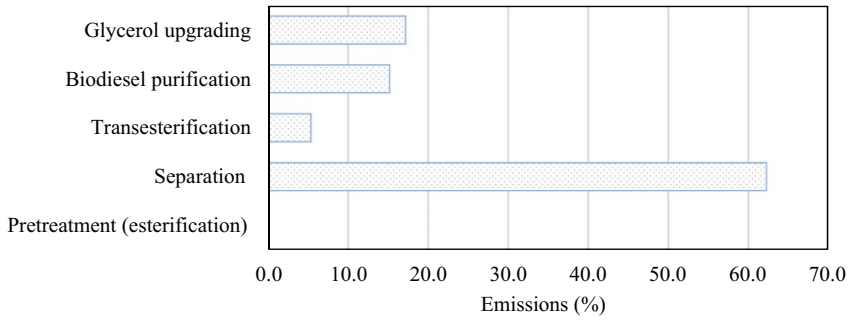

Fig. 31.5
Exergy emissions of process steps (case 1).

The results of this analysis showed that a total of 7631.4 MJ/t BD corresponds to the exergy emissions of the process. Hence, mass integration techniques are recommended, giving special attention to the separation stage, since it is the unit with the highest contribution in the process emissions.

31.6.2 Case 2: Biodiesel production from C. vulgaris microalgae through a pretreatment with $ZnCl_2$ and basic transesterification with ethanol

The procedure to develop the exergy analysis was the same as that employed in the previous case. Fig. 31.6 shows the process simulation. A process that uses 198.75 t/day of MAO *C. vulgaris* microalgae with 10% of FFAs to obtain biodiesel was simulated. Ethanol was used as the alcohol for this case. In order to predict the thermodynamic properties properly, and due to its high usefulness in the equilibrium phase, RK-Soave equation was used for liquid–liquid separation equipment, while NRTL was used for the rest of equipment such as reactors, heat exchangers, mixers, distillation towers, and others.

The stages of this process are pretreatment, transesterification reaction, biodiesel separation, glycerol adequacy, and biodiesel purification steps. The pretreatment is carried out with $ZnCl_2$ in order to reduce the MAO acidity below 2% (wt), to avoid soap formation in the subsequen tExergy analysis applied to microalgae-based processes and products basic transesterification with ethanol stage. This pretreatment is carried out at 200°C in the reactor RX-01, a stream of 2.7 t/day of glycerol and the 19,875 t/day of FFAs from the MAO, in presence of $ZnCl_2$ (1% wt with respect to FFAs) in order to obtain the triglycerides (TG) and water. However, water in the products causes problems for the transesterification reaction. For this reason, the flash distillation stage T-01 was considered to remove all the water. Stream 113 is sent to reactor RX-02 in order to carry out the transesterification at 60°C with ethanol in a molar ratio of 6:1 (ethanol:TG) in the presence of NaOH (1% wt). After the transesterification stage, products are sent to the decanter DEC-01 to separate the glycerol (stream 120), and the resultant stream is then sent to the decanter DEC-02 to separate the TG. The glycerol is neutralized with a sulfuric acid solution. The biodiesel is also neutralized with a sulfuric acid solution and then most of ethanol is removed in the tower T-04. Biodiesel is washed with acidulated hot water in the tank TK-01. The mixture is sent to the decanter DEC-03 in order to separate the acidulated water. Finally, the biodiesel is sent to a distillation tower where impurities are separated, and 211.6 t/day of biodiesel are obtained (Fig. 31.7).

The results provided by the general exergy analysis over the system are given in Fig. 31.8.

Following the same procedure used in the previous case, Figs. 31.9–31.11 show some results.

Fig. 31.9 shows that the process stage with the largest percentage of irreversibilities is the pretreatment. As in the previous case, it may be explained by the fact that here there is a stream that is a residual of the process.

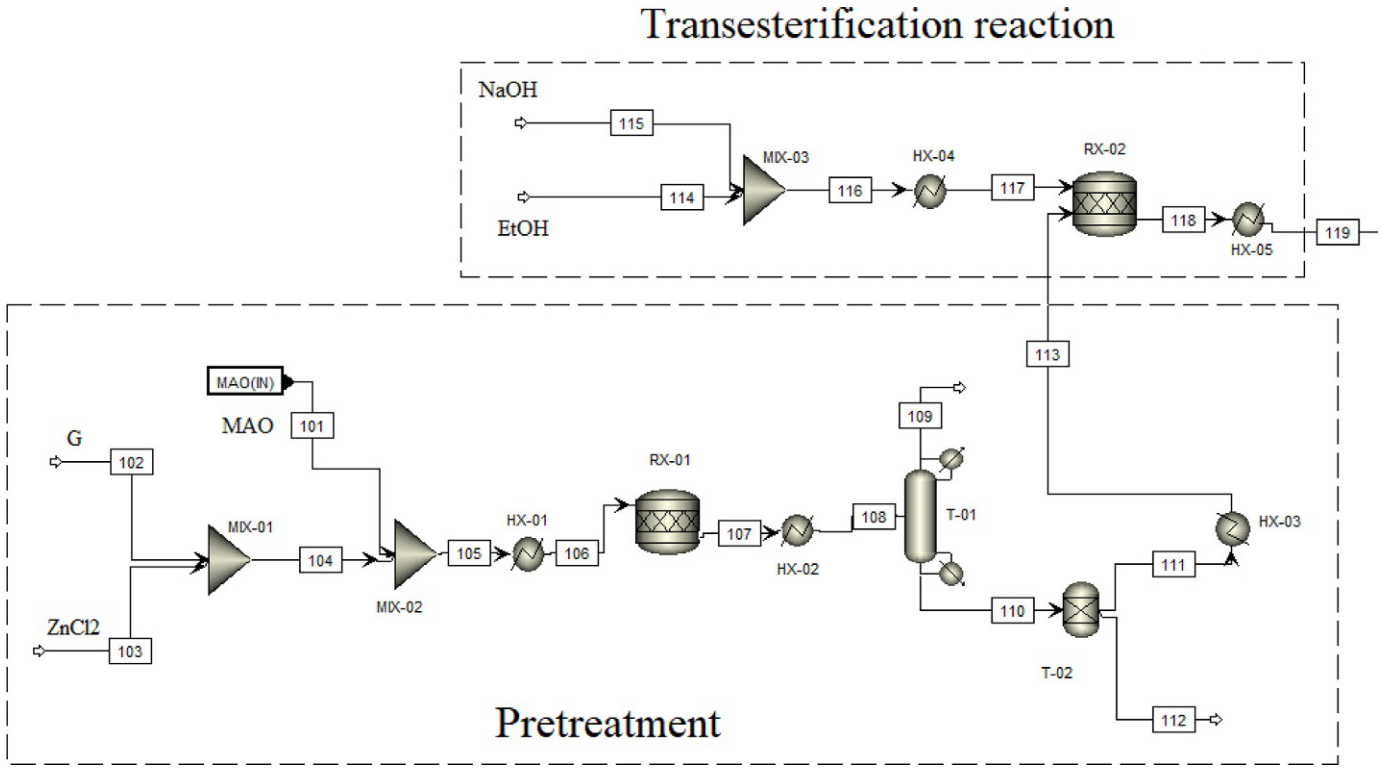

Fig. 31.6
Biodiesel production process (case 2): Transesterification and pretreatment stages.

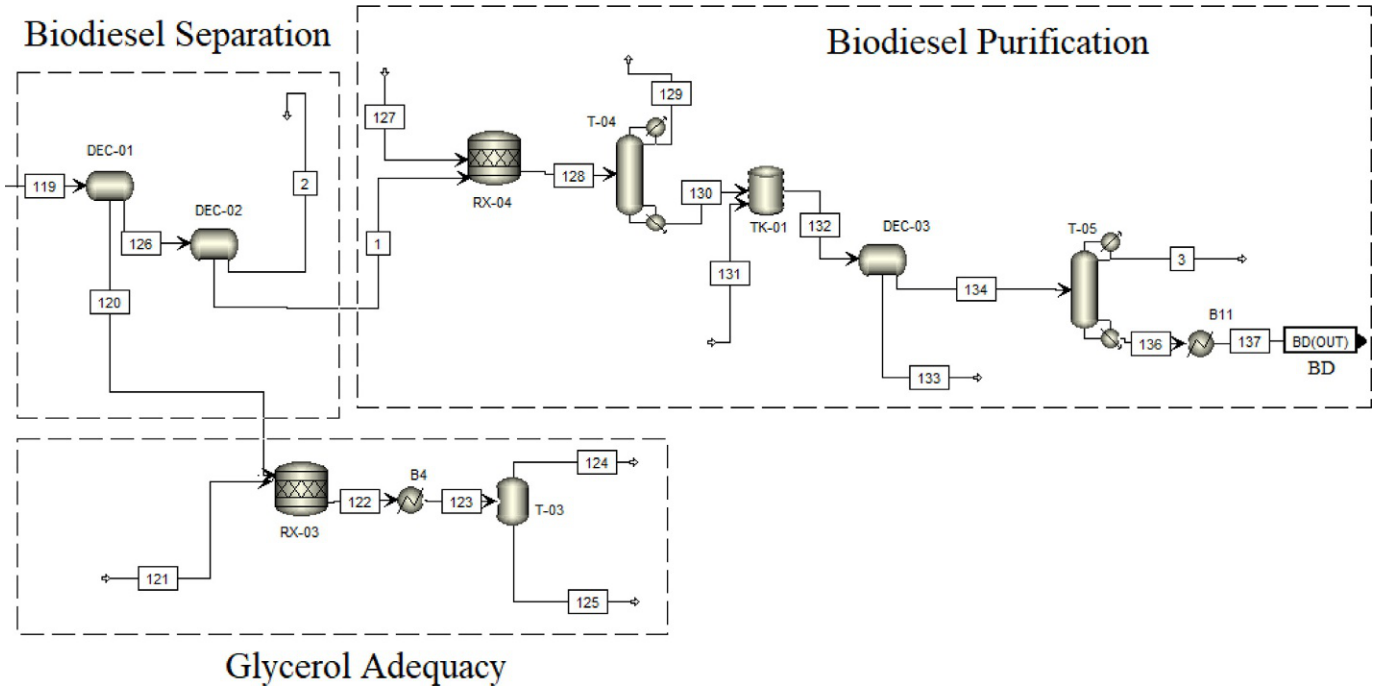

Fig. 31.7
Biodiesel production process (case 2).

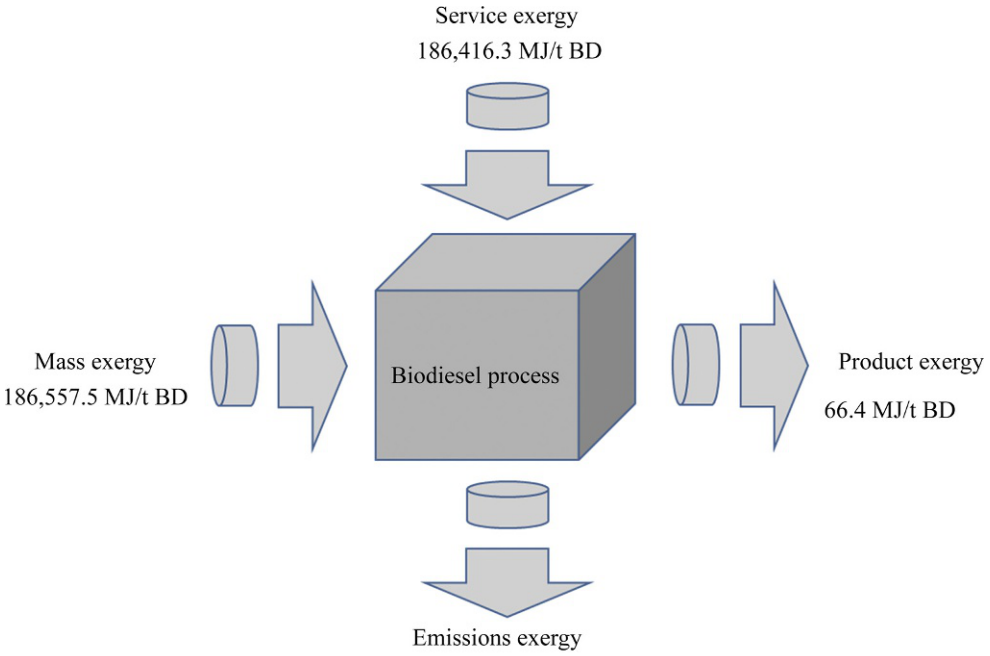

Fig. 31.8
Global exergy balance over the biodiesel process (case 2).

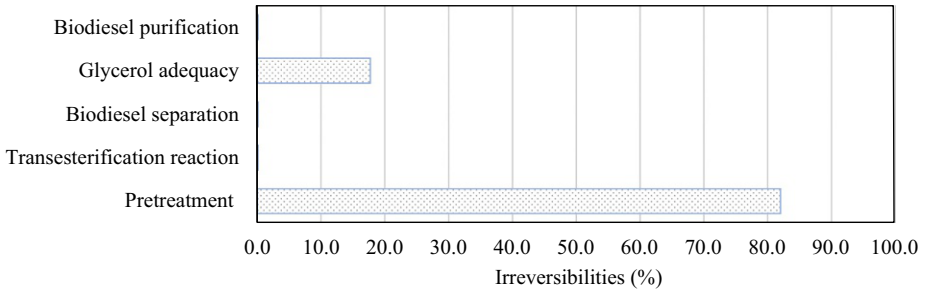

Fig. 31.9
Percentage of steps contribution to the process irreversibilities (case 2).

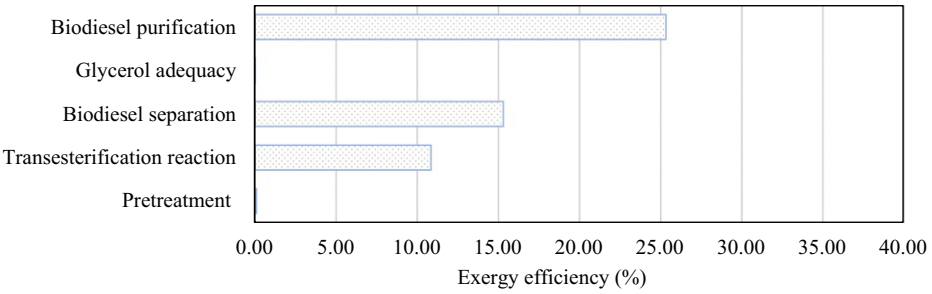

Fig. 31.10
Process steps exergy efficiency (case 2).

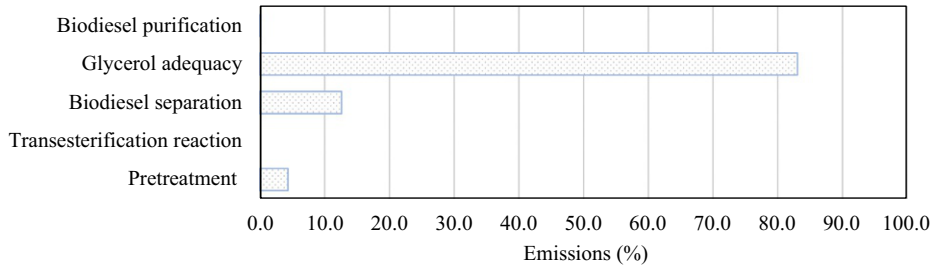

Fig. 31.11
Exergy emissions of process steps (case 2).

Fig. 31.10 shows that the stage with more exergy efficiency is biodiesel purification. This can be explained by taking into account that the principal product, biodiesel, is entering with some other compounds. Therefore, this stage has a high exergy in the inlet stream, and at the end, the biodiesel is almost pure, hence its exergy has less value.

Fig. 31.11 shows that the process stage with more emissions is glycerol adequacy. Therefore, the emission stream is mostly made up of ethanol, which has a high chemical exergy value (1251 kJ/kmol), while the other emissions streams are made up of compounds with smaller chemical exergies, such as water, zinc chloride, and others.

Considering the efficiencies of the two processes analyzed, it is recommended to implement case 1 for an industrial plant. This is supported by the fact that the global process exergy efficiency of this case is 82%, while for case 2 it is 50%. In addition, it has better performance in all stages.

In conclusion, it can be stated that exergy analysis offers an effective tool to calculate the efficiency of a process as well as the irreversibilities and environmental emissions of the process stages. The application of exergy analysis offers a way to compare between processes and allows the identification of stages that require special attention to overcome their lack of efficiency or to improve it. In this sense, process systems engineering and process integration methodology are complementary tools that enhance the application of exergy concepts in a biorefinery based-platform design.

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CHAPTER 32

Scale-up of microalgae-based processes

Niels-Henrik Norsker

Bioprocess Engineering, Wageningen University, Wageningen, The Netherlands

Chapter outline

32.1 Basic microalgal metabolism 861
- 32.1.1 Autotrophic production 863
- 32.1.2 Heterotrophic production 864
- 32.1.3 Mixotrophic production 864

32.2 Physical processes in microalgal reactors 866
- 32.2.1 Mixing 866
- 32.2.2 Mass transfer in photobioreactors 867
- 32.2.3 Shear stress in photobioreactors 869
- 32.2.4 Light in photobioreactors 870

32.3 Scalability of cultivation systems 874
- 32.3.1 Tubular photobioreactors 874
- 32.3.2 Flat panels 875
- 32.3.3 Closely spaced flat panel photobioreactor 876
- 32.3.4 Raceway ponds 876
- 32.3.5 Cascade flumes 876

32.4 Scale and productivity 877
- 32.4.1 Large diameter tubular reactor 877
- 32.4.2 Small diameter tubular reactor 877
- 32.4.3 Medium diameter tubular reactor 878
- 32.4.4 Flat panel 878
- 32.4.5 Tube length and diameter in tubular reactors 878

References 880

32.1 Basic microalgal metabolism

Microalgal production may be autotrophic, heterotrophic, or mixotrophic, depending on the origin of cell energy and carbon.

Autotrophic refers to light being the source of energy while the carbon source is CO_2 or -HCO_3. With heterotrophic growth, an external substance(s) provide the cell with energy and carbon. Mixotrophic, however, is a less clear-cut term (see Section 32.1.3).

