Md. Asraful Alam · Zhongming Wang
Editors

Microalgae Biotechnology for Development of Biofuel and Wastewater Treatment

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Part I
Microalgae Strain and Culture Technology

Chapter 1
Recent Trends in Microalgae Research for Sustainable Energy Production and Biorefinery Applications

Naim Rashid, Bongsoo Lee, and Yong-Keun Chang

Abstract Microalgae are convincing biomaterials furnished with tremendous potential of performing environmental services and energy recovery to promote carbon neutral bio-economy. They have the ability to fix atmospheric CO_2, water reclamation, bioremediation, and production of biomolecules, which offer distinguished features for biorefinery applications. However, several technical challenges in microalgae bioprocesses impede their application at large scale. The most notable challenges include low bioconversion efficiency and biomass productivity, susceptibility to harmful microorganisms, high-energy input in the form of light and nutrient supply, and high cost accounted for biomass harvest. In the framework of microalgae-based sustainable bio-economy, technology integration turns out to be the only viable solution. The integration of microalgal technology with other related field would unveil their meritorious attributes and would offset the cost of biomass processing. The focus of this chapter is to identify the recent environmental technologies which can be integrated with microalgae biorefinery to drive the objectives of resource-efficient bio-economy. The prospects of these technologies are presented to realize their future potential and sustainability outlook.

Keywords Microalgae · Biorefinery · Sustainability · Technology integration · Coculture · Extremophile · Value-added bioproducts

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1 Microalgae Research Outlook

The potential of microalgae has been studied since 1960s in the perspective of environmental and ecological services. Microalgae are considered as a convincing material to promote resource-efficient bio-economy and meet the regulatory drivers of environmental sustainability. Despite many distinct features of microalgae, they are mainly studied as an alternative source of clean and sustainable fuel (Gouveia et al. 2017; Shuba and Kifle 2018). In the last two decades, microalgae industry sensed a great momentum potentially due to hike in oil prices and realization of diminishing resources of conventional fuels. A number of industries and research centers were established across the world to explore the potential of microalgae and asses their potential for large-scale application. In academia, the scientists directed their research focus on microalgal biofuels, algal biotechnology, and mass cultivation. Intensive research on microalgae was noted between 2006 and 2011 (Chen et al. 2015). World's leading countries including the USA, China, Spain, Korea, and Australia actively researched microalgae to develop a sustainable biorefinery model. According to a study, microalgae research was spanned in 80 countries elucidating the realization of their potential. A comprehensive report has been published by the International Energy Association (IEA), demonstrating the global perspective on microalgae-based research projects, industries, and research facilities. A bloom of microalgae research remained almost for a decade. Eventually, however, a decrease in microalgae research was observed in 2012 and the highest decrease after 2014. The potential of microalgae biorefinery became apparent, and it turned out that their exclusive use for biofuel production is not sustainable. Furthermore, a sharp decrease in oil prices after 2014 hampered microalgae research and pushed the research and policy-makers to redirect their focus. Recently, the focus of microalgae has been shifted to value-added bioproducts, water reclamation, and bioremediation (Rashid et al. 2018). Now, the scientists are of the view that the biofuel production can be a long-term goal; however, the near-term and sustainable microalgae research is relying on its use for wastewater treatment and feed source. Moreover, innovation in microalgae bioprocesses and technology development can lead to offset the overall cost of microalgae processing and reorient the research focus in the future (Deconinck et al. 2018).

2 Rationale of Microalgae Biorefinery

Microalgae are unicellular microorganisms, which have unique ability to fix atmospheric CO_2 and convert waste organic materials into valuable biorefinery products. They fix CO_2 through photosynthesis and convert them into biomolecules. According to an estimate, they efficiently use 9% of the solar radiations and can

produce 280 tons of dry biomass per hectare per year (Chen et al. 2018; Dhillon and von Wuehlisch 2013) by consuming 513 tons of CO_2. The microalgae biomass can be used as a potential feedstock for fuel and food, after downstream processing. Despite tremendous inherited potential of microalgae, their large-scale application is delayed due to some technical and economic issues. Microalgae are encountered with pressing challenges of contamination, slow growth rate, low biomass productivity, high energy input, and the cost required for dewatering. The biomass productivity can be enhanced by reducing respiratory losses and capitalizing CO_2 emission (Singh and Olsen 2011). In this perspective, mixotrophy and heterotrophic cultivation can be a promising choice. However, the availability of organic carbon source and other nutrients including nitrogen and phosphorous, to feed microalgae, and high risk of bacterial invasion limit their applications. The limitation of nutrient supply can be eliminated by growing microalgae in wastewaters which are rich source of nutrients required for microalgae growth (Benemann et al., 2018). The contamination issue can be resolved by employing extremophile microalgae (Graverholt and Eriksen 2007; Manirafasha et al. 2016; Moon et al. 2014) which grow at low pHs (1.0–2.0) and high temperature (20–40 °C). Still, these techniques are not ideal due to seasonal variations across the globe. More recently, the use of microalgae coculture has been realized as an efficient method of microalgae cultivation. In a coculture system, autotrophic microalgae are grown with a heterotrophic microalgae or bacteria. A symbiotic relationship between the species is developed. Autotrophic microalgae capture CO_2 and store them as organic carbon. The organic carbon serves as food for heterotrophic species, which can convert them into CO_2. And this CO_2 is utilized through autotrophy again in a synergetic manner. The coculture system offers unique advantages over monoculture system (Amavizca et al. 2017a; Rashid et al. 2018). The most notable advantages include high biomass productivity, less contamination risk, high carbon uptake and bioconversion, diverse biomass composition, and bio-flocculation. It is important to remark that bio-flocculation is the most exciting feature of coculture system; therein, microalgae are self-flocculated by their interaction with organic matters produced during cultivation.

Coculture cultivation system provide distinct feature over monoculture system; still it is not economical and sustainable for commercial application. The sustainability of microalgae system can be promised by technology integration. In this context, the focus shift of microalgae bioprocesses from the biomass productivity to ecological services would be highly rewarding. As mentioned earlier, microalgae have tremendous potential for biofuels, bioproducts, bio-fixation, bioremediation, water reclamation, and upcycling waste into a valuable commodity. They can be employed in fuel cell technology, biohydrogen technology, biogas industry, and many other environmental and biochemical industries. The focus of this chapter is to provide insight about the integration of these environmental technologies with microalgae to reinforce the objectives of sustainable energy production and biorefinery applications. Also, it addresses the challenges of microalgal technology integration and provides solutions evolved through recent research trends.

3 Sustainability Prospects of Microalgae Bioprocesses

Microalgae undergo several necessary bioprocesses before its final use. These bioprocesses largely impact the economics and sustainability of microalgae biorefinery. The bioprocesses encounter several technical challenges, which limit scale-up application of microalgae biomass. The following section provides the prospects of major bioprocesses and proposes some solutions evolved through recent research trends and technology development. Below is the chain of microalgae bioprocesses and bioproducts (Fig. 1.1).

3.1 Strain Isolation and Selection

This is the primary yet the most important step of microalgae bioprocesses. Microalgae thrive in diverse geographical locations and, thus, entail different characteristics. They live in freshwater, marine water, wastewaters, hot springs, and cold-water bodies. They acquire distinct characteristics due to their habitat, which impact the quantity and quality of bioproducts obtained from the biomass (Moon et al. 2014; Singh and Olsen 2011; Velea et al. 2017). For example, marine water algae show high robustness and tolerance to the salinity, while freshwater algae are sensitive to salt concentration but have high bioremediation potential. The bioproducts obtained from the microalgae biomass provide a clue about microalgae habitat and food source. For example, the microalgae living in oil-rich habitat would potentially produce more oil. Thus, the purpose of bioproducts to be obtained from the biomass would dictate the choice of site to isolate microalgae species. However, regardless of the purpose of microalgae bioproducts, robustness is the basic criterion in strain selection. Robust microalgae are tolerant to the environmental (abiotic and biotic) cultivation conditions including temperature, light, water quality, nutrients, pH, media composition, and the pattern of nutrients and light supplements. There are more than 30,000 known species of microalgae, and they respond differently to the environmental and ecological conditions. Ultimately, they return huge variation in

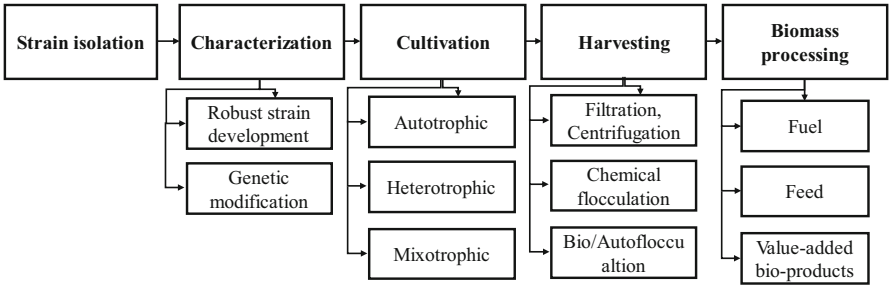

Fig. 1.1 Chain of microalgae bioprocesses and bioproducts

their biomass productivity and the composition too, which impacts the overall economics of microalgae bioprocessing. For an instance, freshwater microalgae show high biomass productivity but low oil yield and harvest efficiency; marine algae exhibit high oil productivity and harvest efficiency but slow growth rate. Likewise, the oil extraction efficiency of the microalgae having cell wall is higher than without cell wall. Thus, there is a trade-off between microalgae selection and the economics of microalgae bioprocesses. Life cycle and techno-economic analysis are the classical tools to determine the right selection of microalgae specie.

3.2 Cultivation

Cultivation is another key step of microalgae bioprocesses. Cultivation is controlled by a number of factors including carbon source, light intensity, light duration, nutrient concentration, media composition, culture pH, carbon influx, etc. Cultivation is categorized based on energy and carbon source, namely, autotrophic, mixotrophic, and heterotrophic cultivation (Graverholt and Eriksen 2007; Singh and Olsen 2011). Autotrophic cultivation is the most common mode of cultivation in which the carbon source is inorganic and light serves as an energy source. In mixotrophic cultivation, both organic and inorganic carbon source can be employed. In heterotrophic cultivation, organic carbon serves as energy as well as carbon source. Each mode of cultivation has distinct advantages and disadvantages (Table 1.1). Autotrophic cultivation returns slow growth rate and biomass productivity due to limited efficiency of photosynthetic apparatus in microalgae and respiratory losses too. The respiratory losses can be overcome by mixotrophic

Table 1.1 Comparison of different microalgae cultivation systems

Autotrophic	Mixotrophic	Heterotrophic
Use CO_2 as a carbon source	Can use both organic and inorganic carbon	Use organic carbon
Low bioconversion efficiency	High bioconversion efficiency	High bioconversion efficiency
Difficult to grow in wastewater	Can grow in wastewater	Suitable to grow in wastewater
Effective to reduce global warming	Effective to balance respiratory losses	Effective for waste reduction
Low biomass productivity	High biomass productivity	High biomass productivity
Result high purity of biomass	Results diverse biomass composition	Result low purity of biomass
Low tolerance to environmental conditions	Fair tolerance to environmental conditions	High tolerance to environmental conditions
Low bio-flocculation potential	High bio-flocculation potential	Very high bio-flocculation potential
Limited downstream applications	Diverse downstream applications	Diverse downstream applications

cultivation (Li et al. 2014). Since both organic and inorganic can be used in mixotrophic cultivation; therefore, the cells consume CO_2 during photosynthesis and organic carbon during respiration cycle. Thus, the cell machinery is engaged during the entire cycle of photosynthesis-respiration to produce new cells and thus, develop biomass. Heterotrophic cultivation gives the highest cell growth and biomass productivity among other cultivation conditions (Velea et al. 2017). High growth rate in heterotrophic cultivation makes it less prone to the contamination. Actually, microalgae cells grow with much faster rate and outperform any other invading bacteria. Less chances of contamination and the use of organic carbon source as a food and energy source warrant them to employ for waste mitigation and wastewater treatment. Wastewater is a rich source of organic carbon and other essential nutrients required for microalgae growth. Growing microalgae in wastewater also reduce water and carbon footprints. A wide variety of wastewaters have been reported for microalgae cultivation including municipal wastewater, textile wastewater, food industry wastewater, biogas, and sugar industry wastewaters. The use of wastewater for microalgae cultivation is promising if employed without dilution and pretreatment. Unfortunately, most of the wastewaters require pretreatment to avoid contamination. In this regard, robust microalgae species should be identified.

Contamination is the most staggering issue of microalgae cultivation. A number of techniques have been developed to control it (Lam et al. 2018). Chemicals are being introduced which are added into the medium to control the growth of contaminating bacterial. Wasif et al. have found that organo-clay is an effective agent to control contamination (Farooq et al. 2013). Sonication is another technique to kill the microorganisms. However, it can rupture microalgae cells to split oil. High strength of sonication can also cause the death of microalgae cells. Apparently, the best of contamination control is to employ extremophile microalgae which can survive under harsh cultivation condition. For example, *Galdieria sulphuraria* and *Cyanidioschyzon merolae* can grow at pH 1.0–2.0, and *Chlorella sorokiniana* can grow at pH 11.0–12.0 (Edgar Amavizca et al. 2017b). At these pH values, bacteria and other microorganism can't grow; thus, monoculture system can be maintained. Detail about extremophiles is provided in Sect. 3.5.