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Autotrophically growing algae also exhibit respiratory or fermentative activity. In darkness, microalgae derive energy from respiration (either oxygenic or nitrate, sulfate or iron respiration) or fermentation of stored carbon compounds. The loss of biomass due to night respiration can be considerable: between 1% and 22% of the biomass was lost overnight in *Nannochloropsis salina*, *Picochloris*, and *Chlorella sorokiniana* (Edmundson and Huesemann, 2015); in outdoor *Spirulina* cultures, protein synthesis was shown predominantly to take place in the dark under respiration of storage carbohydrate. Under such conditions, up to 35% of the carbohydrate accumulated during daytime could be lost overnight (Torzillo et al., 1991). Between 3% and 8% of Phaeodactylum biomass was lost overnight with varying biomass density and tubular PBR diameter (Molina Grima et al., 1996). Mean monthly *Spirulina* biomass loss was 3.6%–6.0% of the biomass, reached at the end of the day light period at the optimal temperature, 35°C (Torzillo et al., 1991). High biomass density in the photobioreactor resulted in a higher fraction of the daily productivity being lost during the following night (Torzillo et al., 1993). Night biomass loss due to respiration may be related to the photosynthesis rate experienced during the day and to biomass growth rate (see Fig. 32.1). Night biomass loss remains poorly investigated; in some instances, it is possible to reduce the night biomass loss by cooling the culture during the night (Torzillo et al., 1991; Edmundson and Huesemann, 2015), although this is not always the case (Michels et al., 2014). As a consequence, it is necessary to distinguish between gross productivity (daytime) and net productivity (24 h) of an outdoor culture (Silva Benavides et al., 2013). The night biomass

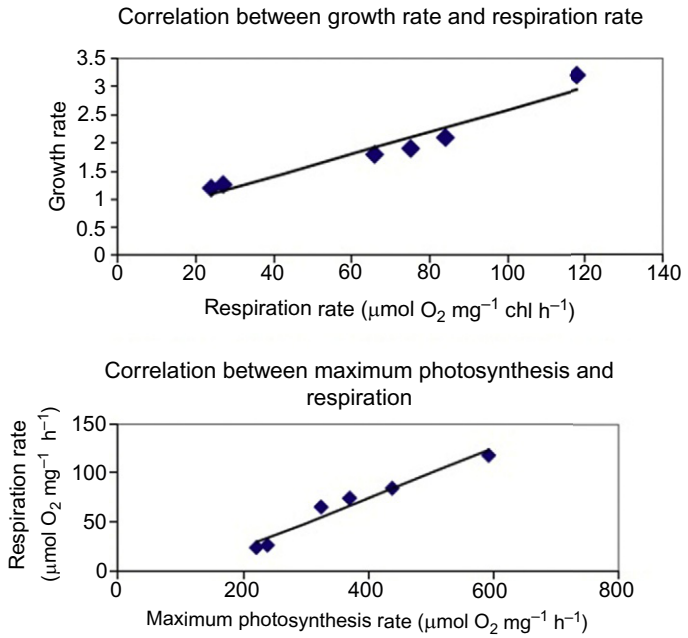

Fig. 32.1
Dependence on respiration of growth rate and maximum photosynthesis rate (Wilhelm and Jakob, 2011).

Table 32.1: Superficial gas velocity and k_La in various vertical sparged photobioreactors.

Photobioreactor	Superficial gas velocity ($m\,s^{-1}$)	k_La (s^{-1})	Source
Flat panel airlift	0.02	0.042	Reyna-Velarde et al. (2010)
Rectangular airlift	0.06–0.20	0.06–0.14	Siegel and Merchuk (1988)
Concentric tube airlift	0.055	0.027	Contreras et al. (1998)
Bubble column	0.00054–0.0082	0.0017–0.0043	Merchuk et al. (2000)
Bubble column, membrane sparger	0.005–0.03	0.013–0.05	Poulsen and Iversen (1998)
- Distilled water	0.005–0.03	0.027–0.13	
- 0.25 M NaCl			

loss cannot necessarily be eliminated though process optimization. To some extent, it is related to circadian carbon flux modulation and cell cycle synchronization.

Fermentative breakdown of stored carbon compounds is referred to as *auto-fermentation* to distinguish it from fermentative breakdown of assimilated carbon compounds. Auto-fermentation has been suggested as a process to produce biofuel, for example, ethanol fermentation by *Chlorococcum littorale* (Ueno et al., 1998). The process has not, to the knowledge of the author, had any scale-up attempts made yet.

32.1.1 Autotrophic production

Autotrophic microalgal biomass may be produced either in open ponds or flumes or, on the other side, in closed photobioreactors, whether naturally or artificially illuminated. Photobioreactors are tanks that are designed to optimize the light transfer to the algal biomass. Although open systems have also been referred to as photobioreactors, it is useful for the discussion to maintain this distinction (Molina Grima et al., 1999).

Light transfer to the photobioreactor is the critical process in the design of photobioreactors that limits the scale attainable. Two other processes need to be provided for: provision of carbon and removal of the resulting oxygen.

Many microalgae have the capacity to assimilate carbon for anabolic purposes (synthesis of cell matter) from organic substances while energy is still derived from photosynthesis. This process is referred to as photoheterotrophy. A small group have retained the capability to live fully heterotrophically, including the genera *Amphora*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorella*, *Chlorococcum*, *Crypthecodinium*, *Cyclotella*, *Dunaliella*, *Euglena*,

Nannochloropsis, *Nitzschia*, *Ochromonas*, and *Tetraselmis* (Perez-Garcia and Bashan, 2015 and sources therein) using either glucose, acetate, or glycerol as a carbon source. With autotrophic microalgal production, the carbon source is inorganic: either CO_2 or -HCO_3. With large-scale autotrophic production, CO_2 is normally used for cost reasons.

32.1.2 Heterotrophic production

Heterotrophic microalgal biomass is being produced at a considerable scale; in 2014 the global production of microalgal omega-3 rich oil was 7280 tonnes (van der Voort et al., 2017). Microalgal omega-3 oils are predominantly produced heterotrophically in fermentors, due to both rapid biomass yield and high DHA content in the produced biomass. Strains used belong to the genera *Schizochytrium*, *Ulkenia*, and *Crypthecodinium* (Enzing et al., 2014), the dominating organism (2014) being *Schizochytrium*. There are few published data on current industrial production, but in a 15 m^3 fermentor, *Schizochytrium limacinum* yielded in 96 h 81.84 g dw L^{-1}, with lipid (oil) and DHA 43.13 and 19.2 g L^{-1}, respectively (Song et al., 2015). Applying these figures, the 7280 tonnes of oil would correspond to roughly 15,000 tonnes of biomass. In March 2017, it was announced that Veramaris, a joint venture between DSM and Evonik, planned in 2 years' time to cover 15% of the omega-3 oil requirements of the salmon aquaculture market (Food Ingredients First, 2017; Sprague et al., 2017). In July 2019, it was announced that the factory had opened in Blair, Nebraska and was ramping up production (Veramaris, 2019).

32.1.3 Mixotrophic production

Mixotrophy is traditionally a broad term that covers metabolization of dissolved and particulate organic matter along with photosynthesis, taking place potentially simultaneously. Photoheterotrophy is a term that covers obligate autotrophic energy supply, combined with assimilation of organic substances for anabolic (carbon source) purposes (Perez-Garcia and Bashan, 2015). Photoheterotrophy may be considered a special category of mixotrophy. While mixotrophy has traditionally been considered an unusual and potentially useful trait in predominantly autotrophic microalgae, one should remember that assimilation of exogenous organic substances is the original trait of the eukaryote cell from which the microalgae developed and that the chloroplast was acquired later by ingesting a cyanobacterium and integrating it as the chloroplast. With increasing knowledge of algal metabolic pathways, mixotrophic flagellates are now believed to be quantitatively more important in the marine carbon cycle than obligate autotrophic microalgae (Mitra et al., 2014). Although the general opinion is not quite unified on the subject, uptake of dissolved organic substances is believed to be ubiquitous among photosynthesizing microalgae, and it is therefore not considered useful to keep assimilation of dissolved organic substances (osmotrophy) as a special physiological mixotrophic category. From a practical point of view, supplementing an

autotrophic culture with complex substances that it would otherwise have to synthesize may theoretically result in increased productivity; for example, nonessential vitamins are frequently added for this reason. The practical applications of supplementation are limited if the culture is intended to be predominantly monoseptic but is not strictly axenic; however, short-term supplementation with carbon sources may be used in nonaxenic batch processes if the concomitant proliferation of bacteria is manageable and acceptable. Cultivation of nonspecific algal biomass in wastewater is sometimes presented as mixotrophic algal production, but that is outside the scope of this chapter.

If mixotrophic production of microalgal biomass should be justifiable on a large scale (and to the author's knowledge, there is as yet no industrial production of mixotrophic microalgal biomass), the scenario should presumably be that supplementation with light to a basically heterotrophic culture should be advantageous or a precondition—not the other way round. Furthermore, the advantage should be considerable to justify the expense of supplementing light to a stainless steel fermentor. This is costly because the glass surfaces must be able to withstand steaming. An option is glass tubes, inserted in the culture, which for steaming can be approved to a maximum diameter of 200 mm (see Fig. 32.2). The cost per m^2 irradiation surface is about €40,000, so there is a strong case for optimizing high light intensity conversion in the algal culture.

It is a question whether fully additive mixotrophic growth of microalgae can be accomplished—with *Chlamydomonas*, which has the capacity to grow fully autotrophically and heterotrophically, light and darkness induces differentially and reversibly enzymes required for autotrophic and heterotrophic pathways (NAD-isocitrate dehydrogenase, NADP

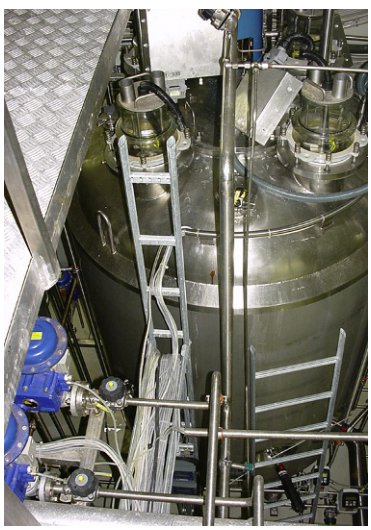

Fig. 32.2
A microalgal photofermentor with Pyrex glass lighting tubes inserted into the reactor from above.

isocitrate dehydrogenase and isocitrate lyase) (Martínez-Rivas and Vega, 1993). It thus remains a question whether these pathways can be manipulated to work simultaneously at their full.

32.2 Physical processes in microalgal reactors

32.2.1 Mixing

Mixing refers to:

- raceway ponds
  - paddle wheels
  - impellers
- flume tanks
  - pumps
  - air lift pumps
- PBRs
  - impeller pumps
  - air lift pumps
  - air sparging

Mixing of an algal culture is one of the large operating costs; for example, with a 1 ha GWP2 flat panel plant, an 8-month growing season produced 36 tonnes of *Tetraselmis* biomass at a total cost of €446,367 with an electrical power cost for aeration of the cultures of €22,537, i.e., 5.1% of the total costs or ¢ 63 per kg biomass. Mixing represented 18% of the operating costs of the plant. The panels were aerated with 0.22 NL/L min^{-1} during the day and 0.12 NL/L min^{-1} during the night (Tredici et al., 2015, 2016). This represents a significant improvement of practice over the preceding decade: in 2011, a desk study based on current practice at the time (1 m tall panels, aeration 1 NL/L min^{-1} @ 24 h) calculated aeration costs to ¢240 per kg biomass (Norsker et al., 2011).

The purpose of mixing:

- Mass transfer of CO_2 and O_2
- Mixing biomass between illuminated and dark zones
- Breaking nutrient micro gradients around cells
- Macro-scale mixing of nutrients and biomass between zones of the reactor
- Breaking temperature gradients in the reactor, enabling heat transfer
- Preventing biofilm buildup on solid surfaces in the PBR.

32.2.2 Mass transfer in photobioreactors

The feeding and removing from dissolved state of two gaseous compounds, CO_2 and O_2, from the algal broth, is referred to as mass transfer.

Both of the two gaseous compounds can exert a rate-limiting effect on the biomass productivity of photobioreactor culture and are addressed carefully in scale-up and operation of microalgal reactors. The mass transfer efficiency for a particular reactor design is indicated by the k_La coefficient and can be found empirically. Two methods to do this are presented in the following, and are based on dissolved oxygen (DO). CO_2 is equally relevant but is more complicated to measure because of the slow chemical solubility reactions.

The oxygen transfer rate (OTR) is given as:

$$\mathrm{OTR} = k_La*(\mathrm{DO}' - \mathrm{DO}).$$

with DO being the dissolved oxygen concentration in the liquid and DO′ being the equilibrium value of dissolved oxygen with headspace air. k_La is the product of the mass transfer coefficient, k_L, and the specific bubble area, a. Dissolved oxygen removal represents a large fraction of the operating costs of microalgal production, and optimizing the photobioreactor design to achieve maximum oxygen removal at minimum energy cost is important in the scale-up process. Siegel and Merchuk obtained oxygen removal efficiencies ranging from 2.5 to 6.0 kg O_2 per kWh in a 300 L airlift reactor with various separator and sparger designs, which was superior or equal to industrial aeration devices, including deep shaft and fine bubble aeration (Siegel and Merchuk, 1988). With a rubber membrane sparger, an oxygen removal efficiency of 8 kg O_2 $kW\,h^{-1}$ was obtained in a 15 cm bubble column (Poulsen and Iversen, 1998). k_La depends on the geometry of the photobioreactor, the medium composition and the aeration rate; k_La may be directly proportional with the riser superficial gas velocity in an air lift reactor or with the riser volumetric pneumatic power (Siegel and Merchuk, 1988). Important design elements include (riser) geometry that reduces bubble coalescence, the sparger, and the gas separator. Superficial gas velocity and k_La for a number of sparged reactor designs are given in Table 32.1.

32.2.2.1 Measurement of k_La

Mass transfer is normally measured with a pure medium in the reactor; for CO_2, measurement is complicated by the inorganic carbon reactions in water and a ratio between k_La for CO_2 and k_La O_2 is sometimes used to predict k_La for CO_2 on the basis of measurements done with O_2 (Contreras et al., 1998); a theoretical ratio of 0.90, based on molecular radius ratio was tested with two different aeration systems, and actual values of 0.74 and 0.96 times the theoretical ratio were found (Aitchison et al., 2007).

For O_2, the k_La may be measured in different ways; two simple methods are:

- the outgassing method (Rainer, 1990); and
- continuous reduction with sulfite (N.-H. Norsker, unpublished).

32.2.2.1.1 The outgassing method

The outgassing method is based on purging out the dissolved oxygen of the medium in the reactor with, for example, N_2 and measuring the rate at which the oxygen accumulates in the medium when aeration is reestablished. Calculation of k_La is done by measuring dissolved oxygen values in the medium in the reactor during the reoxygenation and depicting ln (DO′ – DO) against time. k_La is then found as the (negative) slope of the trendline (see Fig. 32.3). Depending on the operating conditions and the reactor, the response time for the DO electrode may bias the result; this corresponded to a k_La of 0.015 s^{-1} (3.8% seawater, 25°C, aeration rate 1 min^{-1}).

32.2.2.1.2 The continuous method

The reaction is:

$$2SO_3^{2-}(aq) + O_2(g) \rightarrow 2SO_4^{2-}(aq)$$

and is catalyzed by Cu^- ions.

A continuous method was adopted in which the oxygen transferred to the panel was reduced with sulfite that was pumped into the reactor at a certain rate. At steady state, the oxygen transfer rate corresponds to the sulfite solution pumping rate providing that the reaction is complete with respect to sulfite. The k_La can thus be calculated from the pumping rate, sulfite concentration, stoichiometric ratio between sulfite and O_2, volume of reactor, and steady-state DO concentration in the reactor.

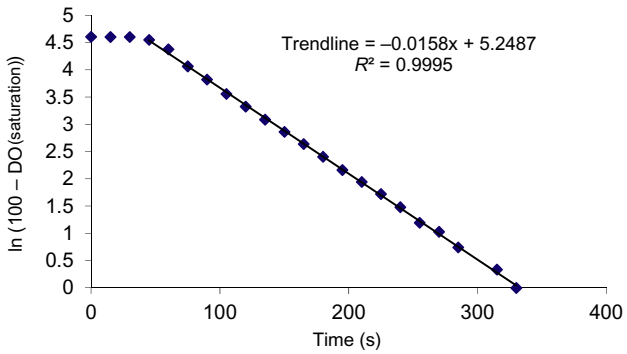

Fig. 32.3

Mass transfer in 1.7 L flat panel with 3.8% seawater at 25°C at an aeration rate of 1.0 min^{-1} as measured with the outgassing method. Superficial gas velocity was 0.04 m s^{-1}. Resulting k_La (slope of line) is 0.015 s^{-1}. DO electrode placed in 1 mL chamber with recirculation rate 42 mL min^{-1}.

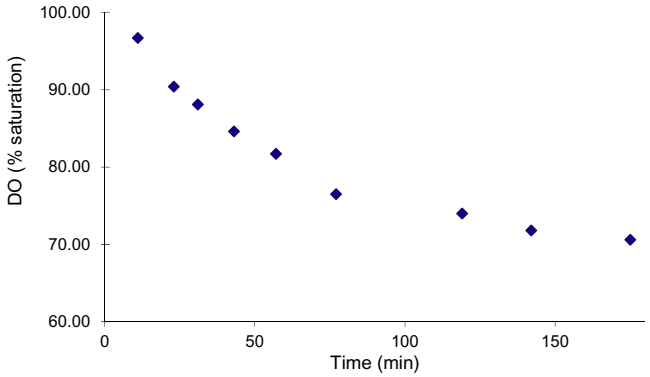

Fig. 32.4

Dissolved oxygen (in % saturation) as measured with the continuous sulfite reduction method. Conditions otherwise as in Fig. 32.3. Cs = 0.468 M, $F = 39.6$ mL/h, $Q = 2$, and $V = 1.7$ L. OTR $= 0.468 \times 39.6/60 \times 0.5/1.7 = 0.0908$ mmol O_2 min^{-1} L^{-1}. $k_La = 90.8\,\mu M\,min^{-1}/(250\,\mu M - 0.70 \times 250\,\mu M) = 1.2\,min^{-1}$ or $0.02\,s^{-1}$.

At steady state, the OTR can be calculated from $C_s \times F \times Q/V$, denoting the concentration of sulfite in the reaction solution, the flow rate of the reaction solution, the stoichiometric coefficient (O_2/SO_3^{2-}), and the volume of the reactor, respectively (Fig. 32.4). With $DO' = 250\,\mu M$ (3.8% seawater, 25°C):

$$DO = DO_{sat} \times 250\,\mu M. k_La \text{ is then calculated as : } k_La = OTR/(DO' - DO).$$

32.2.3 Shear stress in photobioreactors

When a fluid—here a microalgal culture—moves across a surface, the flow velocity (V) exhibits a gradient from the bulk liquid toward the surface where x is a distance parameter, normal to the surface.

$$\tau = -\eta \frac{\delta V}{\delta x} = \eta\dot{\gamma}$$

$$\tau\,[N\,m^{-2}]\ (\text{or Pa}) : \text{shear stress}; \eta\,[N\,s\,m^{-2}] : \text{kinematic viscosity}; \dot{\gamma}\,[s^{-1}] : \text{shear rate}.$$

The shear stress, or tangential force affecting the fluid, is thus defined by the curvature of the gradient. In the case of a Newtonian liquid, the kinematic viscosity is constant with respect to the shear rate, resulting in a linear velocity gradient.