The economic sustainability of microalgae cultivation system is inevitable for their large-scale applications. The one possible way is to manipulate the bioprocesses of microalgae cultivation which can govern high biomass productivity. The other possibility is the consideration of water footprints and nutrients recovery. Several studies have demonstrated the reuse of spent cultivation medium. The reuse of spent medium would cut- down the cost rendered on nutrients and water supply. However, the reuse of spent media can pose negative impact on the cultivation too, as it can contain bacteria and other pathogens which can hamper the subsequent cultivation of microalgae. Thus, it would need to go through sterilization or filtration, which is not economically affordable. Also, continuous use of spent media can have elevated levels of nutrients and other metabolites which can impact microalgae growth. In this context, cascade cultivation can be employed. In cascade cultivation system, microalgae are first grown autotrophically, by which microalgae release

carbohydrates/sugar into the aqueous media. Subsequently, the aqueous media containing sugar can be employed for the mixotrophic or heterotrophic cultivation. Mixotrophic or heterotrophic would show high sugar and nutrients uptake, give high biomass productivity and higher tolerance to invading pathogens. Moreover, for high nutrients and water recovery, hydrothermal liquefaction (HTL) has received considerable attention (Laurens 2017). In this process, the algae biomass undergoes thermal treatment after solid-liquid separation. The biomass can be used for the production of bio-oil, whereas the liquid stream can be further used to extract bio-crude and other gaseous fuels. The liquid obtained after bio-crude process can be further recycled for cultivation.

3.3 Harvesting

Microalgae culture needs to dewater/harvest to obtain solid biomass for its downstream processing. Microalgae exist in the aqueous medium in a dilute concentration (1–3 g/L), which needs to be concentrated up to 100 times. A number of techniques have been introduced to harvest microalgae including coagulation/flocculation, membrane filtration, electroflotation, and centrifugation; however, none of them can be declared an ideal yet for scale-up application. These techniques are energy-intensive, interfere with downstream bioprocess, and not efficient up to the level of commercialization. Recently, the research has been directed to prompt flocculation without any chemical aid, called auto-flocculation or bio-flocculation (Manheim and Nelson 2013; Alam et al. 2016; Ummalyma et al. 2017). In fact, bio-flocculation is a classical form of well-known flocculation process, in which the cultivation conditions of microalgae are controlled in a way that it urges microalgae particles to self-flocculate (Alam et al. 2014). Generally, bio-flocculation is induced by the release of polysaccharides and their interaction with microalga particles within the cultivation matrix. Microalgae release polysaccharides during their growth under certain circumstances. The polysaccharides have an ability to attach with microalgae cells, make flocs, and finally settle due to gravitation. A number of studies have been carried out to identify the role of polysaccharides and unravel the cultivation condition which support bio-flocculation mechanism (Alam et al. 2016). In general, it is considered that the cells produce polysaccharides under stress conditions induced by light, nutrients, and gas limitations during the cultivation. Unfortunately, preferential studies have been carried out in this regard to support this supposition. In literature, a huge controversy exists about the role of polysaccharides in bio-flocculation mechanism. Some of the studies have proved the positive impact of polysaccharides in inducing bio-flocculation, while the others show the opposite results (Beuckels 2013; Lee et al. 2016). Probably the role of polysaccharides in bio-flocculation is species-dependent, for example, *Chlorella* sp. shows flocculation inhibition in the presence of polysaccharides, while *Ettlia* sp. returns high flocculation in their presence (Lee et al. 2016; Yoo et al. 2013). It is not known yet if the properties of polysaccharides change over the course of cultivation and how it

impacts bio-flocculation. Future studies should be directed to identify the precise role of polysaccharides by investigating their composition, morphological changes, and chelating behavior in different stages of cultivation. The bio-flocculation phenomenon can also be propagated through a co-cultivation system. In this system, an autotrophic microalga is grown with a heterotrophic microalgae/bacterium. This system offers higher production of polysaccharides than the single culture system, and thus, returns high bio-flocculation efficiency (detail in Sect. 3.4).

Moreover, the use of wastewater for microalgae harvesting is also being investigated. It has been reported that wastewater (municipal) contains binding agents which serve as flocculants. In fact, microorganisms present in wastewater produce polysaccharides, during their growth, and serve as a bio-flocculant. The microorganisms also produce other useful hormones and the chemicals which support microalgae growth. The biggest challenge in promoting this concept is to identify microorganisms offering the potential of bio-flocculation. Developing a synergetic relationship between bacterial and microalgae growth is another challenge. Attached growth system is a modified concept of bio-flocculation. In this system, the cultivation conditions are controlled so that the cells could produce excessive polysaccharides. The polysaccharides bind the cells together and serve as a niche as like in natural environment. Also, this system would displace the need of harvesting. So far, attached growth system is mainly tested for monoculture system. However, it can offer distinguished advantages in coculture system too. A number of studies have been reported that attached growth system showed higher potential for wastewater treatment, pollutant removal, and valuable metabolites' productivity than the typical cultivation of microalgae in aqueous media.

The advancement in this concept can bring major breakthrough in microalgae bio-industry. It can cut- down microalgae harvesting cost to a significant level as no flocculant is required in this process.

4 Microalgae Sustainability Through Technology Integration

4.1 Biogas Industry Waste for Microalgae Feed and Single-Cell- Protein Synthesis

Anaerobic digestion (AD) is a widely known technology, which can convert organic waste resources of wastewater into biogas. It is a promising technology from economical and ecological viewpoint. It is reported that Germany had established 10,020 biogas plants till 2014, generating 144 PJ of energy. China set up 41.5 million digesters, generating 293 PJ of energy (Vaneeckhaute et al. 2017; Xia and Murphy 2016). Biogas industry receives widespread attention since it entails numerous advantages. In biogas technology, a wide variety of waste resources including municipal wastewater, animal manure, agriculture waste, poultry waste, food waste,

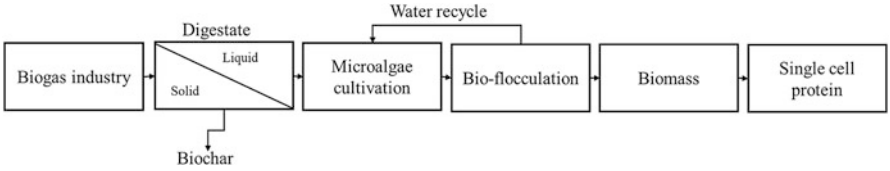

Fig. 1.2 The concept of integrating biogas industry with microalgal technology

and other organic waste can be used as feedstocks. Biogas technology is promising from economical and sustainability viewpoint since it recovers energy from waste resources. However, it confronts with a technical challenge, i.e., handling of its effluent called *digestate*. The digestate contains N, P, C, and other trace metals (depending on input waste), which are considered as waste (according to EU legislation) in high-nutrient region, since they can't be directly applied as fertilizers or disposed into the environment. The conversion of digestate into fertilizers causes nutrient loss and nutrient imbalance in soil ecosystem. Thus, the digestate would require additional treatment cost for its safe disposal into the environment. Resultantly, the overall cost of biogas technology crosses the threshold economic limit. This aggravated challenge can be overcome by coupling it with microalgae technology. Microalgae can use digestate as a nutrient source to promote their growth. Only liquid stream of digestate can be used as a feed. It is reported that biomass obtained through this cultivation is suitable for aquaculture, animal feed, and for biofuels production. The digestate contains N, P and C, K, Ca, Mg and other trace metals which are readily available for the uptake of microalgae in the cultivation matrix. Microalgae use these nutrients and propagate their biomass (Fig. 1.2). The biomass is composed of lipids, carbohydrates, and single-cell protein (SCP). SCP is a value-added product, which can be used as a feed for animals, aquaculture, and the human too. The cultivation conditions of microalgae can be manipulated to increase the fraction of SCP and reduce other bio-chemicals. It is a classified route of SCP synthesis offering cutting- edge advantages over other methods. SCP production through microbial resources can reduce energy requirements from 4000 MJ/Kg-N-Protein (through conventional route) to 230 MJ/Kg-N-Protein only (via microbial growth). Other technologies for N, P mitigation are energy- intensive, for example, nitrification-denitrification (45 MJ/Kg-N), struvite precipitation (69 MJ/Kg-N), air stripping (90 MJ/Kg-N), electrodialysis (65 MJ/Kg-N), and anammox and Haber-Bosch process (54 MJ/Kg-N) (Vaneeckhaute et al. 2017; Xia and Murphy 2016). Microalgae have been rarely studied in the perspective of SCP synthesis.

The use of microalgae biomass directly for biogas production has been studied. Initial investigations showed high-energy recovery up to 287 and 611 L/kg of volatile solids. However, the yield is highly dependent on the composition of biomass and the microalgae species themselves. The biomass containing high concentration of carbohydrates and lipids would give high biogas yield. The promising avenue of extracting biogas from microalgae is that it would not require special

attention to achieve the desired biomass composition since both the carbohydrates and lipids can be converted into biogas. The biogas yield can be species-specific because of diversity in cell structures of different species. Some species possess cell wall and the others don't, which impact the biochemical processing and the yield too. The use of microalgae for biogas production might lead to high carbon footprints. However, a life-cycle analysis should be carried out to reflect these changes. The economic sustainability of microalgae-based biogas production is not assessed yet. Yet, research should be focused to reduce the overall process cost. This can be achieved by valorizing the potential of spent (fuel-extracted) microalgae biomass. Studies have been carried out to successfully recover energy from the spent algal biomass by producing biogas out of it. Another method to improve the sustainability of this technology is to adopt cascading technique (Laurens 2017). In the first step, the biomass undergoes for high-value biogas production, and in the following step, the residual biomass is employed for low-value biogas production. This technique can be helpful to reduce carbon footprints too.

4.2 Microalgae Technology Integration with Microbial Fuel Cell

Microbial fuel cell (MFC) is an electrochemical device driven by the microorganisms to transport electrons and produce electricity. In MFC, microorganisms produce electrons by decomposing organic compounds. Organic compounds can be in the form of pure chemicals or extracted from waste materials. Pure organic compounds are not economically supported. Alternately, waste materials are investigated to replace them. A wide variety of wastes can be used as anodic feedstocks. Wastewater is one of them. A number of wastewaters have been reported to be used for electricity generation in microbial fuel cells. The use of microalgae in MFCs is a way beyond its typical use. Microalgae-driven MFCs have added advantages over others. Microalgae can be used in MFCs either as anodic or cathodic feedstocks. At anode, dry microalgae biomass is fed along with wastewater and nutrients medium. The microorganisms degrade organic substances under anaerobic condition producing CO_2 and electrons. The electrons move through external circuit to generate electric current. The use of microalgae at cathode is more interesting. Fresh and live microalgae cells are used as cathodic feedstocks. CO_2 released through metabolic degradation of waste at anode is guided toward cathode to feed microalgae. Studies showed that in this system microalgae can grow successfully up to 2 g/L (Cui et al. 2014; Velea et al. 2017). The microalgae biomass obtained at cathode can be used for biorefinery products. Thus, in this system, electricity generation, wastewater treatment, and microalgae biomass production can be carried out simultaneously. Preliminary investigations have proven this idea. However, the efficiency of the overall system is very low, and it is expensive too. To address this challenge, it is proposed to upcycle waste microalgae biomass. Microalgae obtained at cathode are

first used to produce biofuels (bio-hydrogen/biodiesel). The fuel-extracted biomass can be further recycled by feeding it to the anode. Spent microalgae biomass serves as an anodic feedstock. MFC can also be employed for the improvement of bio-hydrogen yield. In bio-hydrogen production process, some intermediate by-products like acetic acid, propionic acid, butyric acid, and valeric acid are produced rather than hydrogen, which lower its yield. The mixture of these by-products can be fed to the fuel cell at anode. Fermentative bacteria like *Enterobacter cloacae*, *Rhodobacter* sp, and *Rhodobacter sphaeroides* can be employed to degrade these products and turn them into protons, electrons, and carbon. Electrons move through external circuit to generate electricity, while protons and carbon can be used for the growth of microalgae at cathode (as described earlier).

4.3 Microalgae in Bio-hydrogen Technology

Microalgae are unicellular microorganisms which have the ability to do photosynthesis. In natural environment, they use sunlight as a light source and atmospheric CO_2 as a carbon source. During the process of photosynthesis, they accumulate various compounds in their body including carbohydrates. During this process, they also produce hydrogen and oxygen in trace amount. However, hydrogen and oxygen mix with each other and produce water. Thus, in bio-hydrogen process, concomitant discharge of oxygen and hydrogen is not desirable. To overcome this limitation, two-staged hydrogen production is adopted. In stage 1, microalgae photosynthesis is carried out to acquire carbohydrates, a feedstock for hydrogen. In stage 2, they are subjected to anaerobic condition to degrade carbohydrates and produce hydrogen.

There are a number of intermediate steps involved between photosynthesis and anaerobiosis. Sulfur deprivation is one of the important and necessary steps. In sulfur deprivation (s-deprivation), the cells are harvested (dewatered) by flocculant/coagulant aid, washed two to three times, and then re-suspended in sulfur-free medium (Sharma and Arya 2017). Nitrogen gas is also purged into the medium for few minutes to remove dissolve oxygen. The cell can be transferred into s-deprived medium in two ways: (1) directly as free cells and (2) the cells after solidifying. In free cells transfer, the cells are collected in exponential phase and simply transferred to s-deprived medium after nitrogen purging. The other method of s-deprivation is to use the cells after solidifying in agar, called immobilization. Immobilized cells furnish distinct features over free cells. Immobilized cells are viable for a long time, are easy to recycle, and produce up to three times more hydrogen than the free cells. After immobilization, the cells undergo a necessary step of fermentation (Fig. 1.3). In this step, the stored organic carbon is degraded into hydrogen ions and then combined with electrons through enzymes to form hydrogen molecule. Nitrogenase and hydrogenase are responsible enzymes to couple the hydrogen ions and the electrons. Fermentation can be carried out in dark or light condition. In dark fermentation, 2–4 moles of hydrogen are produced per mole of glucose. Obligatory

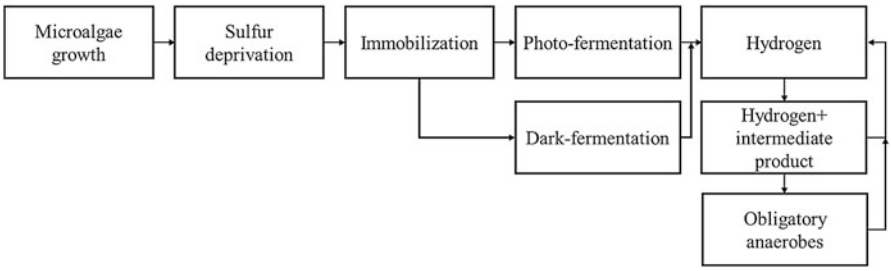

Fig. 1.3 Methods to improve bio-hydrogen yield form microalgae

anaerobes produce 4 moles, while facultative can produce only 2 moles of hydrogen. Photo-fermentation is more efficient than the dark, as it can produce up to 12 moles of hydrogen. Considering the economics of these processes, photo-fermentation causes high cost due to the supplement of light throughout the period of anaerobiosis. Dark fermentation on the other hand is less efficient. In this context, mixed culture approach can be employed. In this approach, the cells are first subjected to anaerobic condition under dark to produce 4 moles of hydrogen and acetic acid or other intermediate products. Now, these intermediate products are fed to special bacteria, which further degrade them to produce another 4 moles of hydrogen. Light can also be applied to degrade these intermediate products. The alternative and efficient approach to obtain the maximum yield of hydrogen at low cost is to manipulate the effect of photoperiod. It is reported that high- light condition (above 200 umoles/m^2/s) can also help in attaining anaerobic condition. It is typical in hydrogen production process that anaerobic condition is achieved after 72 h. However, the experiments have proved that high light condition for 24 h only is enough to reach anaerobic condition. Thus, the advantage of using high light condition is that it has to be provided for short time (24 h) only, while in the rest of the period (70–100 h), the cells are subjected to dark condition (Sharma and Arya 2017).

$$\textbf{Photo} - \textbf{fermentation} : C_6H_{12}O_6 + 6H_2O + \text{light} \rightarrow 12H_2 + 6CO_2$$

$$\textbf{Dark} - \textbf{fermentation:} C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + 2CO_2 + 2 + CH_3COOH$$

$$\textbf{Mixed culture after dark fermentaion} : CH_3COOH \rightarrow 2H_2 + 2CO$$

The modulation of fermentation techniques can improve hydrogen up to some extent; still hydrogen technology is not sustainable for commercial application. Several other techniques can be adopted for the economic sustainability of this technology. Restoring the photosynthesis process after fermentation step and subsequently producing hydrogen can significantly save the material cost vested on obtaining biomass. The recycling of spent growth media can greatly reduce the water footprints and the nutrients cost too. Several studies have reported using spent microalgae biomass for energy recovery in the form of hydrogen, which would

also lead to sustainability of this technology. High cost of bio-hydrogen is mainly because of the provision of carbon and light. To this end, mixotrophy and heterotrophy cultivation methods can address these challenges. In these cultivation systems, a wide variety of cheap organic sources can be employed including wastewaters to feed microalgae. Also, they displace the need of providing light. Future research should be designed to investigate the potential of mixotrophy/heterotrophy cultivation-based hydrogen production and its integration potential with wastewater technology.

4.4 Microalgae Coculture System

Studies on microalgal technology mostly rely on monoculture system. The monoculture system is promising and has distinct attributes. However, there are some technical barriers including low biomass productivity, low bioconversion efficiency, and high risk of contamination. Unfortunately, despite adequate studies and technological developments, it is still hard to overcome these challenges. To this end, coculture system has emerged as potential alternative which can provide economical and sustainable solutions to these challenges. In a coculture system, microalgae coexist with other microorganisms in a symbiotic relationship to share resources provided in the form of nutrients and energy and produce useful metabolites for biorefinery applications (Chwenk et al. 2014; Demuez et al. 2015; Domozych et al. 2005: Fouchard et al. 2005). Microalgae have the ability to grow in a niche of microorganisms including bacteria and fungi. In an ideal coculture system, autotrophic microalgae develop a symbiosis with a heterotrophic microorganism in a cooperative manner. The autotrophic microalgae fix carbon through photosynthesis and convert it into organic carbon; the heterotrophic microorganisms utilize the carbon source and convert into CO_2. The released CO_2 is then used by autotrophic algae (Fig. 1.4). In this way, the metabolite cycle continues and facilitates both microorganisms to grow optimally. Numerous studies have been reported to claim higher biomass productivity in coculture system than the monoculture system. High biomass productivity is just one intriguing aspect of coculture system; it offers many other unique advantages. Coculture system exhibits high self-flocculation efficiency to harvest microalgae. Actually, in coculture system, the cells produce excessive polysaccharides which aid in flocculation. It is also reported that coculture system produces such metabolites which protect it from contamination. Coculture system shows high pollutant removal efficiency and water reclamation potential than the monoculture system. As mentioned earlier, the coculture can be composed of microalgae-microalgae, microalga-bacteria, or microalgae-fungi/yeast. It is not established yet which of the coculture system is best suited to perform environmental services and biorefinery applications. Future studies should be directed to realize the potential of coculture system and to declare their definite superiority over monoculture system.

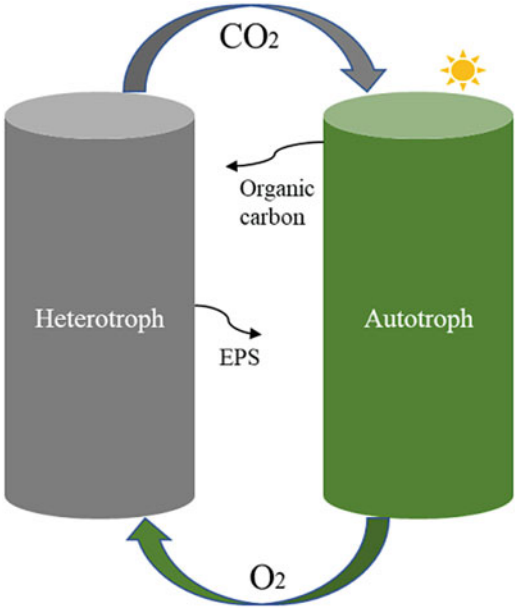

Fig. 1.4 Illustration of microalgae coculture system

4.5 Extremophile Microalgae

Contamination is the biggest challenge of microalgae bio-industry. Unwanted microorganisms outcompete the growth of microalgae cells, consume nutrients, decrease the metabolites productivity, and change the proximate composition of the biomass. This aggravated problem can be partially resolved by exploiting the potential of extremophile microalgae. They thrive in harsh environmental conditions. They can survive in a broad range of temperature (20–55 °C) and acidic pH (1.5–2.0). Majority of the microorganisms can't tolerate such harsh conditions (Sakurai et al. 2016; Schmidt et al. 2005; Wan et al. 2016).

Thus, they are not prone to contamination under such conditions. Regardless of contamination, extremophiles possess distinct features which differentiate them from other microalgae species. They can grow under various cultivation conditions including autotrophic, heterotrophic, and mixotrophic. Under heterotrophic conditions they grow fast (16 h doubling time) and result high biomass productivity (~30 g/L). They are an ideal microorganism for metabolite production and biorefinery applications. Metabolites are categorized into macro- and micronutrients based upon their weight percentage in the biomass composition. Macronutrients include protein, carbohydrates, and lipids. Micronutrients are mainly composed of vitamins, carotenoids, and phycobiliproteins. The composition and productivity of metabolites depend on a number of operating and environmental conditions.

The productivity of macronutrients is somewhat proportional to the biomass productivity. The biomass productivity and its composition depend mainly on cultivation conditions. According to a study, an extremophile microalga, *Galdieria*

*sulfuraria (*GS), in heterotrophic condition grows two times higher than the autotrophic condition. Graziani et al. (2013) measured the doubling time of 20 different stains on GS under heterotrophic and autotrophic condition. Among them, GS 064/309 showed the shortest doubling of 16 h under heterotrophic condition, whereas the same strain returned 39 h doubling time in autotrophic condition. The corresponding biomass productivity was 29 g/L under heterotrophic and 5.7 g/L under autotrophic condition. The lipid contents were extremely low, 1.4% under heterotrophic and 1.1% under autotrophic conditions. The lipid profile of GS was highly dependent on growth condition. Unsaturated fats were higher in heterotrophic than the autotrophic conditions. Overall, polyunsaturated fats were more than the monounsaturated fats. About 69% of GS was composed of carbohydrates. Interestingly, 54% of total carbohydrates were comprised of insoluble dietary fiber. It should be noted that insoluble dietary fiber poses positive impact on human health by improving human intestinal system. Wan et al. (2016) reported 8% lipids, 24% protein, and 51% carbohydrates under heterotrophic condition. Sakurai et al. (2016) observed the proximate composition of metabolites in GS under autotrophic, heterotrophic, and mixotrophic conditions (Sakurai et al. 2016). They found high biomass productivity under mixotrophic condition; however, lipids and glycogen contents were low. Furthermore, the lipids were observed under mixotrophic and heterotrophic conditions only. The lipids were mainly (43%) composed of diacylglycerol. The lipid contents could be increased by adding glucose. However, lipid increase was high in heterotrophic condition as compared to mixotrophic. The dominant lipids were C16:0, C18:1, and C18:2 in all growth conditions. An increase in C18:0 under mixotrophic and C18:3 were observed under heterotrophic conditions.

Extremophile is a promising source of micronutrients which include carotenoid, pigments, chlorophyll, and vitamins. A study showed vitamin B_2 and B_3 (30–32 mg/Kg) under heterotrophic condition; a lower concentration (1–20 mg/Kg) was noted in autotrophic condition. Carotenoids were found (only in autotrophic condition) in small fraction only in GS (1.2% of dry biomass). Astaxanthin and lutein were the major components of carotenoids. Phycobiliproteins were also found under autotrophic conditions as well as heterotrophic conditions. Allophycocyanins were high (79 g/Kg) under autotrophic condition while phycoerythrins under heterotrophic condition (6.5 g/Kg).

Phycobiliproteins have received widespread attention in recent years due to their versatile applications. They are being used in fluorescent, cytometry, gel electrophoresis, chromatography, and medical, food, cosmetic, diagnostic, and other biotechnology applications. They have antioxidant and anti-inflammatory properties. Their anticarcinogenic and nontoxic nature makes them a promising choice in cosmetic industries. Phycobiliproteins are water-soluble fluorescent protein, present in cyanobacteria and red algae. Generally, they are classified into three categories: (1) C-phycocyanin(2) R-phycocyanin, and (3)allophycocyanin. They function as light-harvesting antenna in microalgae. They help in light capture and energy transfer. C-PC is identified as a major pigment in microalgae. Cyanobacteria have been considered as a rich source of C-PC. Recently, C-PC presence in red algae is

also being investigated. The use of red algae for C-PC production offers unique advantages over other microalgae species because of their tolerant nature and versatility to grow under different cultivation conditions. C-PC production in several microalgae has been reported under autotrophic, heterotrophic, and mixotrophic conditions. Graverholt found C-PC in GS. Wan et al. (2016) found that C-PC concentration was very low (0.09% of dry biomass) in GS under heterotrophic condition (Graverholt and Eriksen 2007). Schmidt et al. (2005) found C-PC productivity of 27 mg/L/day; Graverholt and Eriksen (2007) found 183.9 mg/L/day in GS under heterotrophic condition (Schmidt et al. 2005). Wan et al. (2016) achieved 2209 mg/L/day (13.88% of dry biomass) by exposing the cells under high light conditions (250 μmoles/m^2/s), which is the highest C-PC productivity reported so far. They correlated C-PC productivity with inoculation density of microalgae culture. The highest C-PC productivity was observed at 0.6 g/L of initial inoculation density. Further researches should be carried in the framework of value-added bioproducts from algae to drive more about the sustainability of microalgae biorefinery.

5 Conclusions

The sustainability of microalgae-based biorefinery can be promised by improving the overcall economics of microalgae chain processes. With current available technology, a limited improvement in microalgae bioprocesses can be made, and yet, they are not economical. It emerges the need of redirecting the focus of microalgae industry. Exploring the potential of extremophile microalgae, mixotrophy cultivation, and coculture system to obtain value-added by-products can essentially offset the cost of microalgae bioprocesses. Moreover, the scope of microalgae should be extended to environmental and economical services, bioremediation and wastewater ecology, and food industry. Recent researches have proved that microalgae can be successfully integrated with bio-hydrogen, fuel cell, and biogas and biofilm technology for high-energy recovery and sustainable biorefinery.

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Chapter 2
The Culture Technology for Freshwater and Marine Microalgae

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Abstract Microalgae are promising eco-friendly source of food, feed, biofuels, and chemicals. There has been substantial progress at the lab and industrial scales to develop efficient and sustainable microalgae culturing techniques. However, several constraints must be addressed to make the overall process economically viable. Chemo-genetics elements can play a pivotal role in achieving the commercial goals because microalgae grow more efficiently in high concentrations of essential nutrients like nitrogen, phosphorus, and carbon in addition to enhance by-product formation. Moreover, alteration in culturing conditions also activates lipid accumulation. Recent strategies have combined these approaches to enhance lipid accumulation and along with enhanced biomass productivity. It is necessary to optimize inoculum production and culture management to avoid contamination, especially at commercial scales. Furthermore, prevailing outdoor conditions of rainfall, variable temperature, and irradiation, which are entirely different from small lab-scale facilities, pose additional challenges during outdoor cultivation. This chapter highlights the nutritional requirements of culturing media and their impact along with possible challenges on microalgae cultivation to ensure the stable and high productivities of large-scale cultures. Media recycling not only reduces the dependency on freshwater but also increases the economic viability of the process. Recent advances regarding media recycling and strategies to control biological contaminants are also discussed.