Shear stress is considered one of the major concerns with scale-up of photobioreactors and affects dimension of pumps, tube velocity, and tube length in tubular photobioreactors. Few pump specifications have any indications of shear rate, and a computational fluid dynamic (CFD) analysis of a proposed system is recommended in a scale-up process. Centrifugal pumps exert the largest shear stress around the tip of the turbine blades and having a large distance between blade tips and housing is recommended to avoid high shear stress (Torzillo and Zittelli,

2015). Slow rotating, large diameter turbines also minimize the shear stress but have disproportionately higher capital costs. It is a normal misconception that peristaltic pumps are low-shear pumps and hence safe to use with microalgae cultures. Whereas the large part of the surfaces in the tube are not subjecting the algae to high shear stress, the compression zone does, so a small fraction of the culture volume may be damaged on each pass through the pump.

32.2.3.1 Sensitivity to shear stress

Shear stress sensitivity among microalgae is variable with sensitive species being damaged at as little as 0.7 Pa (Michels et al., 2010).

Generally, small cells (diameter around 3 μm) are more resistant to shear stress than large cells.

32.2.4 Light in photobioreactors

A few concepts need to be defined here: light path, transparency, and translucency.

32.2.4.1 Light path

The light path denotes the actual distance that the light will penetrate into the depth of a photobioreactor before it is fully attenuated. It is, in other words, a variable entity, depending on a combination of light intensity, impinging on the reactor, depth from window to bottom, biomass concentration, and light absorption coefficient of the culture. The latter is influenced by the operating conditions of the reactor.

32.2.4.2 Translucency, transparency

Transparency means that light rays will pass through a surface without being scattered. This means that an image can be perceived by the eye through the surface. Translucency means that a large proportion of the light rays will pass through the reactor wall without being absorbed, but image transmission does not necessarily take place. The distinction has a couple of practical applications in photobioreactor management: a photobioreactor vessel may be made from a poorly transparent polymer material and still transmit a significant amount of light impinging on the surface, although highly transparent materials are normally used for polymer-based photobioreactor construction. Shading with white plastic film or cotton fiber fabric may be surprisingly ineffective. On the other hand, white plastic film may be manufactured to reflect up to 90% of the light.

32.2.4.3 Materials for photobioreactor construction

For tubular photobioreactor construction, glass is currently (2019) the dominating material of choice in Europe. This may be related to the current orientation toward high-value products where robustness and cleanability have higher priority than photobioreactor cost. For flat panels, polymer film, most often low-density polyethylene, is used with large-scale installations and has a service time of a few months. UV and thermal degradation are serious

problems with polymer film materials for photobioreactors, but biofouling management can also be an issue because of limited options for in-place cleaning and sanitizing; in particular susceptibility to oxidizing agents is a problem. There is currently very little ongoing research in the field of photobioreactor manufacturing materials. New promising developments include wavelength-shifting film materials for photobioreactor construction that appear to be able to enhance productivity and product formation (Burak et al., 2019).

32.2.4.4 Enhancing light flux into photobioreactors

There are various approaches to increase the photon flux density (PFD) of the daylight falling on a photobioreactor. A flat panel reactor may be tilted toward the sun to optimize light capture, either in a manually adjustable position or with tracking devices (Qiang et al., 1996, 1998a). It is not yet clear whether increased productivity justifies the extra expenses and complications with automatic tilting as opposed to stationary vertical panels.

Another strategy to obtain volumetrically specific irradiation is to concentrate the daylight, for example, with linear Fresnel lenses combined with solar tracking systems (Masojídek et al., 2003, 2008; Zijffers et al., 2008). The concentrated beam irradiation from linear Fresnel lenses may then be projected directly on to tubes of the reactor (Masojídek et al., 2003, 2008) or distributed to the cultures via light prisms (Zijffers et al., 2008) or fibers (Ogbonna and Tanaka, 2000). In an experimental system with $9\,m^2$ linear Fresnel lenses, established at Nove Hrady (48.5°N, 114.5°E) in the Czech Republic, noon tube surface light intensity was up to 3.5 times that of the ambient horizontal light intensity. Optimal biomass concentration (*Arthrospira*) was judged to be $1.2\,g\,dw\,L^{-1}$ (Masojídek et al., 2003). *Arthrospira* appears to be able to adapt to the high noon irradiation and recover from quenching on a diel base. It was shown that *Arthrospira platensis* can grow under highly concentrated irradiation without being irreversibly photoinhibited. Photosynthetic efficiency of the culture was rather low. A cost of a 65-L experimental system, including $9\,m^2$ of linear Fresnel lenses, 24 m of glass tubes, frame and tracking system for the tubes, pump, degasser, heat exchanger, sensors, and control (pH, light, temperature) was indicated to total €8700 (Masojídek et al., 2003).

To achieve high volumetric productivity and high photosynthetic efficiency at the same time, biomass concentration and mixing must be carefully optimized.

As was shown in a series of works at Algal Biotechnology Group around the turn of the millennium, it is possible to utilize high light intensity with high productivity and high photosynthetic efficiency. The work with continuously dual-side illuminated, laboratory flat panel *Spirulina* culture is a most impressive demonstration of the reach of this approach (Qiang et al., 1998b). An area productivity of $15\,g\,dw\,m^{-2}\,h^{-1}$ was obtained at a combined dual-side irradiation of $8\,mmol\,m^{-2}\,s^{-1}$, corresponding to a photosynthetic efficiency of about 6% (PAR, photosynthetically active radiation) (see Fig. 32.5). The daylight equivalent photosynthetic efficiency is about 2.5%, which in itself is not a lot, but at a PFD corresponding to about four times the maximum direct solar PFD it is impressive. At a PFD that corresponds

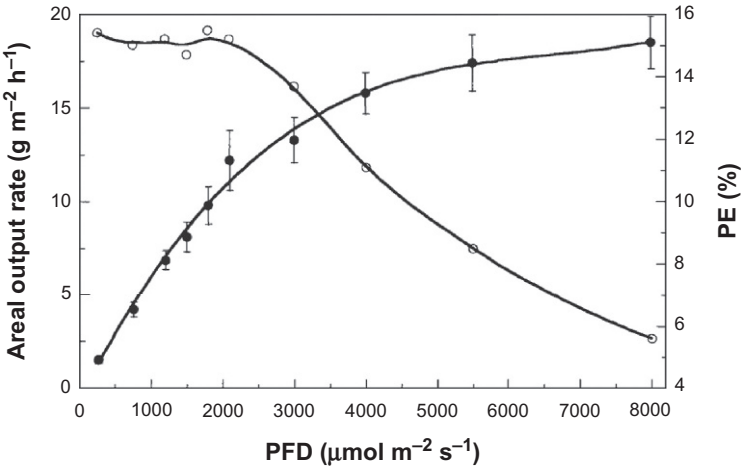

Fig. 32.5

Photosynthetic efficiency and biomass productivity of *Spirulina platensis* in a dual-side illuminated flat panel photobioreactor at varying flux density. The flux density is the sum of the flux density from the two sides. PE (%) is based on the photosynthetic active radiation (PAR) (Qiang et al., 1998b).

to the maximum direct solar PFD ($2000\,\mu m\,m^{-2}\,s^{-1}$), productivity of about $11\,g\,dw\,m^{-2}\,h^{-1}$ was obtained, corresponding to a PAR efficiency of 15% (Qiang et al., 1998b). Equivalent solar efficiency would be 6.3%.

Translating these data to outdoor photobioreactor conditions, however, would result in exaggerated productivity projections.

One obstacle in reproducing this scheme under outdoor conditions is the principle to adjust the biomass concentration carefully to suit the irradiation. This is difficult to achieve under outdoor conditions because of the diel variation in irradiation, not even under cloudless conditions, and even less with changing cloud cover and the resulting dynamical changing light regime.

A general problem with irradiation of photobioreactors is that the light is rapidly absorbed in the algal biomass, depending on biomass concentration and light absorption coefficient. There is an inverse relationship between photosynthetic efficiency of the absorbed light and light intensity; and with high light intensity, sustained exposure may even lead to irreversible photoinhibition.

32.2.4.5 The influence of discontinuous irradiation

Microalgae in outdoor photobioreactors are subject to irradiation fluctuations, with frequencies ranging from 0.00001 to 10 Hz owing to diurnal cycles, cloud cover, canopy shifting, and mixing (Graham et al., 2017). Frequencies thus cover the range from diurnal cycle to turbulent movements of algal cells between zones of different light intensity in the PBRs.

Since its discovery in the 1950s by Emerson and Kok, microalgal plant physiologists have had a long fascination with the *flashing light effect* and how to apply it in photobioreactors: short duration flashes of high intensity, up to 10,000 ftc may be utilized with the same efficiency as the equivalent quantum continuous irradiation at low intensity, 10,000 ftc corresponds to full sunshine, 2500 $\mu mol\, m^{-2}\, s^{-1}$. To obtain high efficiency, the light flashes must be of short duration (<3–4 ms) at moderate light intensity, separated by dark periods of 10 times longer duration. At very high intensities, 1100 $W\, m^{-2}$, flash times <1 ms were required to approach full efficiency (Kok, 1953). The photosynthesis rate in response to flashing at longer light dark cycles, 13–87 s, were investigated by Janssen et al. with *Chlamydomonas*, and it was concluded that light energy cannot be accumulated during the light period to cover the photosynthesis use during the subsequent dark period (Janssen et al., 1999). More recently, Vejrazka demonstrated that *Chlamydomonas* cultures flashed with LED light of an intensity of 1000 $\mu mol\, m^{-2}\, s^{-1}$ administered in 10 ms light flashes intersected by 100 ms dark periods converted the light with the same efficiency as continuous light at 100 $\mu mol\, m^{-2}\, s^{-1}$ (Vejrazka et al., 2011).

However, Grobbelaar et al. demonstrated with *Scenedesmus obliquus* that although the photosynthetic efficiency increased with the frequency of the flashing up to 10^4 Hz, the effect of increasing the length of the dark periods between the flashes from one to two times the flash length diminished the photosynthetic performance of the cultures (Grobbelaar et al., 1996).

An intermittent irradiation pattern may be produced in a photobioreactor by turbulence, shifting the algae between the photic zone and dark zone. This was explored by Laws in Hawaii in 1983, mounting airplane wing-like foils in a 48 m^2 flume tank with *Phaeodactylum tricornutum*. The foils produced transversal vortices that were documented with an anemometer-like instrument. Photosynthetic efficiency increased from 1.5% to 3.3%, comparing data from two adjacent weeks.

Swirl elements, such as aperture discs or bafflers that create swirling flow, may be inserted in tubular reactors. With an annular tubular reactor, Muller-Feuga et al. experimented with tangential inlet with different constriction discs and obtained a 7% increase in productivity and a 26% decrease in time required to reach steady state (Muller-Feuga et al., 2003). Perner-Nochta and Posten undertook a CFD simulation of the effect of inserting static mixers (axial-helical contour elements) in the inlets of tubes of a tubular PBR and showed that light–dark periods between 3 and 25 Hz could be obtained at an axial flow velocity of 0.5 $m\, s^{-1}$, which is normal for tubular PBRs (Perner-Nochta and Posten, 2007). Other flow simulation studies, such as Wu et al. (2010) also suggest that swirl elements or tangential fluid injection could introduce an effective flashing light environment by establishing light integration.[a]

[a] A situation where the photosynthetic performance of a culture reflects average light intensity rather than local light intensity.

The challenge implementing the flashing light effect this way, however, is that it is energy demanding to produce a suitable turbulence pattern. There is also the concern that the shear stress caused by turbulence can be detrimental: micro eddies at a size (as calculated by the Kolmogorov equation) of the diameter of the algal cells may damage the algal cells.

Negative effects of turbulence on growth were found in *Scenedesmus quadricauda*, cultivated in a Couette reactor at different rotational speeds. Characteristically, flocs, consisting of dead and live algal cells, formed over time and this was believed to be a result of shear stress (Hondzo et al., 1997).

Increased mixing has been shown to lead to higher photosynthetic efficiency which was attributed to an improved light regime, but the inference is complicated by the fact the increased mixing per se may also benefit photosynthetic efficiency (Grobbelaar et al., 1996), for example, by increasing CO_2 and O_2 mass transfer. With the objective to separate mass transfer and light regime effects, Kliphuis et al. investigated the effect of rotational speed on the productivity and photosynthetic efficiency in a Couette reactor under high irradiation. It was concluded that the turbulence had little effect on the photosynthetic efficiency (Kliphuis et al., 2010).

32.3 Scalability of cultivation systems

In, for example, Koller (2015), scalability was defined as the feasibility of designing large systems of repeating PBR units.

Reactor unit size is critical in a number of aspects: production safety, instrumentation requirements, and labor requirements. With small repetitive units, labor and instrumentation costs are relatively high whereas large reactor units are demanding in terms of inoculation volume and the risk of contamination is more critical.

Scalability is also defined by the limitations to dimensions of the individual reactor units in the various production systems.

32.3.1 Tubular photobioreactors

32.3.1.1 Tube length

Maximum applicable tube length depends on biomass concentration dissolved oxygen tolerance, flow velocity, and degassing efficiency. If the dissolved oxygen tolerance (as it is customary to assume) is of 300% saturation and degassing to 150% saturation and a flow velocity of about $0.5\,m\,s^{-1}$, the tube length in tubular PBRs may reach maximal length (before repeated degassing) of about 250 m for oxygen accumulation reasons. Oxygen tolerance is not a definitive characteristic, but the acceptable oxygen threshold may be set as a compromise between energy expenditure, construction cost, and productivity.

Normally, the maximum recommendable length is smaller; for tube diameters 5–10 cm, the recommended length is 80–120 m (Molina Grima et al., 1999). With low to medium diameter tubes, shear stress concerns may limit the length further because pressure drop in the long tubes will necessitate a larger initial force.

The Dutch tubular PBR manufacturer LGEM applies tubes of 65 mm diameter in lengths of up to 150 m, but the system applies two-phase flow, undertaking mass transfer in the tubes.

With high diameter tubes, such as the Cellana system, the length of the tubes may be larger (245 m) without excessive oxygen accumulation because of the lower biomass concentration (and possibly also because a headspace is providing some mass transfer in the tubes).

32.3.1.2 Tube diameter

Downwards, the diameter is limited by the pressure drop, building up over the tube. The *Biofence* tubular PBR from Varicon Aqua applies narrow 2.8 cm diameter (i.d.). A module will have a tube length of 11 m, so the pressure drop is not of immediate concern. For a large installation, however, it would be uneconomical in terms of mixing energy, and the Biofence is therefore now mainly used indoors. Upwards, there is a limitation exerted by low biomass density or productivity. The Cellana system with its large tube diameter (38 cm) is harvested at a low biomass concentration, but that is acceptable for the application as the output is used as a whole-broth inoculum without need for harvest.

32.3.2 Flat panels

32.3.2.1 Height

For economic sparging of flat panel reactors, which is a major cost factor in the operating economy of the panels, it is convenient to keep the panels no higher than 50 cm. This is because it allows energy-efficient, low pressure blowers to be used (Norsker et al., 2012). Reducing the height of the green wall panel from 100 cm in the GWP1 to 70 cm at the design of the GWP2 (Fotosintetica & Microbiologica S.r.l., Italy), also allowed a lighter, and therefore more economical, support construction without a wireframe encasing being required. In a sustainability context, the embedded energy in the photobioreactors accounts for a large fraction of the energy requirements; in the 1 ha installation described earlier, it is the same size as the electrical power used (Tredici et al., 2016). The Solix Biosystems panel is 0.3 m high (Quinn et al., 2012).

32.3.2.2 Depth

The depth of the panels normally depends on whether the panels basically are solitary and designed for minimum self-shading; in this case, the depth and biomass density are set to ensure that the light is fully attenuated but a major dark zone is avoided. The actual depth is, of course, a matter of choice; examples of depth of flat panels that have been tested

successfully on a large scale are Fotosintetica & Microbiologica at 4.5 cm (Tredici et al., 2016) and Solix Biosystems at 5 cm (Quinn et al., 2012).

32.3.2.3 Width

The width of the panels is a practical choice; the Solix panel is 17.3 m wide while the GWP2 measures 1.24 m.

32.3.3 Closely spaced flat panel photobioreactor

In contrast to the basically solitary (not mutually shading) panels, a very different approach is the closely spaced flat panel photobioreactor, where mutual shading and reflection between panes is used to create light dilution and hence improved photosynthetic efficiency. An example is the ProviApt photobioreactor, made by Proviron Industries in Belgium. The distance between the panels is 20 cm and the panel depth is 1 cm. The optimal biomass concentration is about 3 g dw L^{-1}. Summer daytime (gross) photosynthetic efficiency is 3.2%–3.8% (Norsker et al., 2019).

32.3.4 Raceway ponds

32.3.4.1 Area

The largest commercial paddle wheel-mixed raceway ponds are from 0.5 to 1 ha in area and used for cultivation of *Spirulina* (Borowitzka, 2005). The pond area is limited by the maximum acceptable depth and minimum acceptable flow velocity.

32.3.4.2 Depth

A flow velocity of 20–30 cm s^{-1} is normally required to keep the biomass suspended and prevent thermoclines, which for a 0.5 ha raceway pond requires a minimum depth of 30 cm. The dependency on depth of the minimum acceptable flow velocity is explained by the physical mechanism that creates the flow: the static height difference between the front side and the back side of the paddle wheel (Chisti, 2013).

Experience shows that the photic depth in a raceway pond should be two-thirds of the actual depth for optimal photosynthetic efficiency; hence a maximum biomass concentration of 0.3 g dw L^{-1} can be achieved in a 30 cm deep pond (Borowitzka, 2005).

32.3.5 Cascade flumes

Cascade flumes may be considered a special case of raceway ponds: by establishing flow over inclined surfaces (1.7%), culture depths of 6–7 mm can be attained and average net summer areal productivity 38 g dw m^{-2} day^{-1} was achieved with *Chlorella* sp. at Trebon, Czech

Republic. Photosynthetic efficiency was 9% PAR (Doucha and Lívanský, 2009), equivalent to 3.9% solar.

Although these results are impressive, the cascading flumes have not found industrial use, probably because the inclined surfaces are inherently costly to establish; the construction costs for cascade reactor are five times higher than for a raceway pond (Zittelli et al., 2013).

32.4 Scale and productivity

There are not many large-scale data sets available from industrial microalgal installations; however, a few public development programs have contributed comprehensive production data. In order to give compare the effect on productivity of light paths, three tubular photobioreactors with different light path were compared: small diameter (28 mm), medium diameter (60 mm), and large diameter (380 mm). In addition, one medium light path flat panel photobioreactor was included.

32.4.1 Large diameter tubular reactor

From Hawaii, Cellana has published productivity data from a 0.5 ha pilot plant unit at their 2.5 ha demonstration facility at Kona, Hawaii. The unit was a so-called hybrid plant, where large-diameter tubular photobioreactors (38 cm tube diameter) were used to start open raceway ponds that were used only for a short grow-out step (2 days). Biomass densities in the tubular reactors at transfer were 0.3–0.70 g dw L^{-1} (depending on nutrient load), and volumetric productivity was about 0.15–0.35 g L^{-1} day^{-1} (calculated from Huntley et al., 2015). Data were collected from production of 110 *Staurosira* sp. batches and >60 *Desmodesmus* sp. batches from April through July. Solar irradiation in Hawaii is rather uniform throughout the year, with average daily irradiation of 5.72 kW h m^{-2} day^{-1} (equal to 20.6 MJ) (Solar_Energy_Local, 2019).