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1 Introduction

Algae are a diverse group of industrially important organisms found in every nook and corner of the world. They may be smaller in size (unicellular) or larger (kelps) habituating in the marine or freshwater environment. Microalgae have gained remarkable attention owing to their potential to accumulate lipids (70%) and carbohydrates (60–65%) (Afzal et al. 2017; Gill et al. 2016). Additionally, they can accumulate essential amino acids (~50% of total biomass) and pigments like chlorophyll, carotenoids, and phycobilin in trace amounts (Afzal et al. 2017). Owing to their high lipid content, they seem promising for the biofuel (mainly biodiesel) production, and some microalgae containing higher carbohydrate content have also been employed to produce bioethanol (Alam et al. 2017; Aziz et al. 2017). Microalgae are being used as an alternative food source since the 1960s due to their nutritional value. For instance, *Dunaliella salina* (a marine microalga) is rich in β-carotene. In aquaculture, microalgae have been used for feed purposes, where 30% of the globally produced microalgae are consumed as animal feed (Shang et al. 2018; Sirakov et al. 2015). Microalgae are also investigated for their medicinal properties. For instance, *Spirulina* has been reported for the prevention of cardiovascular diseases, viral infections, and cancer due to their immunogenic properties. Similarly, *Chlorella* (freshwater microalgae) have the potential to reduce blood cholesterol and sugar and are believed as hemoglobin and immune enhancers. Microalgal pigments have also shown anti-inflammatory, antibacterial, antifungal, anticancerous, and antioxidant properties (El Gamal 2010). Biopigments obtained from microalgae are routinely used in food, textile, and paper and pulp industry due to the presence of carotenoids, flavonoids, chlorophyll, etc. In the food industry, they give the characteristic color to jam, jellies, bubble gums, etc. Furthermore, marine microalgae are a good source of vitamins (A, B-complex, E), astaxanthin, polyunsaturated fatty acid, and β-carotene and are commercially produced as nutraceuticals and food additives (Suleria et al. 2015). They have been used in the cosmetics industry for eyeliners and lipsticks. Microalgal extracts have UV protection, antiaging, and skin-tightening ability and are extensively used in the cosmetics industry (Bueno et al. 2017).

Though freshwater and marine microalgae have been extensively studied, in order to achieve the "green revolution" by "blue biotechnology," there are some areas which need to be focused (Barra et al. 2014) including (i) isolation, identification, and characterization of novel freshwater or marine microalgae strains, (ii) evaluation of nutritional and abiotic factor to enhance the growth and metabolite content, (iii) development of alternative culture technologies to upscale the biomass production while keeping the cost as low as possible, and (iv) development of PBR maintenance strategies to enhance the overall efficiency of the procedure (Fig. 2.1).

One important aspect is the selection of suitable cultivations strategy. Different cultivation methods are in practice for microalgae growth, including photoautotrophic (photosynthetic fixation in the presence of light), heterotrophic (carbon utilization in the absence of light), and mixotrophic (combination of both previously mentioned methods) (Shahid et al. 2017). Additionally, at mass cultivations, open

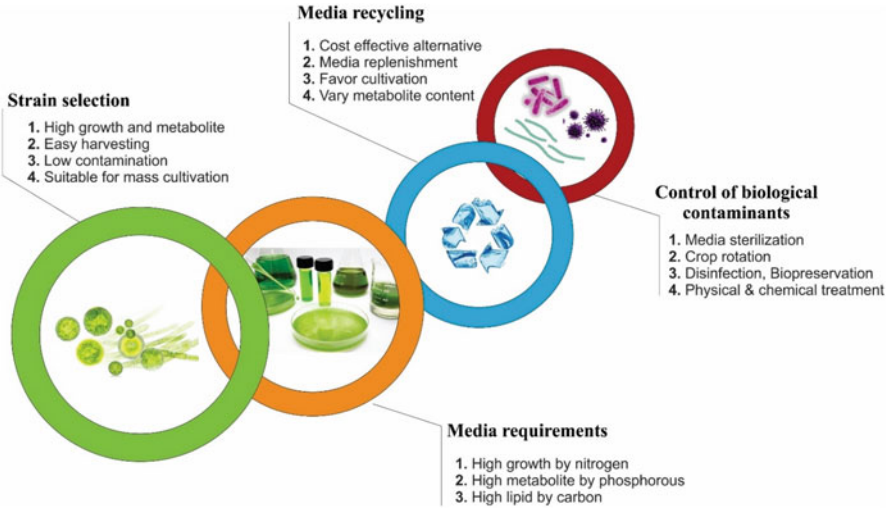

Fig. 2.1 Areas need to be focused on freshwater and marine microalgae culture technologies

pond system (natural ponds, lakes, lagoons, or artificially designed shallow ponds) is preferred due to the ease of use and cost-effectiveness (Alam et al. 2015). Closed pond system is used when microalgae have to be grown in specific conditions as it offers more control over environmental parameters. Most preferred closed cultivation systems include flat-panel and tubular photobioreactors (PBR), while later one is most desirable for outdoor cultivation (Bibi et al. 2017). Hybrid cultivation system is utilized to combine the open and closed cultivation systems in order to achieve the maximum benefits of two methods while limiting the demerits (Shahid et al. 2017). In general, microalgae-based products are quite expensive due to extensive downstream processing (Alam et al. 2016). One possible solution in this regard is to focus on the biorefinery aspect of microalgae cultivation (Gill et al. 2013). In mixotrophic cultivation substrate cost can be reduced by integration of biomass production with wastewater treatment (Gill et al. 2016) as wastewater from various sources like domestic, dairy, piggery, etc. contain a high amount of essential nutrients. So, it is important to investigate the effect of nutrients on biomass growth and metabolite composition to exploit the full potential of the process (Chandra et al. 2016).

This chapter discusses various culturing aspects to enhance the biomass productivity and content of other value-added products at the lab and commercial scales along with the associated challenges and opportunities to ensure the economic viability of the process. The culture medium, nutritional requirements, and impact of these nutrients on fresh- or marine water microalgae are also discussed. Mass-scale cultivation of microalgae has several problems which raise the cost of production. Cost of the media is one of the major economic barriers because media and huge amounts of freshwater are always required regardless of the selected strain, environmental conditions, etc. So, the possibility of media recycling and use of wastewater as an alternative low-cost media is also discussed.

2 Nutritional Requirements of Culturing Media

Considering the potential of microalgae for commercial uses such as health foods (Milledge 2011), pigments (phycobiliproteins) (Chandra et al. 2017), fatty acids (Wijffels 2008), animal feed (Raja et al. 2008), stable biochemicals (Spolaore et al. 2006), human food (Borowitzka 2006), and biofuel production (Suganya et al. 2016), optimized conditions are required to obtain the maximum biomass productivity (Chen et al. 2017). Different compositions of culturing media strongly influence the biomass productivity and composition (Chen et al. 2011) under varying culturing conditions like temperature, irradiance, nutrients, CO_2 supply, pH, and inoculum size (Kim et al. 2014a; Yen et al. 2014). Presence of different nutrients, namely, nitrogen (N), carbon (C), phosphorus (P), and minerals, in the culturing media or water bodies strongly affects the growth and biochemical composition of microalgae (Bartley et al. 2016). Under normal circumstances, lipids, proteins, and carbohydrates are being produced in well-adjusted manners by microalgae, while environmental stresses can direct the carbon flux toward lipid production (Xin et al. 2010).

2.1 Impact of Nitrogen on Growth and Lipid Productivity

Nitrogen is the fundamental nutrient because it is the main component of structural and functional proteins, enzymes, energy currency molecules, chlorophylls, and other genetic materials of microalgae (Kim et al. 2016). Lipid profile, cell growth, and carbohydrate content of the microalgae are directly influenced by the concentration of nitrogen in the culturing media (Arumugam et al. 2013). Different studies have demonstrated that lower concentration of nitrogen in the culture medium decreases the cell growth rate (Sharma et al. 2012) while increasing the lipid/carbohydrate content (Shang et al. 2018). In fact, under nitrogen-depleted environment, microalgae minimize the routine cell protein synthesis and start accumulating the lipids. The extent of lipid accumulation under stress conditions differs among various strains of microalgae, so as their potential for biodiesel production (Sharma et al. 2012). The strains with higher lipid content and faster growth are most suitable strains for biodiesel production, but unfortunately, such strains are very rare in nature. Generally, with reference to lipid and growth productivity, the microalgae with higher lipid content usually have lower cell growth rate such as *Botryococcus braunii* which is known to have 70% lipid content but lower biomass productivity (Dayananda et al. 2007). Alternatively, the strains with higher cellular growth often have minimum lipid productivity, for example, *Chlorella vulgaris* which have 19 h doubling time but can accumulate only 20–30% lipids (Griffiths and Harrison 2009). Microalgae can utilize nitrogen in both organic and inorganic forms including nitrites, nitrates, ammonia, nitric acid, nitrogen dioxide, and urea (Cai et al. 2013). As assimilation of ammonium requires less energy, so many strains of microalgae

prefer to reduce nitrogen from any source into the primary form of fixed nitrogen. During fattening conditions, such as nitrogen starvation, cell proteins are being transformed to lipids or carbohydrates. However, the duration for nitrogen deprivation should be carefully monitored, as *S. obliquus* was observed to accumulate more carbohydrates than lipids when the duration of nitrogen depletion is increased (Ho et al. 2012). In microalga *Neochloris oleoabundans*, lipid and carbohydrate content increased dramatically, while levels of chlorophyll a and proteins decreased in the cell with increasing duration of nitrogen deprivation (Sun et al. 2014). Unlike other microalgae, *B. braunii*, *Dunaliella tertiolecta*, and some *Chlorella* spp. utilize nitrate instead of ammonium for cell growth (Ruangsomboon 2015; Muthuraj et al. 2014). Studies have shown that nitrogen starvation also affects cell size, the thickness of cell wall, and mechanical strength of microalgae. The microalgal strains *Nannochloropsis* sp., *Chlorococcum* sp., and *Chlorella* sp. were found to have increased cell size and cell wall thickness during nitrogen depletion in media (Yap et al. 2016). Recently, a study reported the difference between the standard and mutant (lipid-rich) strains of *Tisochrysis lutea* when grown in nitrogen-rich and nitrogen-depleted environments. Both strains exhibited lower cell growth rate and accumulate triglycerols (mutant strain) and alkenones (standard strain) as reserved lipids in the nitrogen-depleted environment (da Costa et al. 2017).

2.2 Impact of Phosphorus on Growth and Metabolite Content

Phosphorus is another major nutrient required for the growth of microalgae with a strong impact on the process of photosynthesis and respiration (Elser 2012). It plays an important role in the formation of phospholipids, energy currency molecules, nucleic acids, and signal transduction. Without phosphorus, the cell growth is impossible, as it is the most fundamental nutritional factor (Solovchenko et al. 2016). The inadequacy of phosphorus can reduce the levels of phospholipids in the cells and consequently non-phosphorus sulfolipids and glycolipids are formed. Microalgae form cellular components such as phospholipids to assimilate phosphorus in its biomass. The other route also is known as luxury uptake, where microalgae can store surplus phosphorus in the form of inorganic polyphosphates. Some of these polyphosphates can be soluble in the acid, which makes their use possible in cell metabolism, while insoluble polyphosphates can be stored inside the cell when external phosphorus is not available (Schmidt et al. 2016).

The phosphorus limitation in the microalgae *Chlorella* sp. increased its lipid content and biomass productivity (Liang et al. 2013). While, under nitrogen-deficient and phosphorus-sufficient conditions, *C. vulgaris* exhibited lipid productivity of 58.39 mg L^{-1} day^{-1} which was higher when compared to the lipid productivity of algal strains cultured under phosphorus-deficient conditions. This increase in the lipid content is due to the fact that excess phosphorus provides the cell with sufficient energy to synthesize essential cell materials which help to boost the

growth and lipid accumulation in microalgae (Chu et al. 2013). Similarly, under sufficient supply of phosphorus and nitrogen-stress conditions, *S. obliquus* was triggered to maximum lipid synthesis (Chu et al. 2014), and fatty acid methyl esters (FAME) productivity was reached to 55.9 mg L^{-1} day^{-1} (Shen et al. 2015). A similar trend was observed for *C. vulgaris*, which can accumulate both acetate and FAME during nitrogen deficiency and phosphorus sufficiency in the media, with an enhanced productivity of 66 mg L^{-1} day^{-1} (Shen et al. 2016). Cultivating *C. regularis* under limited nitrogen and excess phosphorus conditions during heterotrophic glucose cultivation, lipid contents increased dramatically (up to 310.0 mg L^{-1} day^{-1}) with an increase in phosphorus supply (Fu et al. 2017). With an increase in the phosphorus concentration in media, luxury uptake of phosphorus also was shown to increase by *Nannochloropsis salina,* both in batch and continuous cultivation systems (Sforza et al. 2018).

2.3 Impact of Carbon on Microalgae Cultivation

After nitrogen and phosphorus, carbon is another vital nutrient, playing an indispensable role in the growth of microalgae (Ramaraj et al. 2015). Microalgae play an important environmental role by biologically fixing the atmospheric carbon consequently reducing the risk of global warming (Russel et al. 2018; Zhao and Su 2014). Microalgae contain about 50% carbon in their biomass (Chisti 2006). Besides atmosphere, microalgae can also fix the carbon from exhaust gases of industries or power plants and from soluble carbonates (Cuellar-Bermudez et al. 2015). Irrespective of the sources, carbon is fixed to synthesize polysaccharides, volatile organic compounds, hormones, and organohalogens (Sydney et al. 2010; Ho et al. 2011). A smaller fraction (9%) of incoming solar energy contributes to the dry biomass production of 280 tons per hectare per year while utilizing 513 tons of carbon dioxide (Bilanovic et al. 2009). The source of carbon also has an impact on the biomass productivity of microalgae, when *Chlorella* was cultured in the N-depleted medium supplemented with sucrose as a carbon source, initially at lower concentration growth rate increased along with the lipid production. But with the increasing sucrose in the medium, microalgal cell growth became slow. Because, under nitrogen deprived conditions, protein synthesis is hampered, metabolic flux is diverted to the synthesis of lipids and carbohydrates (Lin and Wu 2015). Culturing of *Scenedesmus obtusus* in the growth media supplemented with CO_2 resulted in the maximum lipid productivity of 15 mg L^{-1} day^{-1}; however, further increase in CO_2 concentration did not increase the lipid content. Moreover, use of glycine and ammonium acetate as carbon sources in the media enhanced the growth (Chandra et al. 2016). Supplementing 15% CO_2 in the media as a carbon source, *B. braunii* showed maximum carbohydrate content, but at 20% concentration, carbohydrate levels reduced, and lower lipid content was observed at a lower concentration of CO_2 in the media (Ruangsomboon et al. 2017). The microalgal species, namely, C. *sorokiniana* and *Asterarcys quadricellulare*, are tolerant to high

temperatures, high CO_2, and high-light intensities and have shown the highest carbohydrate and lipid content when cultured using higher concentrations of CO_2 (Varshney et al. 2018), while *C. sorokiniana* is found to be more extremophilic than the others.

2.4 Impact of Inoculum Size on Lipid Productivity

In particular, inoculum size has a great impact on specific growth rate, metabolite production, biomass content, and lipid accumulation (Ho et al. 2013). Inoculum size reflects the number of cells that will reproduce to increase biomass production (Richmond 2008). Increase in the inoculum size higher than a certain limit negatively affects the cell reproduction due to limited light and nutrient supply (Markou et al. 2014). Studies have shown that a slight increase in inoculum concentration enhance the lipid productivity of microalgae but to a certain limit. Maximum lipid productivity was observed in *Nannochloropsis oculata* at 2.3 gL^{-1} inoculum concentration when the concentration was increased to 2.88 gL^{-1}, a decreasing trend was observed in the lipid productivity (Su et al. 2011). It is observed that the dose of inoculum also affects the FAME content and biomass composition of *C. sorokiniana*, showing maximum FAME content at an inoculum concentration of 2.5×10^5 cells mL^{-1}. With an increase in the inoculum dose, microalgae become more efficient to compete with bacteria present in wastewater for nutritional requirements (Bohutskyi et al. 2016).

Among five different strains of microalgae, *C. vulgaris* 1067 exhibited the potential of maintaining higher growth in response to a larger inoculum size, resulting in an overall biomass productivity of 0.13 g L^{-1} day^{-1} with an inoculum size of 0.103–0.135 g L^{-1} (Bibi et al. 2017). Nitrate and arsenic removal efficiency of microalgae is also influenced by inoculum size. *C. pyrenoidosa* showed higher biomass concentration and arsenic removal efficiency with an inoculum size of just 10%, while the increased concentration of inoculum resulted in an increase in the microalgal growth and arsenic removal efficiency (Podder and Majumder 2016). Similarly, in another study decrease in inoculum size (from 100% to 5%) was reported to be associated with high nitrate removal efficiency, while the increase in inoculum concentration results in decreased microalgal growth (Daneshvar et al. 2018).

3 Maintenance of Cultivation Systems: Challenges and Opportunities

Lipid, carbohydrates, and proteins are the essential biomass components of the microalgae, levels of these components in microalgae go on changing under different conditions. For example, during normal conditions biomass of *C. vulgaris*

mainly consists of 14–22% lipids, 12–17% carbohydrates, and 51–58% proteins (Spolaore et al. 2006). But during changes in the culturing environment, the biomass composition also changes. Four types of culturing systems are commonly used for microalgae, (i) fermentation tank, (ii) open photobioreactors, (iii) closed photobioreactors, and (iv) algal biofilm photobioreactors (Genin et al. 2016). Fermentation is the most common laboratory-scale cultivation method for microalgae, but it has never been used on commercial scales. Open pond microalgal culturing despite its low operational cost faces several challenges (Mata et al. 2010) such as it requires larger areas and is prone to contamination, changes in weather patterns, poor light absorbance, and evaporation.

Monitoring is not easy in open pond system, and its design does not support the effective photosynthesis (Wolkers et al. 2011). A variety of closed photobioreactors are used to culture different strains of microalgae; enhanced surface area-to-volume ratio is required for closed photobioreactors. Slight changes in daily temperature can influence the growth; therefore, the integrated cooling system should be installed in the closed system to avoid elevated temperatures that can halt the microalgal growth (Kunjapur and Eldridge 2010). In comparison to the other cultivation systems, algal biofilm photobioreactors produce highly concentrated microalgal biomass but with poor control of temperature and microalgal species (Genin et al. 2016; Elrayies 2018). The quality and composition of untreated wastewater differ depending upon its source, including municipal, agricultural, and industrial wastewater (Komolafe et al. 2014). The culturing of microalgae using wastewater on lab scale cannot actually reflect the challenges associated with mass-scale cultivation; those may include nutrient composition, heavy metals, microbial interactions, and other physiological factors such as temperature, pH, and light intensity (Cai et al. 2013). Urban wastewater was used to cultivate seven different microalgae species, which actively participated in the removal of 80% concentration of total phosphorus and 87% concentration of total nitrogen, with a biomass productivity of 108–118 mg L^{-1} day^{-1} (Mennaa et al. 2015).

The problems associated with the microalgal cultivation using agriculture wastewater include (i) considerably higher or lower concentration of nutrients (Chen et al. 2015), (ii) lower concentration of carbon for microalgal assimilation (Zhou et al. 2014), (iii) increased turbidity that halts the penetration of sunlight (Olguín 2012; Sahu et al. 2013), and (iv) rigorous use of insecticides, pesticides, antibiotics, and other toxic chemicals which influence the overall growth and biomass quality of microalgae. The wastewater coming from industries has unusually increased levels of biological oxygen demand (BOD), chemical oxygen demand (COD), and total suspended solids (TTS). It can also be deprived of essential nutrients and enriched with heavy metal or toxic compounds which can hamper the growth of microalgae (Kong et al. 2010). Limited nutrient supply in wastewater can be treated with nutrient supplementation to combat the issues of lower productivity in microalgae. Nutrient-rich food wastewater has shown to be very effective for the growth of *S. obliquus* which exhibited substantial lipid productivity and FAME content (Ji et al. 2015). Wastewater from agriculture and municipal sources contains a high amount of heavy metals, toxic compounds, and increased turbidity which

negatively affect the microalgal growth. So, to counter these problems, this high strength wastewater can be diluted with freshwater or low-nutrient wastewater for sustainable growth of microalgae (Zhou et al. 2012). Careful optimization of the dilution rate is required by considering the strength and source of wastewater (Park et al. 2015). Other than microalgae wastewater possesses several bacteria, fungus, and other microbes which interfere with the growth of microalgae by competing for nutrients present in the wastewater (Cho et al. 2011). These microorganisms can be removed or reduced in the wastewater by employing different pretreatment methods, the most popular of them are filtration and autoclaving (Ramsundar et al. 2017).

Other recent techniques for pretreatment involve the acidification of wastewater (up to pH 2.0), ozonation, ultraviolet exposure, chlorination (Qin et al. 2014), employing the high ammonia concentration, and applying short anaerobic periods to halt fungal growth (Gan et al. 2014). Recent advances in algal research enable scientists to overcome the challenges associated with microalgal culturing for its sustainable production (Guldhe et al. 2017). Commercial-scale production of microalgae is highly desirable because it is an efficient platform to produce a variety of useful products like biofuels, bioplastics, biopolymers, biopigments, and other health products (Dixon and Wilken 2018).

4 Media Recycling and Utilization

Water is a major requisite for microalgae as it acts as a medium for nutrient supply and provides thermal regulation. Mass culturing of microalgae requires a substantial amount of nutrients and water which are of particular concern as these commodities are directly or indirectly have been used for human consumption (Murphy and Allen 2011). Moreover, their acquisition contributes to high cultivation cost. Large-scale microalgae production has been criticized for its high-water requirements. It is estimated that approximately 3000 L water is required for the production of 1 L microalgae-based biodiesel (Yang et al. 2011). Even in optimized heterotrophic cultivation condition, the enormous amount of water is essential for microalgae which accounts for 80–85% of water according to cell densities (Lowrey et al. 2016). Culture medium after harvesting accounts for 84% of total water used which is now termed as waste. This loss of water can be reduced to 591 kg of water/kg of biodiesel if the medium is recycled (Table 2.1). Still, water footprint (WFP) of petroleum refineries is comparatively low so, it's necessary to reduce the microalgal WFP.

The use of specific growth medium (especially in closed PBR) contributes to running cost of the process. The used growth medium is often considered as waste, and fresh medium is often supplied to each batch which raises the cost of the process (Fret et al. 2017; Lowrey et al. 2016), while media recycling offers a sustainable and cost-effective approach (Fret et al. 2017). However, spent media also contain microalgal cell debris, organic compounds, and bacterial species which may pose several challenges during media recirculation such as media cleaning, nutrient

Table 2.1 Water footprint of microalgae for different cultivation technologies

Cultivation method	Source of microalgae/ biomass	Description	WFP (kg-water/kg-biodiesel)	References
Open pond	Freshwater	Freshwater (without recycling)	3726	Yang et al. (2011)
		Freshwater (with recycling)	591	Yang et al. (2011)
		Wastewater	400	Kumar et al. (2017)
	Marine	Freshwater (without recycling)	370	Yang et al. (2011)
		Wastewater	399	Maeda et al. (2018)
Photobioreactor	Freshwater	Freshwater (without recycling)	3494	Feng et al. (2016)
		Freshwater (with recycling)	245–2118	Feng et al. (2016)
		Wastewater	219	Feng et al. (2016)
–	Petroleum refining and extraction for gasoline production	–	2–6 L/L gasoline	Kumar et al. (2017)

replenishment, and the negative impact of the released material on biomass growth (Fret et al. 2016; Hadj-Romdhane et al. 2012).

Microalgal cultivation using recycled media is in practice since 1940; however, this approach has been utilized from the last 5 years for biotechnological application of microalgae. Table 2.2 reflects the effects of extracellular compounds and recycled media on the growth of algae (Loftus and Johnson 2017).

Most of the studies have shown a significant reduction in biomass concentration and growth rate in recycled media. However, some microalgae including *Desmodesmus*, *Tetraselmis*, *Arthrospira*, and *Hormotila* showed promising growth potential on spent media (Loftus and Johnson 2017). Impact of media recycling varies depending upon cultivation conditions and microalgae species. For instance, the reduced growth rate was reported for *Nannochloropsis* sp. during water reuse (Rodolfi et al. 2003), while media recycling reported no negative impact on the growth at laboratory and pilot scale (Fret et al. 2017). However, it corresponds to high organic matter accumulation in microalgae (Depraetere et al. 2015).

Table 2.2 Overview of impact of media recycling on growth and metabolite content of fresh and marine water

Microalgal strain	Cultivation scale	Cultivation method	Recycling	Impact on growth/metabolites	References
Chlorella vulgaris	Lab scale, batch	MPBR	>13 times	Limited growth due to the accumulation of counterions and non-limiting nutrients	Discart et al. (2014)
Nannochloropsis gaditana	Continuous	BCPBR	1 time	0.8 gL^{-1} day^{-1} biomass productivity with lipid (40%) and proteins (50%)	González-López et al. (2013)
Nannochloropsis oceanica				Reduced growth from 2 gL^{-1} to 1.6 gL^{-1} with enhanced cell aggregation	Zhang et al. (2016)
Tetraselmis MUR 233	Pilot scale	Raceway pond	–	48–160% enhanced DW biomass	Sing et al. (2014)
Chlorella vulgaris	Lab scale	Erlenmeyer flasks	Daily post-harvest recycling	532.2 mgL^{-1} day^{-1} and 40 $mgL^{-1}day^{-1}$ biomass and biodiesel productivities, respectively	Deng et al. (2018)
Chlorella sp.	Indoor	PBR	Repeatedly 3 times	80% biomass harvesting efficiency (recycling combined with ferrofluid harvesting)	Ho et al. (2017)
Chlorella vulgaris	Lab scale	Erlenmeyer flasks	>3 times	Enhanced biomass and lipid content	Farooq et al. (2015a)
Nannochloropsis oceanica	Industrial	PBR	–	50% productivity loss, possible to enhance productivity by media disinfection	Gaspar (2014)
Scenedesmus sp.	Lab scale	Erlenmeyer flasks	4 times	Slightly reduced biomass growth (0.03 gL^{-1} day^{-1}) as compared to control	Crofcheck and Crocker (2016)

C. vulgaris an industrially important freshwater microalga which was able to grow successfully in nutrient supplemented recycled water (Farooq et al. 2015b). Similarly, it's viable to cultivate *Scenedesmus* sp. in recycled media up to three times with no negative impact on cell growth; however, reduction in metabolite content, especially of protein and lipids, was observed. Moreover, recycled media must be supplemented with essential nutrients (Rocha et al. 2015). *Nannochloropsis salina* was able to grow in recycled media (with the addition of essential nutrients) with little change in biomass productivity, but losses were observed in lipid productivities (Lammers et al. 2017).