Annual biomass yield (ash free dry weight) could thus be estimated to 78 and 77 MT for *Staurosira* sp. and *Desmodesmus* sp., respectively, and lipid yields were 30 and 28 MT ha^{-1} (Huntley et al., 2015).

32.4.2 Small diameter tubular reactor

The Biofence (Varicon Aqua, UK) tubular PBR has a light path of 28 mm (inner diameter) and a relatively close juxtaposition of the tubes (24 mm between the tubes) and two faces of tubes with a distance of 200 mm between them. Long-term outdoor productivity with this type of PBR has been observed during the months April through September at a position at Anglesey, UK, 53°N, 4°W. (Hulatt and Thomas, 2011). The back-surface irradiation constituted on average about 20% of the total irradiation. The reactor was operated with *S. obliquus* and PAR energy input ranged from 4.23 to 21.43 MJ PAR m^{-2} day^{-1} during the

observation period. Daily net productivity over the period varied from 1.39 to 23.67 g dw m^{-2} day^{-1}, with an average 11.37 g dw m^{-2} day^{-1}. Average photosynthetic efficiency during the observation period was 2.18%.

32.4.3 Medium diameter tubular reactor

Two European Union-financed projects, BioFAT and InteSusAl, have generated long-term pilot plant data in Portugal and Italy.

In Pataias, Portugal, with average annual irradiation of 17 MJ m^{-2}, 65 mm tubular PBRs with a total tube volume of 60 m^3 were operated with *Nannochloropsis* and *Phaeodactylum*. Volumetric productivity of 0.3/0.4 g dw L^{-1} day^{-1} was obtained with areal productivity of 18/19 g dw m^{-2} day^{-1} (EUREC_E.E.I.G., 2016). Annual average areal biomass productivity was 9 g dw m^{-2} day^{-1}, and similar to the productivity in a simultaneously operated cascade raceway pond (Biondi et al., 2016). Representative of June–July 2015, average areal biomass productivity was 12.7 g dw m^{-2} day^{-1}, in the 30 m^3 plant operating with biomass density ranging from 0.5 to 1.5 g dw L^{-1} (Fonseca, 2016).

32.4.4 Flat panel

Data from two GWP2 plants in Tuscany, Italy, one of which covers an area of 1500 m^2, were used to assess the productivity of a 1 ha production of *Tetraselmis suecica* for 8 months of the year, using the same technology scale as the pilot plant. The annual yield was 36 tonnes dw. The photobioreactors were 0.7 m tall and stood with a 1 m distance between them. The culture volume was 315 m^3, and the average horizontal irradiation in the period was 17.3 MJ m^{-2} day (Tredici et al., 2015, 2016).

The key data for the four plants are summarized in Table 32.2.

It is interesting to note that the volumetric productivity in the facilities is rather identical, 0.35–0.48 g dw L^{-1}, except for the small diameter tubular reactor (0.64 g dw L^{-1}).

32.4.5 Tube length and diameter in tubular reactors

32.4.5.1 Tube diameter

Currently the most commonly used tubular PBR diameter is around 60 mm; it is very probably a consequence of a series of engineering studies at the Chemical Engineering group of the University of Almeria carried out at the turn of the millennium, which concluded that for the model species used, *P. tricornutum*, 10 cm was the maximum diameter applicable for large-scale reactors, considering scale concerns, turbulence, shear stress sensitivity, dissolved oxygen

Table 32.2: Summary of productivity characteristics in large-scale photobioreactor systems.

	Unit	Tubular PBR, large diameter	Tubular PBR medium diameter	Vertical GWP	Tubular PBR small diameter
Culture depth	mm	380	65	45	28
Radiation in period	$MJ\,m^{-2}\,day^{-1}$	21		17.3	15.7
Dilution rate	day^{-1}	0.5	0.2		0.28
Biomass density	$g\,dw\,L^{-1}$	0.70	3.1		3.5
Areal productivity	$g\,dw\,m^{-2}\,day^{-1}$	35	19	15	14.3
Volumetric productivity	$g\,dw\,L^{-1}\,day^{-1}$	0.35	0.40	0.48*	0.64*
Photosynthetic efficiency	%	4.11		2.10	2.2
Biomass productivity	MT $dw\,ha^{-1}\,year^{-1}$	75		36	
N-quota	$g\,N\,g^{-1}\,dw$	0.19	0.09	0.7 (N-, P-replete)	N-, P-replete
Species		*Staurosira* sp. *Desmodesmus* sp.	*Phaeodactylum* (*Nannochloropsis*)	*Tetraselmis suecica*, F&M M33	*Scenedesmus obliquus*
Source		Huntley et al. (2015)	Eurec (2016)	Tredici et al. (2015) and (2016)	Hulatt and Thomas (2011)

Data pertaining to the period for which irradiation is indicated in row 2. Values calculated from source are indicated with *.

accumulation, and biomass concentration (Molina-Grima et al., 2000). In a later work, 6 cm was selected as a reasonable compromise between areal and volumetric productivity, energy consumption, scale concerns, dissolved oxygen accumulation, and biomass concentration in outdoor tubular reactors (Acién Fernández et al., 2001). A couple of companies adopted the tubular PBR technology with diameter 50–65 mm; in 2012, Georg Fischer DEKA introduced a series of clear lightweight 63 mm (outer diameter) highly transparent PVC pipes and bends for algal cultivation (Schuessler, 2012) in collaboration with the Dutch algal development company LGEM, and in 2014, the glass manufacturer Schott started marketing 54 and 65 mm (outer diameter) glass tubes for algal culture, along with fittings and bends, manifolds, and other equipment (Schott, 2019). The tube dimension 50–65 mm has become commonly used in European tubular PBR construction.

Horizontal tube reactors can now be easily assembled as serpentine or flat array configuration. Vertically mounted tubes have been pioneered by the Austrian company Ecoduna. Partially filled tubes have been pioneered by LGEM with the advantage of facilitating gas exchange and increased turbulence in the tubes. In-tube gas exchange lifts one of the constraints on scale-up of the tubular reactor, namely the dissolved oxygen buildup between degassing events.

32.4.5.2 Tube length

In tubular PBRs without gas headspace, the tube length is limited by the acceptable dissolved oxygen buildup in the tubes. This can be illustrated by an example: in an optimally growing *Spirulina* culture in a horizontal tubular PBR with 5 cm diameter, dissolved oxygen concentration can increase at a rate of 2–3 ppm min^{-1} (Torzillo and Zittelli, 2015). If the degassing, for instance, reduces the dissolved oxygen to 12 ppm and the maximum acceptable concentration at the end of the tube is 30 ppm, the oxygen accumulation in a culture volume, 18 ppm, is thus reached in 9 min (assuming dissolved oxygen increase is 2 ppm min^{-1}). With a velocity of 0.5 m s^{-1}, the maximum distance that a water volume can travel before returning to the degasser is thus 270 m.

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Index

Note: Page numbers followed by *f* indicate figures, *t* indicate tables and *b* indicate boxes.

A

AA. *See* Arachidonic acid (AA)
Acaryochloris marina, 37–38
Acetaldehyde, 670–677*t*
Acetophenone, 677*t*
Acetyl-CoA carboxylase (ACC), 429
Acetyl-CoA hydrolase (Ach), 629–630
Acetyl valeryl, 670–677*t*
Acid/alkali-assisted cell disruption methods, 289–290
Acid catalysts, 351–352, 356–357
Acid pretreatment, bioethanol production, 298
Acyl carrier protein (ACP), 429, 431
Adenosine triphosphate (ATP), 30, 186–187, 576–579
Adsorbent/absorbent materials, 200
Adsorption, VOC recovery, 678–679, 680*t*
African swine fever virus (ASVF), 552–553
Agar, 172
Agriculture, nitrogen for, 13–14
Airlift reactors, 111, 116, 122, 843, 867
Alcohol, 669, 670–677*t*
AlcR-$P_{alc\text{-}A}$ system, 87–88
Aldehyde, 669, 670–677*t*
Algae, 3–4, 799, 803–805
  biology, 842–843
  biomass production, historic timeline of, 208, 209*f*
  biotechnology, 88
  classification of, 331
  commercially grown, 5*f*
  commercially used pigments, 7*f*
  commercial production of, 3–4
  conventional dewatering methods, 209–213, 210*f*
    centrifugation, 210–211
    electrophoresis methods, 213
    filtration, 211
    flocculation, 209–210
    flotation, 212
    gravity sedimentation, 211–212
  conventional drying methods, 213–215, 214*f*
    convective drying, 214
    freeze-drying, 215
    solar drying, 213–214
    spray-drying, 214–215
  cosmetic products, 8*f*
  encapsulation-dehydration, 77
  microalgae (*see* Microalgae)
  polysaccharides used in food, 7*f*
  products, 845–846
  wastewater treatment, 17–18
AlgaGEM, 422–424
Alginate, 172
Aliphatic ketones, 664
Alkaline flocculation, 240
Alkaline pretreatment, bioethanol production, 298–299
Alkalinization, of adsorbent/absorbent materials, 200
Allochromatium vinosum, 611
Allophycocyanin, 31, 38–39, 466, 468–470
α-amylases, 689–692
α-carotene, 467
α-galactosidase, 698–699
α-ionone, 670–677*t*
Alum, 209–210
Aluminum salts, 242–244
Aluminum sulfate ($Al_2(SO_4)_3$), 242–244, 382
Alveolar bioreactor, 633–634
Alzheimer's disease, 469
Amberlyst-15 resin catalyst, 352
Amino acids, 42, 503–505
Ammonia, 42–43, 312–314, 317–319
Ammonia-ammonium system, 172
Ammonium sulfate, 475
Amylases, 689–693
  applications, 690–692*t*
  types, 689–692
Amyloglucosidase, 299, 689–692, 756–759
Anabaena sp., 29, 31–32, 42, 472–475
  A. cylindrica, 614, 617–618
  A. solitaria, 633–634
  biorefinery, 783, 785*f*
  carbon-supply regimes, 620
  H_2 production, 398*t*, 402
Anaerobic digestion (AD), of microalgae, 167, 312–314
  archaea in, 315
  bacteria, 315
  drawbacks, 315–320
    complex cell walls, 315–317
    low C/N ratios, 317–319
    microalgal biomass, humidity of, 319

Anaerobic digestion (AD), of microalgae *(Continued)*
salinity, 319–320
spent microalgae, 320–321
Anaerobic membrane bioreactor (AnMBR), 761
Analgesics, 177
Angiotensin I-converting enzyme (ACE), 499–500
Ankistrodesmus falcatus, 755
Antennae, 465–466
Antiaging activity, 562
Antibacterial activity, 553–554
Antibiotics, 169, 177, 208
Anticancer activity, 557–558
Anticoagulant activity, 560–561
Antifungal activity, 554–555
Antiglycemic activity, 561–562
Antiinfectious agents, polysaccharides (PS)
antibacterial activity, 553–554
antifungal activities, 554–555
antiviral activity, 551–553
Antiinflammatory activity, 559–560
Antilipidemic activity, 561–562
Antimicrobial peptides, 500
Antioxidant activity, 556–557
Antipyretics, 169
Antitussive activity, 561
Antivaccinia virus, 552
Antiviral activity, 551–553
Aphanizomenon flos-aquae, 468–469
Aphanothece microscopica Nägeli, 26–27, 32–33
Apparent digestibility, 506–507
Aquaculture, 10–13, 67–68, 833–834
Aqueous two-phase system (ATPS), 497–498
Arachidonic acid (AA), 6, 355, 426–427
Archaea, 315
Arrhenius equation, 133–134
Arsenic, 173*t*
Arthrospira sp, 7–8, 32. *See also Spirulina* sp.
A. fusiformis, 32
A. maxima, 32, 411
A. platensis, 32, 39, 312–313, 467–469, 471–474, 742–743*t*, 762, 871
asparaginase activity, 700
lipase activity, 695
photosynthetic efficiency, 410–411
protease production, 694
superoxide dismutase activity, 696
biorefinery, 788
Ash free dry weight (AFDW), 230
L-Asparaginase, 699–700
applications, 690–692*t*
production, 700
Astasia longa, peroxidase activity, 693
Astaxanthin, 33–34, 91–92, 285, 286*f*, 320, 467–472, 477, 480–481, 750, 779, 845
in cosmetics, 654*t*
Haematococcus pluvialis, 651
structure of, 650*f*
Atmospheric CO_2 concentrations, 185–186
ATP: citrate lyase (ACL), 428–429
Aulosira sp
A. fertilissima, 634–635
carbon-supply regimes, 622–623
Auto-fermentation, 863
Autoflocculation
bioethanol production, 382
microalgal biomass recovery, 235–242, 236–239*t*
Autotrophic cultivation, 332–334, 620–622
Autotrophic microalgae, 321–322, 861–864
Axial dispersion model, 102–103
Azoreductase, 177

B

Bacillariophyceae, 35
Bacillariophyta, 283–284, 534
Bacillus sp, 315
B. cereus, 611
B. megaterium, 611
Bacteria, 315
Bacterial bioflocculants, 249–250
Bacteroidetes, 315
Base-catalyzed transesterification, 351
Batch reactors, 102–103
Bead milling, 286–287*t*, 287, 749–750
Benzaldehyde, 670–677*t*
Benzothiazole, 670–677*t*
Benzyl alcohol, 670–677*t*
Bernoulli's equation, 138–139
β-amylase, 689–692
β-carotene, 31, 33–34, 91–92, 285, 286*f*, 320, 467–472, 475–476, 480–481, 779
structure of, 650*f*
β-carotene oxygenase (BKT), 91–92
β-cyclocitral, 670–677*t*
β-galactosidase, 699
β-ionone, 670–677*t*
Bidirectional hydrogenase, [Ni-Fe], 398–399, 402
Bioactive peptides, 498–500, 501*t*
Bioadsorption process, 175
BioBricks, 87, 92
Bio-crude oil
chemical compounds, *Spirulina platensis*, 299–300, 300*t*
conversion techniques
hydrothermal liquefaction (HTL), 301–302
pyrolysis, 300–301
Biodiesel production, 87, 294–295*t*, 752–756, 777, 806, 824–825, 829–830, 845–846, 850*f*
Chlorella vulgaris microalgae
acid esterification and transesterification using methanol, 849–853
pretreatment with $ZnCl_2$ and transesterification with ethanol, 853–857
drying microalgae, 345–346
flash drying, 346–347
freeze-drying, 347–348
rotary dryers, 346
solar drying, 346
spray-drying, 347
genetically engineered strains, 342–343

harvesting microalgae, 343–344
centrifugation, 344
chemical flocculation, 344
electroflocculation, 345
filtration, 345
ultrasonic sedimentation, 345
in situ supercritical transesterification, 354–358
lipid/oil extraction, 295*f*, 348–350
direct method, 349
electroporation, 349
ionic liquids, 296
mechanical, 348
microwave-assisted, 349
organic solvent extraction, 293–296
supercritical fluid extraction, 296–297, 349
ultrasonication-assisted, 349
lipid-rich microalgae
cultivation, 332–335, 333–334*t*
as feedstock, 331–332, 332*t*
heavy metals, effect of, 341
light irradiation stress, 341–342
nutrient starvation, 338–339
pH effect, 340–341
salinity induction, 340
temperature stress, 339–340
UV irradiance, 342
microalgal lipids
chemical composition, 354–356
processing parameters, 356–358
water and FFAs, effect of, 358
in photobioreactors, 721
process flow, 754*f*
process parameters affecting microalgae cultivation, 335
carbon nitrogen ratio, 337
carbon sources, 336
cell density, 336
dissolved oxygen, 337
light supply, 336
nitrogen and phosphorus, 337
temperature, 337
transesterification, 297, 297*f*
acid-catalyzed, 351–352
base-catalyzed, 351, 351*f*
catalysts used, 351
lipase-catalyzed, 352
supercritical alcohol, 352–354
Biodiversity, 49, 70–71
Bioeconomy, 800, 814–815*t*, 818*t*
algae production
evolution of, 810–811*f*
green algae, global production of, 812*f*
products and circularity, 803–806
sustainability, 800–802
and trade, 808–819
development of, 801
global export and import trends, 817*f*
supply chain characteristics, 800–802
Bioeconomy Strategy, 802
Bioelectrochemical systems (BES), 411–412
Bioenergy, 717
Bioethanol, 88–90, 756–759, 845–846
advantages, 376–377
disadvantages of, 377
physical and chemical features, 376–377
production, 379, 385–386, 385*t*
distillation process, 378–379, 384
emissions, 377
fermentation, 298*f*, 299, 378–379, 384
first-generation production, 90, 373–374, 377
flow diagram, 379, 380*f*
harvesting and drying, 381–383
hydrolysis, 298*f*, 299
microalgae cultivation, 379–381
pretreatment methods, 298–299, 298*f*, 377–378, 378*f*, 383–384
saccharification, 383–384
second generation production, 90, 373–374, 377
third generation production, 90, 373–374, 377
yields, of feedstocks, 377, 378*t*
Bioethanol-biodiesel-microbial fuel cell, 786
BioFAT, 878
Biofence tubular PBR, 877–878
Biofertilizer, microalgae, 781–782, 808
aquatic microscopic algae, 445–446
biomass valorization, 455
closed systems, 446
cultivation
closed photo-bioreactors (PBRs), 452–454
high-rate algal ponds (HRAP), 452, 453*f*
open ponds, 452
in wastewater, 455–456
cyanobacteria, 445–446
extracellular polysaccharides, 446
growth promoting substances, 446
harvesting
bulk harvesting, 454
thickening, 455
mucilaginous substances, 446
nitrogen fixation, 447–449, 448*t*, 456*t*
open systems, 446
phosphorus, 449–450, 456*t*
plant growth promoters, 451–452
saline soil, reclamation of, 450–451
soil, physical properties of, 450
wastewater treatment facilities, microalgal remediation, 446–447, 447*f*, 457, 458*f*
Biofilms, 64–65, 120
Bio-flocculation techniques, 217–218, 236–239*t*, 247–250
Biofouling, 156, 870–871
Biofuels, 87, 285*f*, 808, 828–833
advantages, 284
benefits of, 842
bio-crude oil (*see* Bio-crude oil)

Biofuels *(Continued)*
biodiesel (*see* Biodiesel production)
bioethanol (*see* Bioethanol)
biogas (*see* Biogas)
biojet fuel, 832
generation, 775
biochemical conversion, 776
fuel cells, 777
thermochemical conversion, 775–776
transesterification, 777
high-temperature thermochemical processes, 832
hydrocarbons generated by *B. braunii*, 830
hydrogen, 833
hydrothermal liquefaction, 831–832
Biogas, 759–760, 833
anaerobic digestion (AD), of microalgae, 312–314
bacteria and archaea, 315
drawbacks, 315–320
spent microalgae, 320–321
biorefinery concept, 323
nutrient and CO_2 recycling, 321–322
production, 806
brown algae production, 811–812
concepts of, 801
upgrading, 321–322
Biohydrogen production
challenges and future perspectives, 409–413
factors influencing
light intensity, 401
nutrient media, 401–402
pH, 400
temperature, 400–401
pathways, 392
biophotolysis, 393–402
dark fermentation, 402–408, 408–409*t*
process intensification (PI), 759–760
Biohythane, 411
Biojet fuel, 832
Biological extraction, 752
Biological pretreatment, 317, 407–408, 412
Biomass Crop Assistance Program, 802
Biomass harvesting, microalgae, 227, 231–232, 231*f*, 233–234*t*, 271
centrifugation, 259–261, 260*t*, 262*t*
challenges, 228–229
filtration methods, 261–267, 263–264*t*, 265*f*
flocculation
aggregates/flakes formation, 235
autoflocculation, 235–242, 236–239*t*
bioflocculation, 236–239*t*, 247–250
chemical flocculation, 236–239*t*, 242–247
physical flocculation, 236–239*t*, 250–253
flotation, 253–254
dispersed air flotation (DiAF), 255–256*t*, 257
dispersed ozone flotation, 255–256*t*, 258–259
dissolved air flotation (DAF), 254–257, 255–256*t*
electrolytic flotation, 255–256*t*, 258
gravity sedimentation, 232–235
research needs, 270–271
selection of appropriate technique, 268–270
techniques, 229–231
Biomass production, microalgae, 132*f*
applications, 226
challenges, in process cost, 226–227
cost of, 360
dissolved oxygen, 134–135
historic timeline of, 208, 209*f*
light, 103, 130–133
overall growth model, 135
pH/CO_2, 134
photobioreactors (*see* Photobioreactors (PBRs))
sunlight, 360
temperature, 133–134
water supply, 359–360
Biomass valorization, 455
Bio-oil, 832
Biophotolysis, H_2 production, 392
direct, 393–395, 394*f*
enzymes involved, 397–400
indirect, 395–397, 396*f*
Bioplastics, 781
Biopolyesters
general aspects, 600–603
raw materials, 603–606
Biopolymers, 245–247
Bioprocess optimization, 473*t*
Biorefinery, microalgae, 323, 828–829
Anabaena sp, 783, 785*f*
applications, 688, 689*f*
Arthrospira, 788
biofuel generation, 775
biochemical conversion, 776
fuel cells, 777
thermochemical conversion, 775–776
transesterification, 777
carbon dioxide mitigation, 774–775
Chlorella
C. protothecoides, 786, 787*f*
C. vulgaris, 784–786, 786*f*
concept of, 772–773
Dunaliella
D. salina, 787
D. tertiolecta, 787
Nannochloropsis sp, 782, 783*f*
Scenedesmus obliquus, 789–790, 789*f*
Spirogyra sp, 788–789, 788*f*
Tetraselmis sp, 790–791
value-added compounds, 777
carbohydrates, 778
lipids, 778
pigments, 778

polyunsaturated fatty acids, 780–781
proteins, 778
wastewater treatment, 774
Biotechnology, microalgae
biofuels, 15
biomass processing, 19
carbon sequestration, 15–17
commercially grown algae, 5*f*
commercial production, 3–4, 19
Earth, life, photosynthesis, and algae, history of, 4–6, 5*f*
harmful algae, 18–19
as human food, 7–13
nitrogen, for sustainable agriculture, 13–14
organic chemicals, 15
photosynthesizing microorganisms, 3–4
plants, 3–4
survival of life, 3–4
wastewater treatment, 17–18
Blackman reaction, 110
Blaussius equation, 138–139
Blue bioeconomy, 801
Botryococcus sp, 321–322, 760–761
B. braunii, 228–229, 333–334*t*
B. sudeticus, lipase production, 690–692*t*, 695
Brassinosterols, 581
British Petroleum (BP) Statistical Review of World Energy, 185–186
Brown algae production, 813, 815*t*
Bubble column photobioreactors, 113–114, 114*f*, 123–124, 843
Bubble size, 199
Bulk harvesting, 454
2,3-Butanedione, 670–677*t*
Butylated hydroxyanisole (BHA), 471
Butylated hydroxytoluene (BHT), 471

C

Cadmium, 169
human body, effect on, 173*t*
removal, by microalgal biomass/cultures, 175–176*t*
Calcium oxide, 760
Calvin-Benson-Bassham cycle, 40, 186–189, 197–198, 576–579
CaMV 35S promoters, 87–88
Candida antarctica, enzymatic lipase, 352
Canthaxanthin, 467, 471–472, 650*f*
Carbohydrate-active enzyme (CAZymes), 290
Carbohydrates, 92, 283–284, 778
Carbon, 718, 727
in biomass, 196–197
culture medium, retained in, 196
dissolved carbon, 195–196
gaseous, 194–195
Carbon Capture and Storage Association (CCSA), 727
Carbon capture systems (CCSs), 761
Carbon dioxide (CO_2) capture, 189–201
carbon balances for evaluation of, 194–197
carbon in biomass, 196–197
dissolved carbon, 195–196
gaseous carbon, 194–195
culture systems for, 192–194
genetic engineering and metabolic modifications, 197–199
global strategies, 197*f*
photobioreactors, 199–201
bubble dynamics, manipulation of, 199
built-in materials for, 200
configurations, 201
hybrid photobioreactors, 201
liquid medium, modifying, 200
physicochemical aspects, 191–192
light and nutrients, 191
mixing, 192
pH, 192
temperature, 191–192
process intensification in, 761–762
sources and physicochemical properties, 189–190
strains, 190–191
Carbon dioxide (CO_2) fixation, 187–189
Carbon dioxide (CO_2) mitigation, 774–775
Carbonic anhydrase (CA), 189, 197–198, 696–697
applications, 690–692*t*
production of, 697
Carbonic gas, 39
Carbon nitrogen ratio, 337
Carbon sequestration, 15–17
Carboxylation, 187, 337, 429
Cardinal model, 133–134
Carotene *cis-trans* isomerase (CRISCO), 91–92
Carotenes, 467, 469, 471
Carotenoids, 36–39, 186–187, 284, 302, 466–467, 469, 479–480, 778, 803, 807–808
astaxanthin, 779
β-carotene, 779
cell disruption methods
acid, alkali, osmotic shock, and ionic liquids, 289–290
advantages and disadvantages, 285, 286–287*t*
bead milling, 287
enzyme-based approaches, 290
high-pressure homogenization, 287–288, 288*f*
microwave, 289
ultrasonication, 288–289
chemical structure, 285, 286*f*
conventional purification methods, 292, 293*f*
extraction methods
organic solvent extraction, 290*t*, 291
supercritical fluid extraction, 290*t*, 291–292
global market, 779
lutein, 779
lycopene, 780
ROS scavenging, 649
structures of, 650*f*
synthetic biology, 91–92
UV protective effects, 649–651
zeaxanthin, 779
Cascade flumes, 876–877
Cellana system, 875

Cell density, 336
Cell disruption methods
carotenoids
acid, alkali, osmotic shock, and ionic liquids, 289–290
advantages and disadvantages, 285, 286–287*t*
bead milling, 287
enzyme-based approaches, 290
high-pressure homogenization, 287–288, 288*f*
microwave, 289
ultrasonication, 288–289
process intensification, 749–752, 750*f*, 753*t*, 845*t*
bead milling, 749–750
biological extraction, 752
high-pressure homogenization, 750
high-speed homogenization, 750
microwave, 751
pulsed electric field (PEF), 752
supercritical fluid extraction, 751
ultrasonication, 751
Cell drying, 748–749, 749*t*
Cell lysis, microalgae, 498
Cell ultrastructure, of microalgae, 27–30
eukaryotic cells, 29–30
prokaryotic cells, 29
Centrifugation method, 208, 210–211, 748
biodiesel production, 344
bioethanol production, 382–383
microalgal biomass recovery, 227, 259–261, 260*t*, 262*t*
Cesium, 175, 175–176*t*
Cetyltrimethylammonium bromide (CTAB), 254–257
C18 fatty acids, 354–355
Charophyta, 33–34
Chemical engineering, 101
Chemical flocculation, 236–239*t*, 242–247, 344
Chemical pretreatments, 383, 407
Chemical reactors, 101–103, 102*f*
China Food and Drug Administration (CFDA), 562–563
Chitosan, 209–210, 245–246, 382
Chlamydomonas sp, 232–235, 236–239*t*, 247, 316–317, 756, 760–761
C. polypyrenoideum, 760
C. reinhardtii, 49–50, 86–88, 89*t*, 91–92, 94, 178*t*, 319–320, 580, 755–759
amylase activity, 692–693
biorefinery, 786–787
carbohydrate accumulation, 405
carbonic anhydrase, 697
genetic modification, 342–343
H_2 production, 398*t*, 401, 406–407, 410–411
hydrogenase in, 395
phosphorus depletion, 338–339
cultures, 873
pH effect, 340–341
Chlorella sp, 7, 33, 128, 146–150, 152, 157–158, 190–191, 200, 216, 232–235, 236–239*t*, 242–246, 254–258, 255–256*t*, 260–267, 260*t*, 301, 314, 316–319, 321–322, 469, 471–472, 479, 742–743*t*, 756, 760–761, 763, 848
C. ellipsoidea, 343, 469
C. marina, 780
C. protothecoides biorefinery, 786, 787*f*
C. pyrenoidosa, 178*t*
C. sorokiniana, 178*t*, 317, 692, 742–743*t*, 752–753, 760
C. stigmatophora, 763
C. vulgaris, 33, 178*t*, 212, 215, 315, 318–319, 323, 469, 471, 581, 742–743*t*, 747, 752–753, 760–761, 827
asparaginase activity, 700
biodiesel production, 849–857
biorefineries, 784–786
carbohydrate accumulation, 404–405
heavy metals, effect of, 341
microwave irradiation of biomass, 406–407
protease activity, 694
C. zofingiensis, 427–428
lipid content
and biomass yield, 332*t*
and productivity, 332, 333–334*t*
microwave-assisted hexane extraction, 349
Chloride salts, 242–244
Chlorogloea fritschii, 607–608
Chlorophyll, 36–39, 482, 778, 780
chlorophyll *a*, 31, 33–35, 37–38, 107–108, 186–187, 214–215, 466–467
chlorophyll *b*, 31, 33–34, 37–38, 186–187, 466
chlorophyll *c*, 35, 37–38, 466
chlorophyll *d*, 31, 37, 466
chlorophyll *f*, 37
Chlorophyta, 27, 28*t*, 30–31, 33–34, 38
Chloroplast carbonic anhydrase (chCA), 189
Chloroplasts, 29–30
Chromatoplasm, 29
Chromium, 169
human body, effect on, 173*t*
removal, by microalgal biomass/cultures, 175–176*t*
Chroococcus sp, 322
Chrysochromulina polylepis, 34
Chrysophyta (gold algae), 575–576
Ciguatera, 34–35
Circular economy, 801–802
Circulation time approach, 124*t*
Citronellol, 670–677*t*
Closed photo-bioreactors, 452–454
Closely spaced flat panel photobioreactor, 876
Clustered regularly interspaced short palindromic repeats associated protein 9 (CRISPR-Cas9), 198–199
Coagulation, 744–747
Cobalt, 175–176*t*
Coccolitophyceae, 34

CO_2 concentrating mechanisms (CCMs), 189
Coelastrella sp, 693
Coenobium, 27
Colorants, 169
  elimination of, 176–177
  removal, by microalgae and cyanobacteria, 177*t*
Compensation irradiance (Ic), 130
Compound annual growth rate (CAGR), 737–738
Computational fluid dynamics (CFD), 211–212, 869–870, 873
Concentration factor (CF), 229–230, 260–267
Condensation, VOC recovery, 678
Conditionally essential (CEAAs), 505
Confidence interval (CI), 816
Convective drying method, 214
Co-pelletization, 248–249
Copper, 169
  human body, effect on, 173*t*
  removal, by microalgal biomass/cultures, 175–176*t*
Co-solvents, biodiesel production, 358
Cross-flow filtration, 211, 262–267
Crude glycerol, 719
Cryobiology, 74–77
Cryopreservation, 70–77, 72*f*
  advantages of, 71*t*
  applications of, 71
  cryoprotectants, 73–74
  disadvantages of, 71*t*
  encapsulation-dehydration process, 77
  sources of damage, 73–74
Crypthecodinium cohnii, 340, 421–424, 431–434
Cryptophyta, 38
Cultivation, microalgae biofertilizer
  closed photo-bioreactors (PBRs), 452–454
  high-rate algal ponds (HRAP), 452, 453*f*
  open ponds, 452
  in wastewater, 455–456
Cultivation systems, scalability of
  cascade flumes, 876–877
  closely spaced flat panel photobioreactor, 876
  flat panel reactors
    depth, 875–876
    height, 875
    width, 876
  raceway ponds
    area, 876
    depth, 876
  reactor unit size, 874
  tubular photobioreactors
    tube diameter, 875
    tube length, 874–875
Culture Collection for Algae and Protozoa (CCAP), 80–81
Culture collections, microalgae, 49–50, 75–76*t*
  across the world, 78–83
  basic laboratory infrastructure, 52–61, 52–53*f*
    climate-controlled rooms, 52–54, 52*f*
    growing facilities, 54*f*
    high efficiency particulate air filter, 61
    horizontal rods, 56*f*
    incubator chambers, 52–54, 53*f*
    larger experimental flasks, 52–54, 53*f*
    lighting, 58–60, 59*f*
    microalgae culture vials, incubator, 55*f*
    photonic flux density, 60
    temperature, 55–57
    transfer chambers, 60–61
  categories, 51
  cryopreservation, 70–77, 72*f*
    advantages and disadvantages of, 71*t*
    applications of, 71
    cryoprotectants, 73–74
    encapsulation-dehydration process, 77
    sources of damage, 73–74
Culture Collection of Algae and Protozoa (CCAP), 80–81
culture identification, 51, 62*f*
culture propagation, 63–66
  curator, 82
  diversification of services, 83
  growing conditions, 66–68
  operation of, 50–78
  Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), 80
  strain quality control and routine problems, 68–70
Cupriavidus necator, 600, 602*f*, 610
Cyanobacteria, 3–4, 26–27, 28*t*, 29–33, 39, 42, 77, 88, 90–91, 94, 188, 419–420, 465–466, 474, 479, 805, 827–828
  biofertilizers, 447–449, 782
  carbohydrate-enriched, 312–313
  cell of, 29
  polyhydroxyalkanoate (PHA) (*see* Polyhydroxyalkanoate (PHA), cyanobacteria)
  polysaccharides (PS)
    antibacterial activity, 553–554
    antifungal activities, 554–555
    antiviral activity, 551–553
  ribosomes in cyanobacteria cells, 29
  SODs of, 696
Cyanobacterial hydrolysate, 834
Cyanophyta, 26–27, 38, 41–42
Cyanothece sp, 398*t*
Cycloartenol synthase (CAS), 579
Cyclohexanes, 574
Cyclohexanol, 670–677*t*
Cyclopentaneperhydrophenanthrene, 574
Cytosolic carbonic anhydrase (cyCA), 189

D

DAF. *See* Dissolved air flotation (DAF)
Dark fermentation, biohydrogen production, 402–404
  challenges, 411
  formate decomposition pathway, 403
  microalgal biomass
    as feedstock, 404–405, 408–409*t*
    pretreatment, 405–408

Dark fermentation, biohydrogen production *(Continued)*
NADH re-oxidation pathway, 403
photofermentation integrated system, 411
Dead end filtration (DEF), 262–267
2,4-Decadienal, 664, 677*t*
Degree of polymerization (DP), 535
1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 576–579
Desmodesmus sp, 178*t*, 877
Dewatering methods, of algae processing, 209–213, 210*f*
centrifugation, 210–211
for commercialization, 216–217, 217*t*
electrophoresis methods, 213
filtration, 211
flocculation, 209–210
flotation, 212
future perspective, 217–218
gravity sedimentation, 211–212
DGAT isoforms, 431, 434–435
DHA. *See* Docosahexaenoic acid (DHA)
Diadinoxanthin, 35
DiAF. *See* Dispersed air flotation (DiAF)
Diarrhetic shellfish poisoning, 34–35
Diatoms, 419–420
Bacillariophyceae, 35
palmitoleic acid, 355
Diazotrophic cyanobacteria, 448
3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), 612–613
Diethanolamine (DEA), 200
Digestates, 322
Digestibility, 506–507, 508*t*
fecal digestibility, 506–507
simulated in vitro enzymatic assays, 506–507, 508*t*
species-specific in vivo digestion bioassays, 506–507, 509–510*t*
Dimethylallyl diphosphate (DMAPP), 661, 668
Dimethyl disulfide, 670–677*t*
2,4-Dimethylheptane, 670–677*t*
3,3-Dimethyl-hexane, 677*t*
2,4-Dimethyl-3-pentanone, 677*t*
Dimethyl sulfide (DMS), 665–666, 667*f*, 720
Dimethyl sulfonic propionate (DMSP), 720
Dimethyl trisulfide, 670–677*t*
4,7-Dimethyl-undecane, 677*t*
Dinitrogenase reductase, 42
Dinoflagellates, 28*t*, 30–31, 34–35, 283–284
Dinophyta, 26–27, 576
Diode array detector (DAD), 586–587
1,3-Diphosphoglycerate, 40
Direct biophotolysis, H_2 production, 392–395, 394*f*
Direct lipid extraction, 349
Dispersed air flotation (DiAF), 255–256*t*, 257
Dispersed ozone flotation, 255–256*t*, 258–259
Dissolved air flotation (DAF), 254–257, 255–256*t*
Dissolved carbon, 195–196
Dissolved inorganic carbon (DIC), 190, 200
Dissolved oxygen (DO), 134–135, 141–143, 142*f*, 148–150, 154, 337, 867
DNA barcoding techniques, 50
Docosahexaenoic acid (DHA), 6, 91, 320, 421–422, 426–427
Dodecane, 670–677*t*
Dodecyl amine hydrochloride (DAH), 212, 257
N-Dodecylpropane-1,3-diamine hydrochloride (DN2), 212
Dodecyl pyridinium chloride (DPC), 212, 257
Downstream processing, 713*b*, 772
Drying methods
for algae processing, 213–215, 214*f*
convective drying, 214
freeze-drying, 215
solar drying, 213–214
spray-drying, 214–215
biodiesel production, 345–346
flash drying, 346–347
freeze-drying, 347–348
rotary dryers, 346
solar drying, 346
spray-drying, 347
Dry weight (DW), 230, 260–267
Dunaliella sp, 127–128, 146–147, 316–317, 468–469, 471, 483, 756
D. marina, amylase activity, 692–693
D. salina, 26–27, 33–34, 318–319, 467–468, 475–476, 481
biorefinery, 787
lipid content and productivity, 333–334*t*
temperature effect, 339
D. tertiolecta, 315, 763
β-galactosidase activity, 699
biorefinery, 787
peroxidase activity, 693
salinity stress, 340
Dynamic flow membrane, 211