Interestingly, contradicting results were obtained for *Tetradesmus obliquus* (freshwater microalga) as an increase in the metabolite content (carbohydrate and lipid) was observed when it was cultivated in batch photobioreactor repeatedly (Massa et al. 2017). Highest biomass, lipid, and biodiesel productivities of 266.66 mg L^{-1} day^{-1}, 46%, and 20.66 mg L^{-1} day^{-1}, respectively, were reported in *C. zofingiensis* (Zhu et al. 2013) when cultivated in nutrient-limiting conditions in recycled media. It was reported that harvested water can be reused up to ten times without any negative impact on growth and composition of algae (*C. kessleri*) with 82–84% water recovery per growth cycle (Igou et al. 2014). Recycled media (nutrient replenished) support the growth of *Scenedesmus* sp. with no deleterious effects (Crofcheck and Crocker 2016).

Media replenishment is important to fulfill the nutrient requirements of microalgae for the growth and metabolite storage when culture medium is reutilized as an economic and ecological alternative. Recultivation of *Neochloris oleoabundans* in nitrogen and phosphorus replenished mixotrophic and autotrophic exhaust media has shown to enhance cell growth and free acid production (Sabia et al. 2015). A 16% enhanced cell growth was observed for *N. oceanica* cultivated in recycled water due to the presence of polysaccharides as a carbon source in this water (Kim et al. 2014b). *Tetraselmis* MUR 233 showed approximately 160% enhanced growth under mixotrophic cultivation (where media was recirculated continuously in raceway ponds) as compared to the control conditions (Sing et al. 2014). Positive effects on the lipid and biomass productivities of *Acutodesmus obliquus* were reported by Hesse et al. 2017 when this specific microalga was cultivated in media obtained after the flocculation. In a study regarding the impact of media recycling on growth and metabolite content of *Chlorella sorokiniana*, 3–18% reduction in growth rate was observed for media recycled up to four times, respectively, while carbohydrate content was shown to be 8–10% along with some inhibitory polyunsaturated acids (Spence 2016). *Arthrospira platensis* cultivated in recycled medium showed a reduction in growth as compared to the control cultures. However, this reduction associated with organic matter accumulation mainly in the form of polysaccharides (Depraetere et al. 2015).

In general, media recycling is not so cost-effective method as it seems because spent medium must be supplemented with additional nutrients to fulfill the biomass's nutrient requirement. Reduction in growth rate and metabolite content are related issues; however, there are some cases where enhanced production was observed when the medium was recycled. Almost similar results were observed at lab-, pilot-,

and industrial-scale levels. Another important aspect which greatly influences the effect of media recycling on biomass is the harvesting process and the number of times media was harvested. A possible solution in this regard is the use of wastewater as an alternative cost-effective media due to its high nutrients content which greatly favors the production and productivities of biomass and lipids.

5 Strategies to Control Biological Contaminants in Culture

Various cultivation systems are being employed at the pilot and commercial scales for microalgae cultivation. The culturing system varies in terms of contamination chances, control of growth parameters, biomass productivity, operation, and capital cost (John et al. 2011).

Algal cultivation, especially at mass scale, is highly susceptible to various biological contaminants including bacteria, viruses, fungi, photosynthetic microbial species, zooplanktons, grazers, and harmful freshwater and marine algal species (Fig. 2.2).

Most common strategy to reduce contamination load is by eliminating unwanted species either applying physical means (sedimentation, filtration) or through chemical treatments (Mata et al. 2010). Maintenance of extreme environmental conditions (pH, nutrients, salinity, light, etc.) is one possible solution to maintain microalgal monocultures (John et al. 2011). Identification of contaminant's nature is a crucial factor for the implementation of management strategies in order to maintain a high yield of desired microalgal strain while removing the contaminant (Wang et al. 2016). Another possible option to minimize contamination risk is by media sterilization, but it increases the input in terms of cost and energy requirements (Lam et al. 2017). The following are the major contamination risks, their problems, and common control strategies.

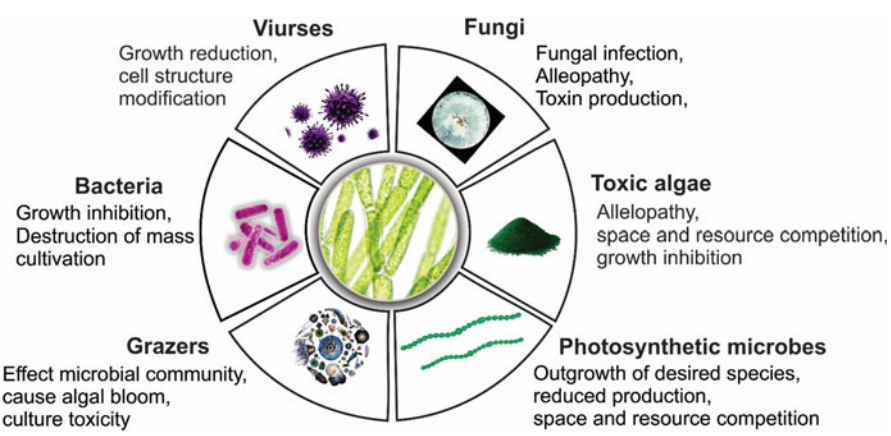

Fig. 2.2 Biological contaminants and their effect on algal cultivation

5.1 Algicidal Bacteria

Bacteria interact with algae and often are used in cocultivation. They are omnipresent in marine and freshwater environments. Most of the algicidal bacteria belong to the group gammaproteobacteria, while some belong to *Firmicutes* and actinobacteria. Marine algicidal bacteria like *Halomonas*, *Thalassospira*, and *Marinomonas* have been applied to control harmful algal blooms (Zheng et al. 2018). However, sometimes they have a negative impact on the desired algal species as they lysed the unicellular algae and stimulate algal blooms of toxic species like diatoms and dinoflagellate, thus inhibiting growth (Meyer et al. 2017).

Open ponds are high targets of bacterial contamination which can be prevented by chemical disinfectants, but this approach can be troublesome at mass-scale cultivation due to high nutrient levels and turbidity. Another more feasible option is the elimination of harmful bacteria by maintaining a suitable ratio of beneficial bacteria that can control the algicidal bacteria (Lam et al. 2017). Moreover, nutrient management and low/high pH of the growth media can also help the microalgae to dominate the environment and to outcompete the bacterial species.

5.2 Viral Contaminants

Viruses are abundant in aquatic environments and frequently associate with prokaryotes (cyanobacteria) and eukaryotes (algae). Most of them are host specific but are able to infect large populations of algae. Their impact is more predominant on algal blooms (Day et al. 2012), which is a kind blessing in disguise. Viral infections are rapid and specific in their activities due to their high multiplication rate and low replication time. They change the algal cell structure, succession, and diversity; hence, a significant reduction is algal growth rate is associated with them (Wang et al. 2013). A common approach is to use biopreservative microalgae to minimize the risk of contamination. Other possible solutions include the physical treatments like filtration and sedimentation or the use of chemical disinfectants. However, virus control methods are least explored area and more research is required (Lam et al. 2017; Wang et al. 2013).

5.3 Fungi

Freshwater microalgae are highly susceptible to zoosporic fungi or fungi-like organisms; however, pathogens of the marine system still are not well characterized. In a freshwater environment, *Chytridiomycota* is the most common parasite with narrow

to wide host range depending upon the algal species (Smith and Crews 2014). Severe loss in *Scenedesmus* production has been reported due to the activity of *Phlyctidium scenedesmi* (chytrid). It's difficult to fully understand the impact of fungi on microalgae cultivation specially at commercial scale. Treatment of algal inoculum with Triton-X or addition of $CuSO_4$ (copper sulfate) in culture media effectively reduced fungal contamination during microalgal cultivation (Carney and Lane 2014).

Algae are known to produce high amounts of abscisic acid as a natural defense against fungal infection. Exogenous application of abscisic acid has proven to be effective in this regard (Carney and Lane 2014). To reduce fungal contamination chances, fungicides are used, but unfortunately, thick-walled cysts of fungus can withstand high levels of fungicides. In this regard, more favorable and environment-friendly option is the cultivation of fungal infection-resistant algal species (Lam et al. 2017).

5.4 Zooplanktonic Grazers

Grazing is a widespread problem in mass cultivation of microalgae. In all aquatic systems, grazers refer to the zooplanktons like amoeba, protozoa, daphnia, ciliates, and rotifers, known to consume the microscopic organisms, and, thus, have a significant effect on the ecosystem (Day et al. 2012; Lam et al. 2017). Nanoplanktonic microalgae species are highly susceptible, while large-sized species are less susceptible mainly due to their size which makes them difficult to consume by these grazers (Day et al. 2017). Some grazers like rotifers and ciliates are larger in size as compared to microalgae and cause algal blooms, rapidly converting microalgae into inedible ones (Lam et al. 2017).

Netting during culturing and harvesting is possible solution to control these grazers. A shift in pH toward acidic conditions for short period and salinity reduction are some other common methods (Day et al. 2012). Another option is to reduce the fecundity of grazer by the release of chemicals (Day et al. 2017). It is suggested to exploit protozoa-resistant microalgal strains like *Tetraselmis* and *Chlorella* as much as possible (Lam et al. 2017). Pesticides like dichlorodiphenyltrichloroethane (DDT), Dipterex, and Parathion have been applied to reduce zooplanktonic contamination in *Chlorella* cultivation at lab-scale but has not been tried for mass-scale cultivation (McBride et al. 2014). Recently, ultrasonication was applied to remove the contamination (protozoa, fungi, amoeba, and ciliate) from mass cultures of *Chlorella* cultivated in open-raceway ponds. Hourly use of ultrasonic waves having 100% amplitude and produced by 495 W power effectively removed contamination (Wang et al. 2018). In another study, SDS (sodium-dodecyl-sulfate) was added as a surfactant to facilitate foam floatation as a possible approach for ciliate removal. It showed ciliate removal efficiency up to 96.3% when applied on ciliate-microalgae culture by reusing SDS and employing multistage floatation (Umar et al. 2018).

5.5 Toxic Freshwater and Marine Algal Species

High lipid-producing microalgal strains like *Tetraselmis* sp., *Chlorococcum* sp., and *Scenedesmus* sp. mainly face the challenges of population crashes and culture contamination (Greenwell et al. 2009) due to the presence of toxic competing microalgae. It is quite difficult to control such type of contaminants because of the similarity in the physical and biological properties of predators and desired species. Such contamination is a logical consequence of algal cultivation because nutrient-rich media support the growth of both desired and undesired strains. However, in most cases mutant (predator) strain outcompetes the original strain (Mooij et al. 2015).

Selection of biopreservative (capable of dominating the environment by outcompeting invading pathogens) algal strains and/or algal strains adapted to high or low pH can be employed in open ponds which have potential to outcompete the contaminating microbes (Lam et al. 2017). Alteration of culturing environment through chemical treatment (supporting target strain) is another possible solution (McBride et al. 2014). Imposition of a physical barrier in closed PBR ensure the axenic microalgal cultures (Mooij et al. 2015). Diatoms and dinoflagellates are major toxic species of marine system, where the control of the later is more difficult. Physical and chemical methods have been employed to control dinoflagellates. Different practices are in use depending on the type of species. Heating is one of the most environmentally friendly and effective methods in this regard. Another option to clear these species is the invasion of natural predators like filter feeders and copepod (Lam et al. 2017). Thick layers of biomass on the surface hinder the light penetration and reduce biomass productivity ensuring volume-to-surface area ratio by biomass removal is a potential solution to solving this issue. To avoid algal sedimentation, airlift devices or mechanical pumps are applied to induce turbulent flow of algae (Płaczek et al. 2017). Toxic weed or marine algal species possess a great threat to the commercial applications of microalgae, so it is important to maintain the proportion of nontoxic algal species by monitoring conditions of culture especially flow rate, nitrogen-phosphorus ratio, and population density (Lam et al. 2017).

Detailed studies are required (1) to evaluate the impact of contamination through life-cycle assessments studies, (2) to identify the diversity among the contaminating organisms, (3) to study their interaction with the microalgae to establish the contamination-free cultivation systems, (4) to identify biopreservative microalgae which could outcompete the contaminative organisms through some biological secretions, and (5) to manage cultivation conditions specifically pH and nutrients that can help to minimize the contamination risks.

6 Conclusion and Future Perspective

The importance of microalgae can't be debated due to their countless applications. It is important to understand the culturing requirements of the microalgae and challenges associated with microalgae growth specifically at commercial scales. There is need to determine the water footprint of marine microalgae, especially in a photobioreactor. To reduce the cost of the process, large dependence on freshwater can be reduced by using the wastewater and recycling the culturing media. Life-cycle assessment should be performed to understand the cost-effectiveness and to analyze the impact of recycled media on growth and metabolite content of microalgae. It is important to determine the reusability efficiency of microalgae especially in pilot-scale and commercial-scale cultivation. It is necessary to analyze the microalgal growth responses in outdoor conditions to fully understand the impact of environmental factors, biological components on recycled medium, and associated inhibitory factors. There is need to identify the microalgae strains which show maximum potential in varying environments and to optimize the conditions for spent medium utilization as a cost-effective approach. The use of wastewater is also a considerable option as it provides the necessary nutrients and environmental conditions required for the enhanced metabolite content of microalgae. The wastewater will be a low-cost growth media and will be better approach when compared to the processing involved in media recycling. Development of maintenance strategies for the culturing system is one major area that is lacking, and extensive research efforts are required to establish the cost-effective microalgal culturing especially on commercial scales.