E

EAA index (EAAI), 505–506
Echinenone, 31, 38, 650*f*
EFBR, 436
Effluent treatment plants (ETPs), 446–447, 457
Eicosapentaenoic acid (EPA), 91, 215, 320, 421–422, 426–427, 773, 780–781
Electrical base process, 747–748
Electro-coagulation-flocculation, 213
Electro-coagulation-flotation (ECF), 213, 747–748
Electroflocculation, 251–252, 345
Electrolytic flotation, 255–256*t*, 258
Electrophoresis, 208, 213, 251–252
Electroporation, lipid extraction, 349
Elemental nitrogen analysis, 503–505

Elimination capacity (EC), 194
Embden-Meyerhof-Parnas (EMP) pathways, 41, 427, 576–579
Emerging pollutants, 169, 177–178, 178*t*
Encapsulation-dehydration process, 77
Encephalomyocarditis virus (EMCV), 553
Enclosed photobioreactors (EPBRs), 435–436, 436*t*
Endopeptidases, 694
Endoplasmic reticulum (ER), 29–30
Energy integration, 715–718
Energy potential, VOCs, 669, 677*t*
Energy return on investment (EROI), 727, 826–827, 830
Enveloped viruses, 551–553
Environmental stress, 428–429, 431–435, 432–434*t*
Enzymatic catalysis, 352
Enzymatic pretreatment, 383–384, 407–408
Enzyme synthesis, 689, 690–692*t*
EPA. *See* Eicosapentaenoic acid (EPA)
Ergosterol, 580–581
Escherichia coli, 86–88, 92, 696
Essential amino acids (EAAs), 505, 507–511
Ester, 670–677*t*
Ethanol, 376–377, 670–677*t*, 805, 853–857
Ethylene diamine tetracetic acid (EDTA), 471
2-Ethylfuran, 670–677*t*
2-Ethyl-1-hexanol, 670–677*t*
Euglena sp
  E. gracilis, 341, 763
  peroxidase activity, 693
Euglenophyta, 28*t*, 30–31, 34, 576
Eukaryotic cells, 29–30
Eukaryotic microalgae, 6, 33–36, 188, 465–466
  diatoms (Bacillariophyceae), 35
  dinoflagellates (Dinophyceae), 34–35
  Euglenophyta, 34
  Eustigmatophyceae, 35
  green algae (Chlorophyta/Charophyta), 33–34
  Haptophyta, 34
  red algae (Rhodophyta), 35–36
Eulerian approach, 122, 124*t*
Europea Marine Observation and Data Networkb (EMODnet), 803–805
Eustigmatophyceae, 28*t*, 30–31, 35
Eutrophication, 167
Evaporation, 144–146
Exergy, 846–848
  applied to biomass processes, 847–848
  case studies, 848–857
  concepts of, 846
  definition, 846–847
  efficiencies, 846–847
  of flow, 846–847
  formulation, 846–847
  in microalgae-feedstock biorefinery, 848
Exopeptidases, 694
Exopolysaccharides, 29
Extracellular polymeric substances (EPS), 201, 235–242, 248–250, 617–618
Extracellular polysaccharides (ECP), 534
Extremophile cyanobacterial PHA producers, 625, 626*f*
  extreme pH conditions, 625
  halophilic cyanobacteria, 624–625
  thermophile cyanobacteria, 623–624

F

Farnesyl pyrophosphate synthase (FPPS), 579
Fast protein liquid chromatography (FP-LC), 500
Fatty acid biosynthesis pathway, 664, 665*f*
Fatty acid ethyl esters (FAEE), 353, 356–357
Fatty acid methyl esters (FAMEs), 321–322, 350, 752
Fatty acids, 91
  compositions and potential applications, 425*t*
  biofuel feedstock, 424–426
  photoautotrophs, 424–426
  polyunsaturated fatty acids (PUFA) production, 421–424, 426–427, 434–435
  in vegetable oil, 354–355
Fatty acid synthase (FAS), 429–430, 434–435
Fecal digestibility, 506–507
Feed conversion ratios, of farmed animals, 10, 11*t*
[Fe-Fe] hydrogenase, 397–398, 409
Fermentation, 40–41, 378–379, 384
Ferredoxin (Fd), 29, 393
Ferredoxin-$NADP^+$ reductase (FNR), 187
Fertilizer, 835
FeSOD, 695–696
Filamentous microalgae, 27
Filtration methods, 208, 211, 261–267, 263–264*t*, 265*f*, 345, 748
Firmicutes, 315
First-generation biofuels, 311–312
FishStatJ, 808
Flame ionization detector (FID), 586
Flash drying, 346–347
Flashing light effect, 110–111
Flat panel photobioreactors, 116, 155*f*, 843
  advantages of, 154–155
  biofouling and outdoor contamination risks, 156
  closely spaced flat panel photobioreactor, 876
  depth, 875–876
  geometry, 136
  height, 154–155, 875
  light dilution effect, 154–155
  mass transfer, 156
  width, 876
Floating bioreactors, 120
Flocculation techniques, 208–210, 227, 454, 744–747
  advantages, 233–234*t*
  aggregates/flakes formation, 235

Flocculation techniques *(Continued)*
autoflocculation, 235–242, 236–239*t*
for bioethanol production, 382
bioflocculation, 236–239*t*, 247–250
chemical flocculation, 236–239*t*, 242–247, 344
disadvantages, 233–234*t*
physical flocculation, 236–239*t*
electroflocculation, 251–252
magnetic separation, 252–253
ultrasound-assisted flocculation, 250–251
Florideans starch, 35–36
Flotation technique, 208, 212, 227, 253–254, 454, 747
advantages and disadvantages, 233–234*t*
dispersed air flotation (DiAF), 255–256*t*, 257
dispersed ozone flotation, 255–256*t*, 258–259
dissolved air flotation (DAF), 254–257, 255–256*t*
electrolytic flotation, 255–256*t*, 258
Flow reactors, 102–103
Formate decomposition pathway, 403
Formic acid, 497
Forward osmosis membrane, 211
Free fatty acids (FFAs)
biodiesel process, 355–356
microalgal lipids, 358
Free radical scavenging, 556–557
Freeze-drying, 215, 347–348
Friction coefficient, 138–139
Fructose 6-phosphate (F6P), 41
Fucoxanthin, 35, 38, 651, 654*t*
Fuel cells, microbial, 777
Fungi-mediated harvesting technology, 248–249
Furan, 670–677*t*

G

Galactooligosaccharides, 699
Galactosidases, 698–699
applications, 690–692*t*
production of, 699
Galdieria sulphuraria, peroxidase activity, 693
γ-carotene, 33–34, 650*f*
Gamma-linolenic acid (GLA), 91, 426–427
Gas chromatography (GC), 194–195, 585–586
Gaseous carbon, 194–195
Gasification, 775–776
Gas-liquid mass transfer, 148–150
Gemini surfactant, 212
Genetic engineering
biodiesel production, 342–343
CO_2 capture, improvement of, 197–199
PHA biosynthesis, cyanobacteria, 627–632
Genome scale modeling (GSM), 422–424
Geosmin synthesis, 661–664, 664*f*, 670–677*t*
Geraniol, 670–677*t*
Gibberellic acid 3 (GA_3), 474–475
Gibbs free energy, 847
Gluconate dehydrogenase, 41
Glucose 6-phosphate (G6P), 41
Glucose-6-phosphate dehydrogenase (G6PDH), 428–429
Glyceraldehyde 3-phosphate, 40–41
Glycohydrolases (GH), 689–692
Glycolipids, 354–355
Glycolysis, 41
Glyoxysomes, 427
Gold, 175–176*t*
Golgi apparatus, 29–30
Gravity sedimentation, 208, 211–212, 227, 232–235, 233–234*t*, 454
Green algae, 33–34, 419–420, 812, 815*t*, 816
Green/biosynthesized nanoparticles, 217–218
Greenhouse gas emissions, 829
Green plastics, 600–602
Green Wall Panel-II (GWP-II) photobioreactors, 721–722
Green Wall Reactor, 156

H

Haematococcus pluvialis, 33–34, 467–468, 477, 481, 484, 651, 742–743*t*, 762, 779
Halomicronema hongdchlores, 37–38
Halophilic cyanobacteria, 624–625
Haptophyta, 28*t*, 30–31, 34
Harmful algae, 18–19
Harvesting techniques, 844
biodiesel production (*see* Biodiesel production, harvesting microalgae)
for bioethanol production, 381–383
bulk harvesting, 454
microalgae production, intensification of, 741–748, 744*f*
centrifugation, 748
coagulation/flocculation, 744–747
electrical base process, 747–748
filtration, 748
flotation, 747
sedimentation, 747
for microalgal biomass recovery, 227, 231–232, 231*f*, 233–234*t*, 271
centrifugation, 259–261, 260*t*, 262*t*
filtration methods, 261–267, 263–264*t*, 265*f*
flocculation (*see* Flocculation techniques)
flotation (*see* Flotation)
gravity sedimentation, 232–235
research needs, 270–271
selection of appropriate technique, 268–270
thickening, 455
Heavy metals, 169
human body, effect on, 173*t*
pollution with, 173

removal of, 172–175, 175–176*t*
Helical flow promoters (HFP), 114
Helicobacter sp, 470
Helioguard 365, 652
Helionori, 652
Henry's law constant, 141–143, 190–192
Heptadecane, 670–677*t*
2,4-Heptadienal, 677*t*
Heptanal, 670–677*t*
2-Heptanone, 670–677*t*
Herpes simplex virus (HSV), 551–552
Heterochlorella luteoviridis, 762
Heterocystous cyanobacteria, 401–402, 447–448
Heterocysts, 43, 94, 395–396
Heterotrophic bioreactors, 712*b*
Heterotrophic cultivation, 334, 437, 610, 620
Heterotrophic cultures, 334–335, 666, 865
Heterotrophic microalgal biomass, 861, 864
Hexadecane, 670–677*t*
Hexanal, 670–677*t*
Hexane, 348
1-Hexanol, 670–677*t*
3-Hexen-1-ol, 670–677*t*
Hexose uptake protein (HUP1), 437
High-density lipoprotein/low-density lipoprotein (HDL/LDL), 561–562
High performance liquid chromatography (HPLC), 586
High-pressure homogenization (HPH), 286–287*t*, 287–288, 288*f*, 498, 750, 762
High-rate algal ponds (HRAP), 18, 168, 452, 806
High-speed homogenization, 750
High-temperature thermochemical processes, 832
Homogeneous base catalysts, 351
Horizontal photobioreactors, 843
Horizontal tubular reactors (HTRs), 192–193
HPH. *See* High-pressure homogenization (HPH)
HRAP. *See* High-rate algal ponds (HRAP)
Human immunodeficiency virus (HIV), 551–552
Humidity, of microalgal biomass, 319
Hybrid photobioreactors, 120, 121*f*, 201
Hydrocarbon, 670–677*t*
  carotenoids, 649
  generated by *B. braunii*, 830
  unbranched hydrocarbon production, 664–665
Hydrochloric acid (HCl), 497
Hydrogen, 391, 833. *See also* Biohydrogen production
Hydrogenase, 94
Hydrogen-producing bacteria (HPB), 392, 402–403
Hydrolysis, 298*f*, 299
Hydrolysis-acidogenesis, 312
Hydrolytic-acidogenic bacteria, 315–316
Hydrolytic enzymes, 407
Hydrophilic phycobiliproteins, 186–187
Hydrophobic pigment-protein complexes, 186–187
Hydrothermal liquefaction (HTL), 301–303, 831–832
Hydrothermal pretreatments, 316–317
3-Hydroxy-2-butanone, 670–677*t*
3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR), 576–579
Hyperbolic model, 131
Hypercholesterolemia, 32
Hyperglycerolemia, 32

I

Ileal digestibility, 506–507
Immobilization, 172
Immobilized bed reactor, 103–104
Immunomodulatory activity, 558–559
Incubator chambers, 52–54, 53*f*
Indirect biophotolysis, H_2 production, 392, 395–397, 396*f*
Indole-3-butyric acid (IBA), 474–475
Inflammatory diseases, 32
Influenza (IFV) A virus, 551–552
Infrared sensors (IRs), 194–195
Inhibition irradiance (Ii), 130
In situ supercritical transesterification, 354–358
In situ transesterification (IST), 297, 297*f*, 352–353, 356–357
Integrated biorefineries, 808
Integrated Multi-Trophic Aquaculture (IMTA), 806
Intergovernmental Panel on Climate Change (IPCC), 186
International Energy Agency (IEA), 329–330
Interrupted tricarboxylic acid cycle, 607–608
InteSusAl, 878
Inulin, 247
Inverse metabolic engineering, 629
Ion exchange chromatography (IEX), 500
Ionic liquids (ILs)
  carotenoids, 289–290
  lipid extraction, 296
Iron chloride ($FeCl_3$), 242–244
Iron sulfate ($Fe_2(SO_4)_3$), 242–244
Irradiance, 130–133, 130*f*, 132*f*
Isoamylase, 689–692
Isobutanol, 670–677*t*
Isochrysis sp
  I. galbana, 214–215, 690–692*t*, 695, 763
  lipid content and productivity, 333–334*t*
Isofuncoxanthinol, 654*t*
Isopentenyl diphosphate (IPP), 661
Isoprenoid biosynthesis pathways, 661, 662*t*, 663*f*
IST. *See* In situ transesterification (IST)

J

Jones factor, 503–505

K

Kennedy pathway, 430, 434–435
2-Keto acid pathway, 661, 661*f*
Ketones, 670–677*t*
Klebsormidium sp., amylase activity, 693
Kolmogorov equation, 874
Kral collection, 49–50
Krebs cycle, 41, 43

L

Laccases, 698
  activity of, 698
  applications, 690–692*t*
Lactase, 699
Lagrangian approach, 105, 122, 124*t*
Lambert-Beer equation, 104–105, 113–114
Lead, 169
  human body, effect on, 173*t*
  removal, by microalgal biomass/cultures, 175–176*t*
Life cycle assessment (LCA), 723–726, 823
  environmental impacts, interventions, 826*b*
  goal and scope definition stage, 824
  impact assessment stage, 825
  interpretation stage, 827
  inventory analysis stage, 825
  peer-reviewed, applications of, 827–836
    biofuels, 828–833
    fertilizer, 835
    in food and feed sector, 833–835
    phycocyanin, 835
    polylactic acid (PLA) blends, extracted microalgal residue for, 835
    wastewater treatment, 835–836
  stages, 823–824
Light dilution effect, 154–155
Light emitting diodes (LEDs), 754
Light integration factor, 133
Light intensity, H_2 production, 401
Light irradiation stress, 341–342
Light saturation irradiance (Is), 130
Limnothrix sp, 474
Linalool, 670–677*t*
Linear Fresnel lenses, 871
Lipase-catalyzed transesterification, 352
Lipases, 690–692*t*, 694–695
Lipid/oil extraction, 295*f*
  biodiesel production (*see* Biodiesel production)
  ionic liquids, 296
  organic solvent extraction, 293–296
  supercritical fluid extraction, 296–297
Lipids, microalgae, 778
  biodiesel production
    autotrophic cultivation, 332–334
    cultivation conditions, 332, 333–334*t*
    as feedstock, 331–332, 332*t*
    heavy metals, effect of, 341
    heterotrophic cultivation, 334
    light irradiation stress, 341–342
    mixotrophic cultivation, 335
    nutrient starvation, 338–339
    pH effect, 340–341
    photoheterotrophic cultivation, 335
    salinity induction, 340
    temperature stress, 339–340
    UV irradiance, 342
  biosynthesis, 427–430
  chemical composition, 354–356
  processing parameters, 356–358
  synthesis, 90–91
  water and FFAs, effect of, 358
Liquefied petroleum gas (LPG), 584–585
Liquid biphasic flotation, 212
Liquid–liquid triphasic system (LTPS), 790
Liquid nitrogen, 71–72
Living biological collections, 49–50
Long-chain polyunsaturated fatty acids (LC-PUFA), 426–427, 429–430
Low energy algae reactor (LEAR), 147–148
Lutein, 38, 285, 286*f*, 320, 467, 469–472, 477–478, 480–482, 779
  in cosmetic industry, 651, 654*t*
  structure of, 650*f*
Lycopene, 91–92, 650*f*, 780
Lyngbya sp., H_2 production, 398*t*, 402
Lysophosphatidic acid acyltransferase (LPAAT) enzyme, 430
Lysosomes, 29–30

M

Macroalgae, 3–4, 331, 803, 805–806
Macrofiltration, 262–267, 265*f*
Magnetic nanoparticles, 216–218
Magnetic separation, 252–253
Magnetite nanoparticles, 216
Malic enzyme (ME), 428–429
Malondialdehyde, 468–469
Malonyl-acylcarrier protein (malonyl-ACP), 429
Maltogenic amylase, 689–692
Manning's equation, 138–139
Mass integration, 718–720
Mass transfer capacity, 141–143
  flat panel photobioreactors, 156
  raceway reactors, 148–150
  tubular photobioreactors, 154
Mastigocladus, 31–32
Mathematical model, 103, 111
Mechanical extraction, lipid, 348
Mehler reaction, 94
Membrane filtration, 262–267
Menthol, 670–677*t*
Mercury, 169, 175–176*t*
Metabolic aspects of microalgae, 36–43
  nitrogen fixation, 42–43
  photosynthesis, 39–40
  photosynthetic pigments, 37–39
    carotenoids, 38
    chlorophyll, 37–38
    phycobiliproteins, 38–39
  respiration, 40–42

Metabolic engineering, 431–435, 432–434*t*
Methanol, 849–853
2-Methoxy-2-methylpropane, 670–677*t*
3-Methylbutanal, 661, 670–677*t*
2-Methylbutanol, 670–677*t*
Methylerythritol phosphate (MEP) pathway, 576–579, 661, 662*t*, 663*f*
6-Methyl-5-hepten-2-one, 670–677*t*
2-Methylisoborneol (2-MIB)
  biosynthetic pathway, 661–664, 664*f*
  comparative analysis, 670–677*t*
Methyl 3-methyl 2-hydroxybutanoate, 670–677*t*
Methyl octanoate, 670–677*t*
2-Methyl-1-pentanol, 670–677*t*
Methyl phenylacetate, 670–677*t*
2-Methylpropanal, 670–677*t*
Mevalonic acid (MVA) pathway, 576–579, 661, 662*t*, 663*f*
Micractinium, 318–319
Microalgae, 85–86, 225–226, 331, 803
  advantages, 226–227
  amino acid profile, 505–506
  applications, 226, 374
  biochemical contents, 375–376, 375*t*
  bioethanol production (*see* Bioethanol)
  biofertilizers (*see* Biofertilizer, microalgae)
  biorefineries (*see* Biorefinery, microalgae)
  biotechnology (*see* Biotechnology, microalgae)
  carbohydrate accumulation, 376
  carbon dioxide (CO_2) uptake, 226
  cell ultrastructure of, 27–30
    eukaryotic cells, 29–30
    prokaryotic cells, 29
  characteristics, 28*t*
  components, 375–376
  cultivation of, 740–741
    closed system, 741
    heterotrophic cultivation systems, 719
    open-pond system, 740
  culture collections (*see* Culture collections, microalgae)
  environmental conditions, 225–226, 374
  feedstock biorefineries, current state of, 845–846
  lipid/carotenoid content of, 376
  mass culturing for aquaculture, 421
  metabolic aspects of (*see* Metabolic aspects of microalgae)
  morphological aspects of, 26–27
  nutrients, uptake of, 226
  open systems, 375
  parameters, 129, 129*f*
  PHA production, cyanobacteria (*see* Polyhydroxyalkanoate (PHA), cyanobacteria)
  pigments (*see* Pigments, microalgae)
  producers of, 807–808
  products, 710–714, 712*f*
    cultivation systems, 712*b*
    downstream processing, 713*b*
    process intensification methods (*see* Process intensification (PI), microalgae-based processes and products)
  scale-up process (*see* Scale-up process)
  single cell oils (*see* Microalgae single cell oils (MSCOs))
  size, 374
  sterols (*see* Sterols, microalgae)
  wastewater remediation, role in, 226
Microalgae single cell oils (MSCOs)
  biosynthetic pathway
    lipid biosynthesis, 427–430
    regulation, environmental stresses and metabolic engineering, 431–435, 432–434*t*
  C. reinhardtii, 422–424
  Crypthecodinium cohnii, 421–424, 431–434
  cultivation systems, 435–437
  current market and challenges, 437–439
  defensive initiatives, 421
  definition, 420
  development, 420–424, 423*f*
  docosahexaenoic acid (DHA), 421–422, 426–427
  eicosapentaenoic acid (EPA), 421–422, 426–427
  eukaryotic microalgae, 419–420, 422–424
  fatty acid compositions and potential applications, 425*t*
    biofuel feedstock, 424–426
    photoautotrophs, 424–426
    in polyunsaturated fatty acids (PUFA) production, 421–424, 426–427, 434–435
  genome-scale metabolic model, 422–424
  heterotrophic microalgae, 419–420, 427
  lipids, 420
  mixotrophic microalgae, 427–428
  MSCO-producing strains (MSCOPS), 422–424
  oleaginous microalgae, 424
  photoautotrophic microalgae, 428–429
  phototrophic microalgae, 419–420
  prokaryotic microalgae, 419–420
  triacylglycerol (TAG), 422–424
Microbial electrochemical cells (MECs), 411–412
Microbial fuel cell (MFC), 411–412
Microcystis
  M. aeruginosa, 250–251
  M. flos-aquae, 668
Microfiltration, 262–267, 265*f*, 748
Micro RNA (miRNA), 198–199
Microscreen harvesting method, 381–382
Microstrainers, 263–264*t*, 267
Microwave
  irradiation, 406–407

Microwave *(Continued)*
lipid extraction, 349
process intensification, 751
Microwave-assisted cell disruption, 289
Microwave-assisted extraction (MAE), 550–551
Microwave-assisted pyrolysis (MAP), 301
Mitochondria, 29–30
Mixotrophic cultivation, 666
lipid-rich microalgae, 335
Synechocystis sp., carbon-supply regimes, 620–622
Mixotrophy, microalgal biomass, 861, 864–866
MMAs. *See* Mycosporine-like amino acids (MMAs)
MnSOD, 695–696
Moloney murine leukemia virus, 552
Moloney murine sarcoma virus, 552
Monod's model, 131
Monoethanolamine (MEA), 200
Monoraphidium dybowskii, 753–755
Moringa oleifera derivatives, 247
Moringa oleifera seed flour, 209–210
MSCOs. *See* Microalgae single cell oils (MSCOs)
Mucilage, 27
Muriellopsis sp, 467–468, 477–478
Mycosporine-glycine, photostability of, 652
Mycosporine-like amino acids (MMAs), 648
photoprotective potential, 651–653
structure of, 651, 652*f*
Myristic acid, 354–355
Myxoxanthophyll, 31, 38

N

NADH re-oxidation pathway, 403
Nannochloris, 178*t*
Nannochloropsis sp, 35, 89*t*, 90, 127–128, 146–147, 150–152, 155–156, 190–191, 321–322, 426–427, 742–743*t*
biorefinery, 782, 783–784*f*
light irradiation stress, 341
N. gaditana, 316–317, 321, 356
N. oculate, 214–215
lipid content and productivity, 332, 333–334*t*
UV irradiance, 342
N. salina, 211–212, 215, 318–319
Nano titanium dioxide-modified hollow fiber membrane, 216, 217*t*
National Bioeconomy Blueprint, 802
National Renewable Energy Laboratory (NREL), 330–331, 424–426
Natural polymers, 209–210
Navicula, 178*t*
Needle-shaped hydroxyapatite nanoparticles, 216
Neochloris oleoabundans, 332, 333–334*t*
Neofucoxanthin, 35
Neoxanthin, 38, 654*t*
Neurotoxic shellfish poisoning, 34–35
Neutral lipids, 215, 330, 354–355
N-3 fatty acids, 834–835
Nickel, 169
human body, effect on, 173*t*
removal, by microalgal biomass/cultures, 175–176*t*
Nicotinamide adenine dinucleotide phosphate (NADPH), 186–187, 576–579
[Ni-Fe] bidirectional hydrogenase, 398–399, 402
[Ni-Fe] uptake hydrogenase, 399
Night biomass loss, 861–863
NiSOD, 695–696
Nitrogen, 168, 191, 719, 725–726
fertilizer, 835
fixation, 14, 42–43, 447–449, 448*t*, 456*t*
limitation, 376
removal of, 169–172
starvation, 339
for sustainable agriculture, 13–14
Nitrogenase, 94, 392, 395–396
reductase, 42
types, 399–400
Nitrogen oxides (NO_x), 377, 726
Nitrogen-toprotein (N-to-P) conversion factor (k_p), 503–505, 504*t*
2,6-Nonadienal, 670–677*t*
Nonanal, 664, 670–677*t*
2-Nonanone, 670–677*t*
Nonessential amino acids (NEAAs), 505
Nonphotochemical quenching (NPQ), 400–401, 410
Nonprotein nitrogenous (NPN) compounds, 503–505
Nostoc sp, 29, 32, 42, 207–208, 472–475
carbon-supply regimes, 619
H_2 production, 398*t*, 400–401
N. muscorum, 615–616, 619, 627
Nucleus, 29–30
Nutrients
carbon dioxide (CO_2) capture, 191
carbon dioxide (CO_2) recycling, 321–322
media, biohydrogen production, 401–402
starvation, 338–339
Nylon 6, 6 nanofiber, 216

O

Ocillatoria, 42
3,5-Octadien-2-one, 670–677*t*
2-Octanedione, 670–677*t*
2-Octenal, 670–677*t*
1-Octen-3-ol, 670–677*t*
4-Octen-3-one, 677*t*
Oedogonium sp
amylase activity, 692–693
peroxidase activity, 693
superoxide dismutase activity, 696
Oil-to-methanol molar ratio, 357–358
Oleaginous microalgae, 420
Olive mill solid waste (OMSW), 318–319
Omega-3 fatty acid, 6, 320

Omega-6 fatty acid, 6
Open pond systems, 335, 436, 452
Open reading frame (ORF), 611
Optical density (OD), 230
Optimal cell density, 336
Optimal temperature, 337
Opuntia maxima, 318–320
Ordinary least squares (OLS) regression, 816
Organelles, 29
Organic chemicals, 15
Organic solvent extraction
  carotenoids, 290*t*, 291
  lipid extraction, 293–296
Oscillatoria sp, 29, 474, 616
Oscillaxanthin, 31
Osmotic shock, 289–290
Outgassing method, 868
Oxidation, 178
Oxidative stress, 94
Oxygenic photosynthesis, 4–6, 186
Oxygen-sensitive enzymes, 94
Oxygen transfer rate (OTR), 867, 869
Oysters, 35
Ozone flotation, 212
Ozonization, 178
Ozonolysis, 407

P

Paenibacillus polymyxa, 747
Palladium, 175
Palmitoleic acid, 339–340, 355
Paralytic shellfish poisoning, 34–35
Paramylon, 34
Pavlova
  P. lutheri
    lipid content and productivity, 333–334*t*
    temperature effect, 339–340
  P. salina, 333–334*t*
Pavlovophyceae, 34
PBRs. *See* Photobioreactors (PBRs)
Pentadecane, 670–677*t*
1-Pentanol, 670–677*t*
2-Pentenal, 670–677*t*
2,3-Pentenedione, 670–677*t*
1-Penten-3-ol, 670–677*t*
1-Penten-3-one, 670–677*t*
2-Penthylfuran, 670–677*t*
Pentose phosphate pathway (PPP), 41, 428–429, 434–435, 576–579
Peroxidases, 690–692*t*, 693
Pervaporation, 679
Pesticides, 177, 781
Petrochemical plastics, 600–606, 604*f*
Phaeocystis antarctica, 342
Phaeodactylum tricornutum, 35, 86, 90–92, 426–427, 631–632, 742–743*t*, 873, 878–879
  lipid content and productivity, 333–334*t*
  peroxidase activity, 693
  superoxide dismutase production, 696
Phaeophyta, 333–334*t*, 576
2-Phenylethyl alcohol, 670–677*t*
2-Phenylpropene, 677*t*
Pheophytin, 393
Phlorotannins, 806
Phormidium sp, 29, 474, 760–761
Phorphyridium aerugineum, 471
Phosphoglycerate (P-PG) molecules, 40
Phosphorus, 168, 449–450, 456*t*, 720
  biofertilizer, 449–450, 456*t*
  removal of, 169–172
Phosphotransacetylase (Pta), 629–630
Photoadaptation, 107–108, 108*f*
Photoautotrophic system, 666
Photobiological H_2 production, 397, 398*t*
Photobioreactors (PBRs), 103, 117*f*, 127–128, 158, 192–193, 199–201, 335–337, 360, 609, 632–634, 647–648, 712*b*, 716–717, 755, 861–863, 863*t*
  advanced models, 122–124
  advantages, 375
  biodiesel production, 721
  biofilm-based photobioreactors, 120
  bubble dynamics, manipulation of, 199
  built-in materials for, 200
  capacity
    fluid-dynamics, 138–140
    geometry, 136–138
    heat transfer, 143–146
    mass transfer, 141–143
  configurations, 201
  flat panel reactors (*see* Flat panel photobioreactors)
  floating bioreactors, 120
  function, 128
  hybrid photobioreactors, 120, 121*f*, 201
  illumination zones, 103–104, 104*f*
  installation, in Iceland, 121*f*
  island of existence of, 113, 113*f*
  k_La, measurement, mass transfer
    carbon dioxide (CO_2), 867
    continuous reduction with sulfite, O_2, 868–869
    outgassing method, O_2, 868
  Lagrangian approach, 105
  Lambert-Beer equation, 104–105
  light
    construction materials, 870–871
    discontinuous irradiation, 872–874
    history, concept of, 105
    light path, 870
    photon flux density (PFD), 871–872
    saturation zone, 103–104
    translucency and transparency, 870
  liquid medium, modifying, 200
  microalgal cultures, configurations for, 193*f*
  natural water reservoirs, 116
  photosynthesis (*see* Photosynthesis)
  raceways (*see* Raceway reactors)
  shear stress in, 869–870
  spherical photobioreactors, 120
  thin-layer reactors (*see* Thin-layer reactors)
  tubular reactors (*see* Tubular photobioreactors)
Photofermentor, 865, 865*f*
Photoheterotrophic cultivation, 335

Photoheterotrophy, 863–865
Photoinhibition, 103–104, 107–108, 111–113, 112*f*, 130
Photon flux density (PFD), 871–872
Photo-oxidation, 178
Photophosphorylation, 187
Photoprotection, 648
Photorespiration, 188
Photosynthesis, 3–6, 26, 36, 39–40, 198, 467
  biomass production, 131
  electron transport, 187
  fluid dynamics, effects of, 108–110
  kinetics of
    photosynthetic factories (PSF), 106–108, 111, 112*f*, 122
    P-I chart, 106
    sophisticated models, 106
  light reactions, 36–37, 186–187
  and ordered mixing, 110–115
  pigments, 37–39
    carotenoids, 38
    chlorophyll, 37–38
    phycobiliproteins, 38–39
  rate *vs.* irradiance, 130, 130*f*
Photosynthetic algal microbial fuel cell (PAMFC), 785–786, 786*f*
Photosynthetically active radiation (PAR), 130, 871–872
Photosynthetic factories (PSF), 106–108, 111, 112*f*, 122
Photosynthetic photon flux density (PPFD), 410–411
Photosynthetic unit (PSU), 106–108, 122, 123*f*
Photosystems I (PS I), 31, 36–37, 186–187
Photosystems II (PS II), 31, 186–187
Phototrophic microalgae, 128
Photovoltaic electro-flocculation-flotation, 213
pH values, 228
Phycobiliproteins, 29, 36, 38–39, 466, 468–469, 472, 474–475, 479, 482, 778, 780
Phycobilisomes, 29, 466
Phycocyanin, 31, 38–39, 466–469, 471–475, 835
  antitumor effects, 468–469
  purification process of, 475
Phycoerythrin, 31, 36, 38–39, 466, 468–469, 471
Phycoerythrocyanin, 466
Phytases, 690–692*t*, 695
Phytoene, 650*f*
Phytoene desaturase (PDS), 91–92
Phytoene synthase (PSY) catalysis, 91–92
Phytohormone production, 451–452, 451*t*
Phytohormones, 474–475
P-I chart, 106
Pigments, microalgae, 91–92, 466*f*, 778
  bioprocesses, 472–478
    astaxanthin, 477
    β-carotene, 475–476
    lutein, 477–478
    phycocyanin, 472–475
  economical interest and challenges, 479–485
    constraints, 482–484
    market size, 480–482
    prospects and solutions, 484–485
  extraction, process intensification in, 762
  industrial application, 468–472
    feed, 471–472
    food and nutraceuticals, 470–471
    pharmaceutical use and prospecting, 468–470
  photosynthesis, 37–39
    carotenoids, 38
    chlorophyll, 37–38
    phycobiliproteins, 38–39
Plant growth promoters, 451–452
Plasmalemma, 29
Plasticizers, 169
"Plate-*versus*-plastics,", 603–605
Platinum, 175
Platocyanin, 29
Plug-flow reactor, 102–104
Point of zero charge (PZC), 228
Polar lipids, 354–355
Pollutants, 168–169
  classical, 166
  elimination of, 177–178
  emerging, 169
Polyacrulonitrile membrane, 211
Polyacrylamide flocculants, 245
Polyaluminum chloride (PAC), 250–251
Polyelectrolyte, 211–212
Polyether sulfone (PES), 262–267
Polyethersulfone polyvinyl-pyrollidone (PES-PVP), 262–267
Polyethyleneimine flocculants, 245
Polyethylene terephthalate (PET), 262–267
Polyferric sulfates, 209–210
Polyhydroxyalkanoate (PHA), cyanobacteria
  autotrophic and mixotrophic cultivation
    Anabaena sp., carbon-supply regimes, 620
    Aulosira sp., carbon-supply regimes, 622–623
    Nostoc sp., carbon-supply regimes, 619
    Spirulina sp., carbon-supply regimes, 618–619
    Synechocystis sp., carbon-supply regimes, 620–622
  biopolyesters
    general aspects, 600–603
    raw materials, 603–606
  carotenoidschlorophylls, 598–599
  cell factories
    bacterial PHA biosynthesis, enzymatic background of, 610–613
    general aspects, 606–610
  cyanotoxins, 599–600
  extremophile cyanobacterial PHA producers
    extreme pH conditions, 625
    halophilic cyanobacteria, 624–625
    thermophile cyanobacteria, 623–624
  factors

Calvin cycle, 616
carbohydrates, intracellular production of, 617
composition, 613–614
extracellular polysaccharide (EPS), 617–618
Gloeothece sp. PCC 6909, 616
Oscillatoria limosa, 616
photoautotrophic cultivation, 617
production and intracellular degradation conditions, 614–616
pseudofermentation, 617
Spirulina maxima, 617
Synechocystis sp. PCC 6803, 617
Synechocytis sp. CCALA192, 617
genetic engineering, 627–632
Lyngbya sp, 599
mixed microbial culture cultivations, 625–627
molecular nitrogen fixing (diazotrophic) representatives, 598
neurotoxins, 599–600
photosynthetic chromophores, 598–599
phycobilins, 599
production scales and techniques, 632–635
white biotechnology, 600
Polyhydroxybutyrate, 32, 845
Polyketide synthase (PKS), 429–430, 434–435
Polylactic acid (PLA) blends, 835
Polysaccharides (PS), microalgae, 91, 778
antiinfectious agents
antibacterial activity, cyanobacteria, 553–554
antifungal activities, cyanobacteria, 554–555
antiviral activity, cyanobacteria, 551–553
bioactivity and structure activity relationships, 555–562, 555–556*t*
antiaging activity, 562
anticancer activity, 557–558
anticoagulant activity, 560–561
antiinflammatory activity, 559–560
antilipidemic and antiglycemic activities, 561–562
antioxidant activity and free radical scavenging, 556–557
antitussive activity, 561
immunomodulatory activity, 558–559
eukaryotic photosynthetic microorganisms, 533–534
exopolysaccharides (EPS)
Charophyta, 536, 537–538*t*
Chlorophyta, 536, 539–540*t*
cyanobacteria, 536, 540–551, 541–545*t*
Myozoza and Ochrophyta, 536, 546–547*t*
extracellular polysaccharides (ECP), 534
heteropolysaccharides, 534–535
perspectives, 562–563
structural diversity of, 534–539
Polyunsaturated fatty acids (PUFAs), 479, 780–781
in microalgal oils, 354–355
production, 421–424, 426–427, 434–435
Polyurethane foam, 172
Polyvinyl alcohol (PVA) polymer, 262–267
Polyvinylidene fluoride (PVDF), 216, 262–267
Porphyridium, 26–27, 36
P. cruentum, lipid content and productivity, 333–334*t*
P. cruentus, 39
P. purpureum, peroxidase activity, 693
PosiDAF, 257
Potassium carbonate, 200
Poterioochromonas malhamensis, 698–699
PPP. *See* Pentose phosphate pathway (PPP)
Praestol, 245
Pressure filtration, 262–267
Pressurized liquid extraction (PLE), 762
Pretreatment
biodiesel production, 853–857
H_2 production, biomass for, 405–406, 408–409*t*
chemical method, 407
enzymatic method, 407–408
physical methods, 406
ultrasonication and microwave irradiation, 406–407
Process integration, 709–710
definition, 715*b*
economic outlooks, 726–728
energy integration, 715–718
environmental and economic indicators, 723–728
economic outlooks, 726–728
life cycle assessment, 723–726
mass integration, 718–720
in microalgae-based biorefinery, 729*f*
types of, 730*t*
water integration, 720–723
Process intensification (PI), microalgae-based processes and products, 737–763
biodiesel, 752–756
bioethanol, 756–759
biohydrogen/biogas, 759–760
carbon dioxide capture, 761–762
cell disruption methods, 749–752, 750*f*, 753*t*
bead milling, 749–750
biological extraction, 752
high-pressure homogenization, 750
high-speed homogenization, 750
microwave, 751
pulsed electric field (PEF), 752
supercritical fluid extraction, 751
ultrasonication, 751
cell drying, 748–749, 749*t*
cultivation, 740–741
extraction, 762

Process intensification (PI), microalgae-based processes and products *(Continued)*
food application, single-cell protein for, 763
harvesting technique, 741–748, 744*f*
centrifugation, 748
coagulation/flocculation, 744–747
electrical base process, 747–748
filtration, 748
flotation, 747
sedimentation, 747
strain, selection of, 738–740
wastewater treatment, 760–761
Prochlorococcus, 31
Prochloron, 31
Prokaryotic cells, 29
Prokaryotic microalgae (cyanobacteria), 31–33, 36–37, 465–466
2-Propanone, 670–677*t*
Proteases
applications, 690–692*t*
definition, 694
production from
Anabaena variabilis, 694
Arthrospira platensis, 694
Chlorella vulgaris, 694
Protein digestibility-corrected amino acid score (PDCAAS), 507–511
Proteins, microalgae, 778, 834
amino acids, 494, 496*t*
bioactive peptides, 498–500, 501*t*
challenges and opportunities, 518–520
conventional proteins, 495–496
extraction, 497–498
health benefits, peptides, 499, 499*f*
nutritional quality of
analytical methods, 503–505
digestibility, 506–507, 508*t*
evaluation and general factors, 501–503, 502*f*
microalgae amino acid profile, 505–506
terrestrial plant proteins, 501–503
Sisyphean algal biofuels initiatives, 493
surface-related and hydrodynamic properties, 495–496
techno-functional properties, 495–496
utilization parameters, 507–518, 512–517*t*
Proteobacteria, 315
Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), 80
ProviApt photobioreactor, 876
Prymnesium parvulum, 34
Pseudokirchneriella subcapitata, 319
PSF. *See* Photosynthetic factories (PSF)
PUFAs. *See* Polyunsaturated fatty acids (PUFAs)
Pullulanase, 689–692
Pulsed electric field (PEF), 752
Pyranometers, 136
Pyrolysis, 300–301
Pyruvate-ferredoxin oxidoreductase (PFOR), 403

R

Raceway ponds
area, 876
depth, 876
Raceway reactors, 116, 127–128, 146*f*
CO_2 absorption, 150
cost investment, 146–147
fluid-dynamics, 138–140, 140*f*, 147–148, 149*f*
geometry, 136–138, 147
mass transfer, 148–150
temperature, 150
for wastewater treatment, 147
Reaction centers (RCs), 393
Reactive oxygen species (ROS), 467, 556, 648
"RecA-null mutation,", 630
Recovery efficiency (RE), for microalgal harvesting, 229–230, 232–235
autoflocculation, 236–239*t*, 240
bioflocculation, 236–239*t*, 248–249
centrifugation, 259–261, 260*t*, 262*t*
chemical flocculation, 236–239*t*, 242–246
dispersed air flotation (DiAF), 255–256*t*, 257
dispersed ozone flotation, 255–256*t*
dissolved air flotation (DAF), 254–257, 255–256*t*
electrolytic flotation, 255–256*t*, 258
filtration methods, 262–267, 263–264*t*
physical flocculation, 236–239*t*
electroflocculation, 251–252
magnetic separation, 252–253
ultrasound-assisted flocculation, 250–251
Red algae (Rhodophyta), 26–27, 28*t*, 30–31, 35–36, 38–39, 575–576, 815*t*
Reduction phase, 187
Regeneration phase, 188
Released polysaccharides (RPS), 536
Removal efficiency (RE), 194
Residence time distribution (RTD), 102–103
Respiration, 40–42
R. eutropha PHA synthesis genes, 630
Reversed-phase high performance liquid chromatography (RP-HPLC), 500
Rhizoclonium sp
amylase activity, 693
peroxidase activity, 693
superoxide dismutase activity, 696
Ribulose-bis-P, 40
Ribulose-1,5-bisphosphate carboxylase/oxygenase

(RuBisCO), 40, 43, 188–189, 197–198
Ribulose-5-phosphate, 41
Ribulose phosphate (ribulose-P), 188
Rotary dryers, 346
R-phycoerythrin, 39, 469, 471

S

Saccharomyces cerevisiae, 484
Saline soil, reclamation of, 450–451
Sand-enhanced electro-flocculation, 213
Sand filtration, 211
Sargassum muticum, 215
Scale-up process
  cultivation systems (*see* Cultivation systems, scalability of)
  microalgal metabolism
    autotrophic production, 861–864
    heterotrophic production, 861, 864
    mixotrophic production, 861, 864–866
  microalgal reactors
    mixing, algal culture, 866–867
    photobioreactors (*see* Photobioreactors (PBRs))
  and productivity
    flat panel, 878
    large diameter tubular reactor, 877, 879*t*
    medium diameter tubular reactor, 878
    small diameter tubular reactor, 877–878
    tube diameter, 878–879
    tube length, 880
Scenedesmus sp, 27, 190–191, 314, 316–322, 742–743*t*, 747, 755–756, 760–761, 763
  carbohydrate accumulation, 405
  filtration, harvesting biomass, 345
  nutrient starvation, 338
  rotary dryers, 346
  S. almeriensis, 321, 467–468, 477–478
    lutein, 779
    zeaxanthin, 779
  S. dimorphus, 322
  S. obliquus, 178*t*, 321–323, 747, 873
    amylase activity, 692
    β-galactosidase production, 699
    biorefinery, 789–790, 789*f*
    hydrogen production, 392, 406, 408–409*t*
  S. quadriculata, 27, 874
Scytonemin, 653
Seafood, 10–13
Seawater integration, 722
Seaweeds, 3–4, 6–7, 7*f*
Secondary carotenoids, 467
Second-generation biofuels, 311–312
Sedimentation, 747
Sedoheptulose-1,7-bisphosphatase, 197–198
Selenastrum capricornutum, 339
Self-shading effect, 410–411
Separate hydrolysis and fermentation (SHF), 384
Sequencing batch reactor (SBR), 626
Service collections, 50
Sewage treatment plants (STPs), 446–447, 457
Sewage treatment works (STWs), 806
SFA, 434–435
SFE. *See* Supercritical fluid extraction (SFE)
Shear stress sensitivity, 870
Shinorine, photostability of, 652
Silence RNA (siRNA), 198–199
Silica gel, 172
Silica nanoparticles, 216
Silicon oxide, 35
Simultaneous saccharification and fermentation (SSF), 384
Single-celled stalks, 26–27
Single cell oils (SCOs), 420. *See also* Microalgae single cell oils (MSCOs)
Single-cell protein (SCP) for food application, 763
Skeletonema, 747
SOD. *See* Superoxide dismutases (SOD)
Sodium dodecyl sulfate (SDS), 212, 498
Soil-paddy-algae system, 448–449
Solar conversion efficiency, 410
Solar drying, 213–214, 346, 383
Solar tracking systems, 871
Solid–liquid separation operations, 844
Solix Biosystems, 875–876
Solvent extraction, 348, 762
Spectrophotometric copper/dye-binding assays, 503–505
Spent microalgae, 320–321
Spherical photobioreactors, 120
Spirogyra sp
  amylase activity, 692–693
  biorefinery, 788–789, 788*f*
  S. longata, 575–576
Spirulina sp, 29, 32, 127–128, 146–150, 207–208, 468, 475, 608, 742–743*t*, 756, 760–761
  carbon-supply regimes, 618–619
  cultures, 861–863, 871–872, 880
  freeze-drying, 347–348
  S. maxima, 617
  solar drying, 346
  S. platensis, 32, 294–295*t*, 296–297, 299–300, 300*t*, 615–616, 780
Spray-drying method, 214–215, 347, 477
Squalene epoxidase (SQE), 579
Squalene monooxygenase enzyme (SQLE), 579–580
Square-kilometer-scale photobioreactors, 721–722
Standard International Trade Classification, Revision 4 (SITC), 808
Staurosira sp, 877
Steam explosion, 316–317
Stefan-Boltzman constant, 144
Sterols, microalgae bioactive compounds, 573

Sterols, microalgae *(Continued)*
bioactivity of, 587–589
biosynthesis, 576–581, 577–578*f*
biotechnology production, 579, 580*f*
β-sitosterol, 582
campesterol, 582
cholesterol, 582
culture modifications, 581
in cyanobacteria, 582–583
environmental variations, 583–584, 588*t*
ergosterol, 582
fucosterol, 582
green algae, 581
industrial production, 589–590
isolated cholesterol, 573–574
1D and 2D nuclear magnetic resonance spectroscopy, 581
Pelagophyceae, 581
stigmasterol, 582
structural aspects of, 574–576
Ulvophycea sterols, 581
Stirred tank photobioreactors, 843
Stokes' Law, 228–229
Subcritical water extraction (SWE), 790
Succinic dehydrogenase, 41
Succinyl-COA-synthetase, 41
Sulfate salts, 242–244
Sulfoquinovosyldiacylglycerol (SQDG), 338–339
Sulfur integration, 720
Sulfur oxide (SO_x), 377, 726
Supercritical ethanol (SCE), 353
Supercritical fluid extraction (SFE), 349, 751, 762
carotenoids, 290*t*, 291–292
lipid extraction, 296–297
Supercritical fluids (SCF) transesterification, 352–354
Supercritical methanol (SCM), 353
Superoxide dismutases (SOD)
applications, 690–692*t*
isoforms, 695–696
production of, 696
Supply chain characteristics, 800–802
Surface renewal model, 113–114
Surfactants, 169
Suspended air flotation, 254–257
SV40 promoters, 87–88
Swirl elements, 873
Synechococcus sp, 31–32, 762
S. elongatus, 700
S. lividus
phytase activity, 695
temperature effect, 339–340
Synechocystis sp
carbon-supply regimes, 620–622
H_2 production, 398*t*, 402
S. pevalekii, 61–62
Syngas, 775–776
Synthetic antioxidants, 471
Synthetic biology
microalgae, use in, 86–88, 89*t*, 93*f*
biofuels, 88–91
carbohydrates, 92
carotenoids, 91–92
challenges, 92–94
fatty acids, 91
photosynthetic efficiency, 94
vs. molecular biology, 86
Synthetic polymers, 209–210, 244–245

T

Tangential flow filtration (TFF), 211, 262–267
Tanks-in-series model, 102–103
Taylor-Couette photobioreactor, 122, 874
Temperature
biodiesel production and, 339–340
biomass production, 133–134
control, 57
H_2 generation and, 400–401
optimal, 337
Terpenes, 669, 670–677*t*
Tetradecane, 670–677*t*
Tetradesmus obliquus, 699
Tetraselmis sp, 212, 321, 742–743*t*
biorefinery, 790–791
T. gracilis, 53*f*, 65–66
T. subcordiformis, H_2 production, 400–401
T. suecica, 146–147, 763, 878
UV-B irradiance, 342
Thalassiosira pseudonana, 77, 333–334*t*, 742–743*t*
Thermal drying, 590
Thermal hydrolysis, 316–317
Thermochemical biofuel production, 832
Thermophile cyanobacteria, 623–624
Thermosynechococcus elongatus E542, 624
Thickening, 455
Thin-layer reactors, 155*f*
advantage of, 156–157
design of, 156–157
dissolved oxygen concentrations, 156–157
oxygen production rate, 156–157
power consumption, 157
production capacity, 157–158
Third-generation biofuels, 311–312
Thylakoid isoprenoids, 805
Thylakoid membranes, 30
Tolypothrix, 31–32
Total suspended solids (TSS), 230, 258–259, 261–267
Trajectory length distribution, 105
Transcription activator-like effector nucleases (TALEN), 198–199
Transesterification, 297, 777
biodiesel production
acid-catalyzed, 351–352
base-catalyzed, 351, 351*f*
catalysts used, 351
Chlorella vulgaris microalgae, 849–857
lipase-catalyzed, 352
supercritical alcohol, 352–354
supercritical fluids (SCF), 352–354
Transfer chambers, 60–61
Transgenic algae, 78
Transketolase, 197–198
Translucency and transparency, 870
Trentepohiales, 6
Triacylglycerols (TAG), 330, 351, 351*f*, 357–358, 420, 430–431, 755
Tricarboxylic acid (TCA), 41, 427–429

Trichodesmium erythraeum, 42–43
Tridecane, 670–677*t*
"True digestibility,", 506–507
Truncated light-harvesting antenna (TLA), 198
TSS. *See* Total suspended solids (TSS)
Tubular photobioreactors, 127–128, 151*f*, 152, 712*b*
  biomass centration range, 150–152
  cost investment for, 150–152
  fluid-dynamics, 115, 115*f*, 138–140, 140*f*, 152–154
  geometry, 136–138, 152, 153*f*
  inclined discontinuous baffles, 114, 115*f*
  large-scale tubular photobioreactor, 119, 120*f*
  mass transfer, 154
  temperature, 154
  tube diameter, 875
  tube length, 874–875
  velocity profiles and dead zones, in U bends, 116, 119*f*

U

Ultrafiltration, 262–267, 265*f*, 748
Ultrasonication, 209–210, 406–407
  cell disruption, 288–289
  lipid extraction, 349
  process intensification, 751
Ultrasonic sedimentation, 345
Ultrasound-assisted extraction (UAE), 550–551
Ultrasound-assisted flocculation, 250–251
Ultraviolet (UV) detector, 586–587
Ultraviolet (UV) irradiance, 342
United Nations' Sustainable Development Goals (SDGs), 799–800
Uranium, 175, 175–176*t*
Urea, 42
UV-absorbing compounds, 649
  carotenoids, 649–651
  industrial applications, 653, 654*t*
  mycosporine-like amino acids, 651–653
  scytonemin, 653
UV-induced skin problems, 648

V

Vacuum filtration, 262–267
Varicella zoster virus (VZV), 552
Vegetable oil, 329–330, 354–355
Vertical tube photobioreactors, 843
Vibrating screen filters, 263–264*t*, 267
Violaxanthin, 35, 38, 467
Viral hemorrhagic septicemia (VHSV), 552–553
Viral infections, 32
Vitrification, 74–77
Volatile organic compounds (VOCs), 659, 724
  application, 668–678
  biosynthesis mechanism
    dimethylsulfide pathway, 666, 667*f*
    fatty acid pathway, 664, 665*f*
    geosmin synthesis, 661–664, 664*f*
    isoprenoid pathways, 661, 662*t*
    2-keto acid pathway, 661, 661*f*
    2-methylisoborneol (2-MIB) pathway, 661–664, 664*f*
  comparative analysis, 669, 670–677*t*
  energy potential, 669, 677*t*
  factors affecting production, 666–668
  global market, 669
  recovery techniques, 679, 680*t*
    adsorption, 678
    application possibilities, 679, 681*f*
    condensation, 678
    pervaporation, 679
Volumetric gas-liquid mass transfer coefficient, 156

W

Wastewater (WW), 843
  definition, 165
  deodorization, 669
  growth of microalgae in, 322
  integration strategy, 725
  microalgae cultivation, 455–456
  treatment, 17–18, 166–178, 171*t*, 774, 824
    colorants, elimination of, 176–177
    emerging pollutants, elimination of, 177–178, 178*t*
    facilities, microalgal remediation, 446–447, 447*f*, 457, 458*f*
    heavy metal removal, 172–175
    life cycle assessment (LCA), 835–836
    nitrogen and phosphorus, removal of, 169–172
    process intensification, 760–761
Wastewater treatment plants (WWTPs), 166, 168, 177
Water integration, 714–715, 720–723
Water-soluble PS (WSPs), 554–555
Wet biomass, 311–312
White biotechnology, 600
Work collections, 50
World Federation for Culture Collections (WFCC), 50

X

Xanthophylls, 31, 34–35, 38, 467, 469–470, 649

Z

Z-carotene desaturase (ZDS), 91–92
Zeaxanthin, 31, 470, 779
  in cosmetics, 651, 654*t*
  structure, 650*f*
Zeta potential (ZP) values, 228
Zinc, 169
  human body, effect on, 173*t*
  removal, by microalgal biomass/cultures, 175–176*t*
Zinc-finger nucleases (ZFN), 198–199
ZnSOD, 695–696
Zymomonas mobilis, 378–379

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Handbook of Microalgae-Based Processes and Products

Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds

Edited by Eduardo Jacob-Lopes, Mariana Manzoni Maroneze, Maria Isabel Queiroz, and Leila Queiroz Zepka

The *Handbook of Microalgae-based Processes and Products* provides a complete overview of all aspects involved in the production and utilization of microalgae resources at commercial scale. Divided into four parts (fundamentals, microalgae-based processes, microalgae-based products, and engineering approaches applied to microalgal processes and products), the book explores the microbiology and metabolic aspects of microalgae, microalgal production systems, wastewater treatment based in microalgae, CO_2 capture using microalgae, microalgae harvesting techniques, and extraction and purification of biomolecules from microalgae. It covers the largest number of microalgal products of commercial relevance, including biogas, biodiesel, bioethanol, biohydrogen, single-cell protein, single-cell oil, biofertilizers, pigments, polyunsaturated fatty acids, bioactive proteins, peptides and amino acids, bioactive polysaccharides, sterols, bioplastics, UV-screening compounds, and volatile organic compounds. Moreover, it presents and discusses the available engineering tools applied to microalgae biotechnology, such as process integration, process intensification, and techno-economic analysis applied to microalgal processes and products, microalgal biorefineries, life cycle assessment, and exergy analysis of microalgae-based processes and products.

The coverage of a broad range of potential microalgae processes and products in a single volume makes this handbook an indispensable reference for engineering researchers in academia and industry in the fields of bioenergy, sustainable development, and high-value compounds from biomass, as well as graduate students exploring those areas. Engineering professionals in bio-based industries will also find valuable information here when planning or implementing the use of microalgal technologies.

Key Features

- Covers theoretical background information and results of recent research.
- Discusses all commercially relevant microalgae-based processes and products.
- Explores the main emerging engineering tools applied to microalgae processes, including techno-economic analysis, process integration, process intensification, life cycle assessment, and exergy analyses.

About the Editors

Eduardo Jacob-Lopes, Associate Professor, Department of Food Technology and Science, Federal University of Santa Maria, Brazil

Mariana Manzoni Maroneze, Researcher, Department of Food Technology and Science, Federal University of Santa Maria, Brazil

Maria Isabel Queiroz, Formerly at School of Chemistry and Food, Federal University of Rio Grande, Brazil

Leila Queiroz Zepka, Associate Professor, Department of Food Technology and Science, Federal University of Santa Maria, Brazil

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