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Chapter 3
Open Pond Culture Systems and Photobioreactors for Microalgal Biofuel Production

Lei Qin, Md. Asraful Alam, and Zhongming Wang

Abstract Microalgal biomass offers sustainable and carbon-efficient alternative for biofuel production. Biofuels from microalgae feature considerable potential to meet future challenges of carbon dioxide-neutral energy supply and storage. To obtain microalgal biomass, major requirements, including the supply of light and nutrients (carbon, nitrogen and phosphorous), maintenance of adequate culture conditions (pH and temperature) and mixing, should be considered. Proper designs of open pond and photobioreactors are the key link that should meet the growth requirement conditions of microalgae. In this review, the identifying characteristic parameters, advantages and disadvantages of open pond reactors and a variety of closed reactors, the design principles and process management for microalgal biomass production were revised. In addition, computational fluid dynamics modelling of both open pond and closed bioreactors was discussed. Technological advancement, automation and manufacturer information were also summarised.

1 Introduction

Microalgae are a broad category that includes eukaryotic microalgae and prokaryotic cyanobacteria. Given the chemical composition of microalgal biomass, microalgae can be used for different applications, including human and animal nutrition, cosmetics, pharmaceuticals and biofuels. Microalgal biomass offers sustainable and carbon-efficient alternative for biofuel production (Bahadur et al. 2013).

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Biofuels from microalgae show notable potential to meet future challenges of carbon dioxide-neutral energy supply and storage (Alam et al. 2017). Although producing fuels from algal cultures is generally proven to be possible, the process development remains at the early stages. Several pilot-scale plants have been successfully tested, but to date, no large-scale facility effectively generates microalgal biofuels in terms of both energy and financial cost.

Despite the advantages associated with microalgal production, several phenomena should be studied before the application of this technology at the industrial scale. Microalgae (according to applied phycology) are photosynthetic microorganisms. Major requirements, including the supply of light and nutrients (carbon, nitrogen and phosphorous), maintenance of adequate culture conditions (pH and temperature) and mixing, should be met to promote microalgal growth (Fernandez et al. 2013). Production of microalgae requires the use of photobioreactors (PBRs), which must be adequately designed, built and operated to satisfy the requirements of microalgae. The geometry of PBRs and their operating conditions should favour microalgal growth to achieve high biomass productivities.

The PBR design involves basic process engineering principles regarding light distribution, mass transfer and hydrodynamics. Different geometries and operating methods were developed depending on local conditions, the product to be produced and economic constraints (Posten 2009). This article summarises the types of microalgal PBRs, design principles, computational fluid dynamics (CFD) of open pond and closed bioreactors and process management for microalgal biomass production.

2 Types of Microalgal PBRs

In most instances, open culture systems have been located outdoors; they rely on natural light for illumination. Although open systems are inexpensive to install and run, they suffer from numerous problems, such as the following. Cultures are non-axenic. Thus, contaminants may outcompete the desired algal species. Predators, such as rotifers, can decimate the algal culture, and weather vagaries can cause difficulties in the proper control of nutrients, light intensity and CO_2. Closed PBRs (CPBRs) have been used to axenically grow photosynthetic microorganisms, such as microalgae, cyanobacteria, plant cells and photosynthetic bacteria, for various research and biotechnological applications. Table 3.1 shows a comparison of open and closed systems for microalgae (Xu et al. 2009).

Table 3.1 Comparison of open and closed systems for microalgae

	Open systems	Closed systems
Contamination risk	High	Low
CO_2 losses	High	Low
Evaporative losses	High	Low
Light use efficiency	Poor	Excellent
Area/volume ratio	Low	High
Area required	High	Low
Process control	Difficult	Easy
Biomass productivities	Low	High
Investment costs	Low	High
Operation costs	Low	High
Harvesting costs	High	Relatively low
Scale-up	Easy	Difficult

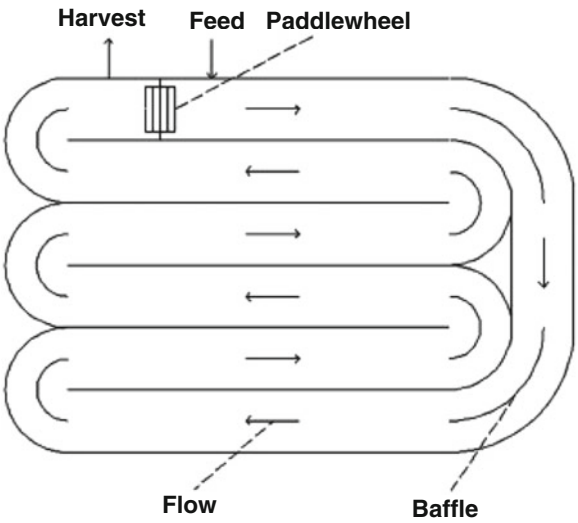

Fig. 3.1 Aerial schematic view of a RP. (Chisti 2007)

2.1 Open Pond Culture Systems

Open ponds are the most applied to cultures at the commercial scale owing to their low capital and operating costs. The most commonly used systems include circular ponds with rotating components for mixing, raceway ponds (RPs), large shallow ponds and tanks. Open ponds are frequently designed similar to RPs. An RP is made of a closed-loop recirculation channel that is typically approximately 0.3 m deep; mixing and circulation are produced by a paddle wheel (Figs. 3.1 and 3.2, respectively). Flow is guided around bends by baffles placed in the flow channel. Raceway channels are built in concrete or compacted earth and may be lined with white plastic. Although raceways are less expensive than PBRs due to the less cost to build and operate, they feature a low biomass productivity compared with PBRs.

Fig. 3.2 View of pilot-scale a RP at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences

Table 3.2 Typical advantages and disadvantages of the three main types of closed reactors (Kunjapur and Eldridge 2010)

Reactor type	Typical advantages	Typical disadvantages
FP	Shortest oxygen path	Low-power consumption
	Low photosynthetic efficiency	Shear damage from aeration
Tubular	High volumetric	Oxygen accumulation
	Biomass density	Photoinhibition
		Most land use
Vertical	Highest gas exchange	Scalability
	Best exposure to light/dark (L/D) cycles	Support costs
	Least land use	
	High photosynthetic efficiency	

Open ponds present significant technical challenges: (1) the presence of competition and predation given the significant difficulty of maintaining a monoculture of a desired algal strain in an outdoor and open environment, (2) loss of water to evaporation hindering the success of open ponds, (3) temperature fluctuation within a diurnal cycle and during different seasons and (4) less efficient utilisation of carbon dioxide than PBRs due to significant losses to atmosphere.

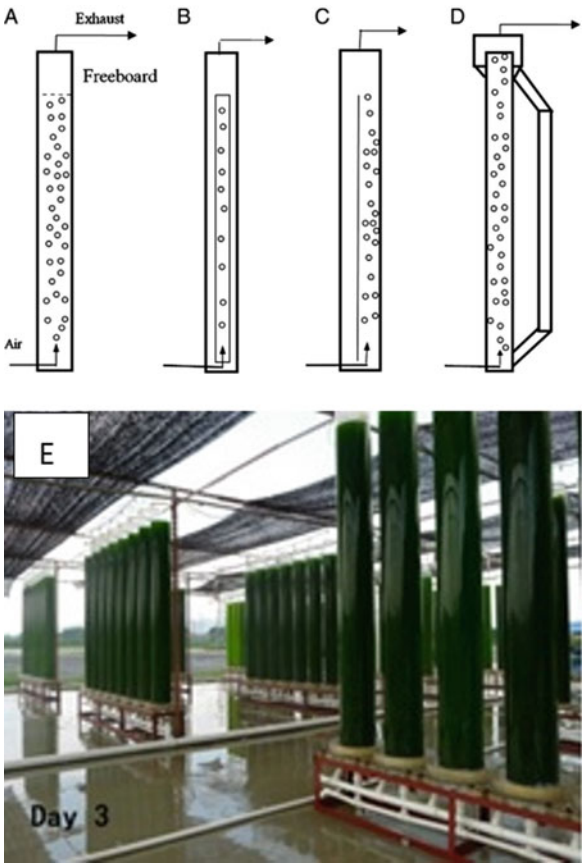

Fig. 3.3 Schematic diagrams of bubble-column PBR (**a**), internal-loop (draft-tube) airlift PBR (**b**), split-column airlift PBR (**c**), external-loop airlift PBR (Wang et al. 2012) (**d**) and bubble-column PBR at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences (**e**)

2.2 PBRs

PBRs can achieve high biomass productivities due to good control of culture variables. Different PBRs, which can be classified according to reactor geometry into vertical column (VC-PBRs), tubular (T-PBRs) and flat panel PBRs (FP-PBRs), have been developed (Table 3.2).

2.2.1 VC-PBRs (Wang et al. 2012)

VC-PBRs usually consist of cylinders with radii of up to 0.2 m and heights of up to 4 m. These columns must possess small radii to increase the surface–volume ratio (S/V ratio). Height restriction is associated with gas transfer limitations and the

strength of transparent materials. VC-PBRs are characterised by their high volumetric gas transfer coefficients. Different types of VC-PBRs, including bubble-column PBR, internal-loop (draft-tube) airlift PBR, split-column airlift PBR and external-loop airlift PBR, are available (Fig. 3.3).

A bubble-column PBR comprises a column with an air sparger located at its bottom. The freeboard regime at the top of the PBR functions for gas/liquid separation. Mixing is achieved by the turbulence created by air bubbles moving upward. Internal-loop airlift PBR typically comprises a transparent column, an internal column and an air sparger. Air or CO_2-enriched air is introduced inside the internal column at the bottom. Degassing occurs in the freeboard regime, which is located at the top of the internal column. Given that the gas hold-up inside the internal column is much larger than that in the degassed liquid outside of the internal column, an upward flow of the liquid/gas mixture will be created inside the internal column, whereas a downward flow of degassed liquid is generated outside. The most significant advantage of this PBR is excellent mixing, allowing good exposure of cells to light radiation with a relatively large column diameter and high cell density. Other benefits include its simplicity and clean ability. In a split-column airlift PBR, a flat plate splits the diameter of the column and separates the column into two parts: the riser and downcomer regions. Air is introduced at the bottom of the riser region to carry the liquid upward. Liquid/gas separation occurs at the top of the column, and the heavy degassed liquid travels downward. Mixing is realised with aeration and liquid circulation. In an external-loop airlift PBR, degassing occurs in a gas/liquid separation region at the top of the column, and circulation of degassed liquid is achieved through an external circulation column.

2.2.2 FP-PBRs

FP-PBRs with a narrow light path are characterised by large illuminated S/V ratios. These PBRs can be oriented into the direct path of light to obtain maximum exposure to solar energy.

The first FP-PBR was described in the 1950s (Burlew 1953). Since then, different types of FP-PBRs have been developed. As shown in Fig. 3.4, these PBRs can be classified into two categories, namely, pump-driven and airlift FP-PBR, according to the means of mixing. Pump-driven FP-PBR depends on the liquid flow created by pumping to generate the necessary turbulence for mixing, whereas airlift FP-PBR depends on compressed air to deliver the power of mixing. A study reported that a 17 L airlift FP-PBR can support *Haematococcus pluvialis* NIES-144 growth with a maximum cell density of 4.1×10^5 cell mL^{-1} and specific growth rate of 0.52 day^{-1}. A similar level of performance was obtained from a 90 L airlift FP-PBR of the same structure with a cell density of 40×10^4 cell mL^{-1} and a slightly low specific growth rate of 0.39 day^{-1} (Issarapayup et al. 2009). Conventional FP-PBRs suffer from deficiencies in culture flow control and the engineering problems encountered in construction of suitably cost-effective panels (Pirt et al. 1983). To solve these problems, Tredici and his coworkers (Tredici et al. 1991; Tredici and Materassi

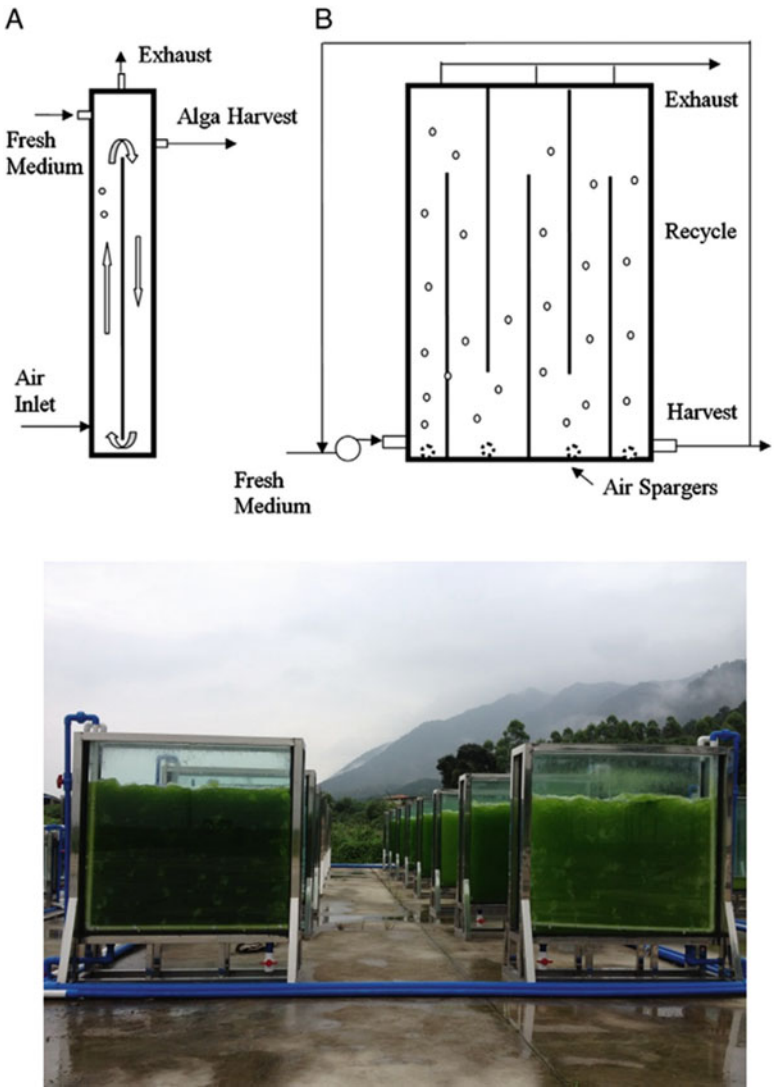

Fig. 3.4 Schematic diagram of the side view of an airlift FP-PBR (**a**); front view of a pump-driven FP-PBR (**b**) (Wang et al. 2012); FP-PBR at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences

1992) proposed a vertical alveolar panel (VAP) PBR made of 1.6-cm-thick rigid alveolar plexiglass sheets with a transparency of approximately 95% to the visible fraction of solar radiation. The VAP yielded a S/V ratio of 80 m^2/m^3 and a culture thickness of approximately 12.5 mm. Net biomass productivity of up to 16 $g/m^2 \cdot day$ was achieved at a biomass concentration of 28 g/m^2 when a VAP with a 5 m^2 panel was used for outdoor cultivation of *Anabaena azollae*. In addition to the relatively

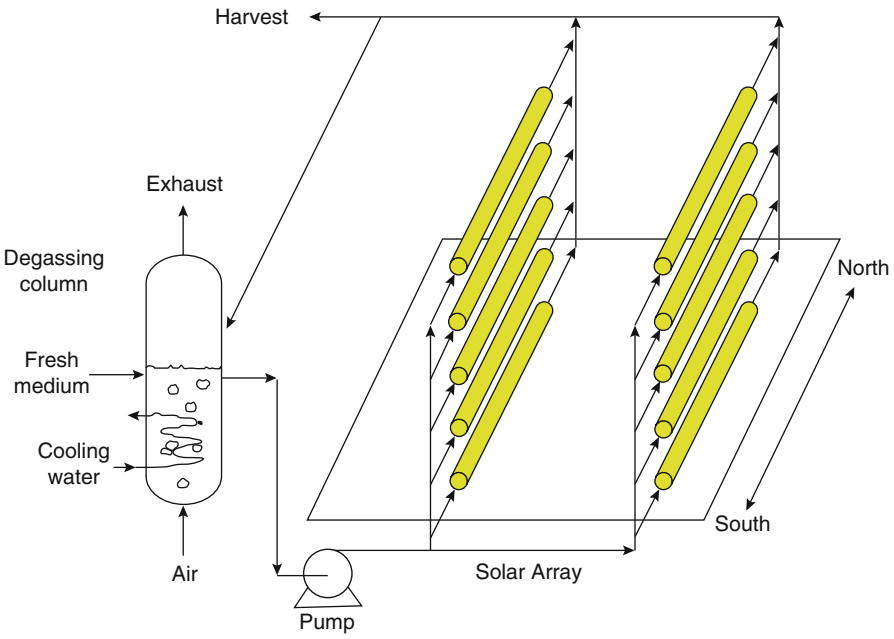

Fig. 3.5 Schematic diagram of a horizontal T-PBR (HTPBRs)

inexpensive manufacturing costs, the VAP offers advantages, such as large S/V ratio, flexibility and good mixing and gas exchange features. However, temperature control and light inhibition are also major challenges in designing such PBRs.

The angle of a FP reactor relative to the ground is referred to as the tilt angle of the reactor. Throughout the year, the optimal tilt of the reactor that allows maximal incident light will change due to the position of the sun. Hu et al. (1998) pointed out that as a general rule, the optimal angle for year-round biomass production is equal to the geographic latitude of the location, and increasing the tilt angle during winter increases biomass production. On a different notion, Zhang et al. (2002) showed that a west–east-facing bioreactor yielded a 1.4-fold higher slope of productivity versus irradiation curve than a south–north-facing reactor at equal solar irradiation. Panel orientation significantly affects productivity, and at increased latitudes, the difference between the north–south and east–west orientations may increase to 50% (Slegers et al. 2011).

2.2.3 T-PBRs

T-PBR is one of the most popular configurations of PBRs. A T-PBR typically includes an array of transparent tubes built in different patterns (e.g. straight, bent

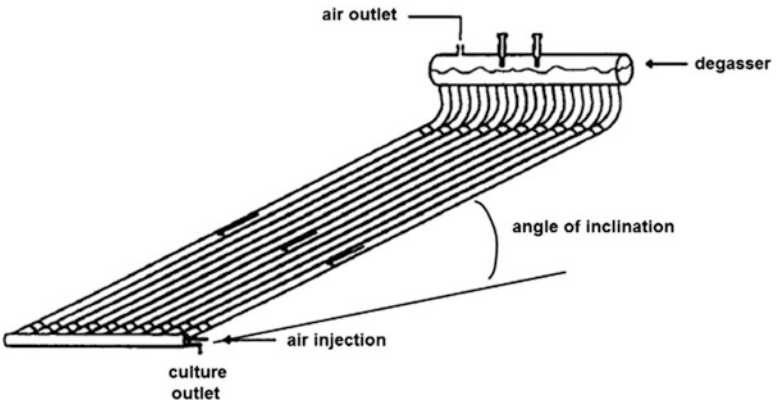

Fig. 3.6 Schematic diagram of a near-HTPBR. (Tredici et al. 1998)

or spiral). A relatively small tube diameter, generally 0.1 m or less, is necessary for ensuring high biomass productivity. T-PBRs can exhibit different orientations, including horizontal, inclined and vertical arrangements. A T-PBR comprises the following components: the solar array for algal growth, the harvesting unit to separate algae from the suspension, a degassing column for gas exchange and cooling (heating) and a circulation pump (Fig. 3.5) (Wang et al. 2012).

HTPBRs can provide a higher S/V ratio than their vertical counterparts owing to their ability to decrease the diameter of the tubes without considering structural integrity. Horizontal bioreactors also present a better angle for incident light compared with vertical tubular reactors, allowing efficient light harvesting. However, this condition also generates considerable amount of heat, occasionally requiring expensive temperature control systems (Richmond 1987). This phenomenon especially causes difficulty in terms of scale-up, wherein large areas present difficulty for temperature control. Often, a heat exchanger is incorporated into the design to maintain an optimum growth temperature (Watanabe et al. 1995).

A new bioreactor, that is, the near-horizontal tubular bioreactor, was designed to improve the HTPBR (Fig. 3.6). This type of reactor consists of a series of thin tubes connected at the bottom by a manifold, which supplies compressed gas, and by a degasser at the top. The tubes are placed on a framework that maintains a certain angle of 6°–12° (typically 10°). In principle, the inclination of the tubes should increase the bubble rise velocities, gas hold-up and gas transfer coefficients (Tredici et al. 1998). Ugwu et al. (2002) investigated the effect of column inclination on the gas transfer characteristics of a near-horizontal TPBR, noting an increased gas transfer coefficient and hold-up time and decreased mixing time with increasing angles. At the extreme, this condition will lead to a vertical column. However, 45° is considered the optimum angle as maintaining the column at angles greater than this value will increase the cost for structural supports.

Another possible design is the helical TPBR of different shapes. This kind of TPBR is a hybrid between a horizontal and vertical TPBR (Fig. 3.7). Pilot plants

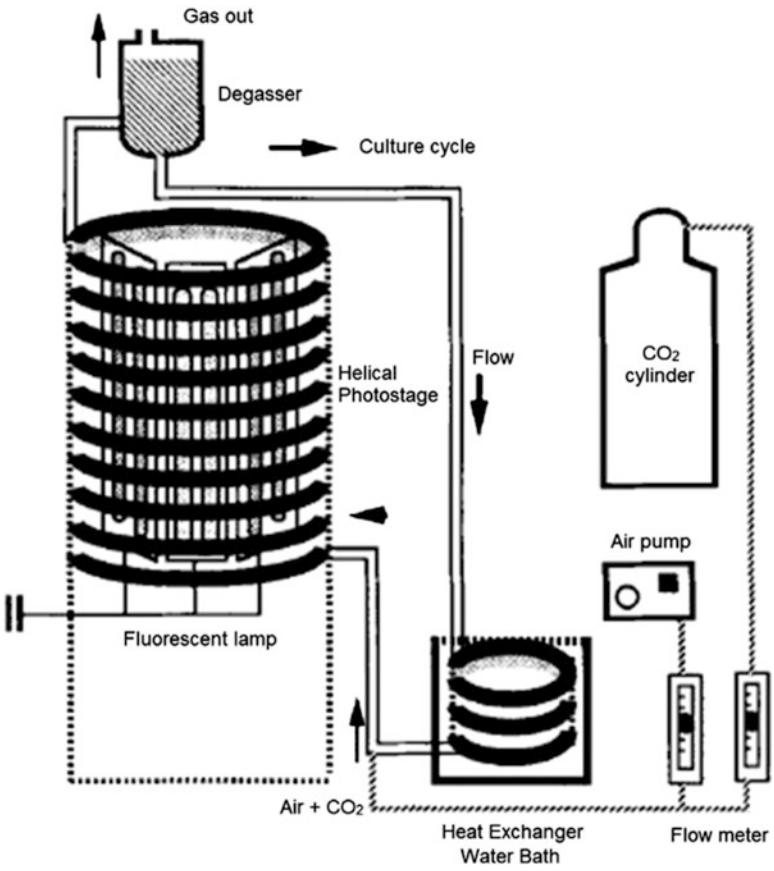

Fig. 3.7 Schematic diagram of a helical T-PBR. (Watanabe and Hall 1995)

(Biocoil) in the UK and Australia were successfully operated using this model (Travieso et al. 2001; Watanabe and Hall 1995).

2.2.4 Other Types of PBRs

2.2.4.1 Membrane PBRs (MPBRs)

MPBRs employ the large surface areas provided by membranes to facilitate gas/liquid mass transfer (Lehr and Posten 2009) while avoiding excessive turbulence or to separate extracellular metabolites continuously to allow long and stable production periods. For instance, Fan et al. (2008) investigated a membrane-sparged helical T-PBR with a cultivation volume of 800 mL. Hollow fibre membranes were

uniformly fitted inside the reactor to function as a gas sparger by producing small bubbles. In another case, a PBR coupled with an ultrafiltration system (immersed membranes) was investigated for the continuous cultivation of the microalga *Haslea ostrearia* to improve pigment (marennine) production and recovery. The system presents a commercial interest as the energetic costs are minimised, and the cells are free of any shear stress due to pumping or circulation. To achieve such a system, a membrane module was placed at the bottom of a cylindrical PBR, and the hydrostatic pressure (the height of water column) was used as a driving force both for the permeation and periodical back flushing steps. The production of biomass and marennine was stable for a 3-week period, with the marennine concentration reaching ~3 times higher than in the conventional batch PBR (Rossignol et al. 2000).

2.2.4.2 Plastic Bag PBRs

Plastic bag PBRs are especially attractive for commercial-scale production due to their low cost and good sterility at start up due to high film extrusion temperatures. These bags can be fitted with aeration systems to improve yields. Large polyethylene bag PBRs of up to 2000 L were once popular in aquaculture operations for the culture of algae as feed but are still used to a limited extent to date. Recently, a new design of vertical FP-PBR consisting of a disposable plastic bag located between two iron frames has been proposed (Sierra et al. 2008), causing a substantial cost reduction to PBR construction. Nevertheless, the disposal of used plastic bags may present a significant challenge in large-scale operations. Vertical FP-PBRs also suffer from inadequate mixing and frequent 'culture crashing' and are inherently fragile. Notably, no evidence shows that scale-up by using large bag volumes constantly leads to increased productivity (Martínez-Jerónimo and Espinosa-Chávez 1994). Disposal of large quantities of used plastic bags in large-scale operations can be another potential problem.

2.2.4.3 Exploration of New Types or Modified PBRs

In order to improve the performance of PBRs, experts and scholars have made great efforts to explore new types or modified PBRs (Table 3.3).

3 Design Principle

Numerous aspects influence the growth and lipid content of algae. Photosynthesis drives the initial conversion of sunlight into stored energy. Therefore, all the components involved in photosynthesis contribute to algal growth. Cultures of autotrophic microalgae are influenced by light distribution, temperature, pH, nutrient qualitative and quantitative profiles, dissolved oxygen (DO) and carbon dioxide

Table 3.3 Summary of various new or modified PBRs and their advantages

Reactor type	Typical advantages	References
X-shaped airlift PBR	Can be practicably utilised for mass production of algal biofuel	Hoang-Minh et al. (2017)
T-PBR with helical blade rotors (HBRs)	HBRs can improve the performance of T-PBR and thus affect positively the cultivation of microalgal cells	He et al. (2017)
Thin-film solar PBR	High biomass volumetric productivity	Pruvost et al. (2017)
Horizontal PBR	High-density cultivation of microalgae	Dogaris et al. (2015)
Floating PBR with internal partitions	Potentials for culturing microalgae by efficiently utilising ocean wave energy into culture mixing in the ocean	Kim et al. (2016)
Filtration PBR	Efficient biomass production	Zhang et al. (2014)

concentrations and the presence of toxic elements (mainly heavy metals) (Pires et al. 2017). The efficiency of PBRs is determined by the integration of light capturing, transportation, distribution and utilisation by microalgae through photosynthesis (Zijffers et al. 2008). An efficient PBR design should achieve the following: (1) harvesting as much sunlight as possible and transporting, channelling and distributing it into a cultivation vessel in such a way that maximum light energy is used for biomass formation; (2) allowing convenient and precise control of important operational parameters to culture cells in an environment that encourages optimum utilisation of light energy; (3) minimising the capital and operational costs; and (4) minimising energy consumption during operations.

3.1 Light

Sunlight is the primary energy source. Autotrophic microalgae and terrestrial plants convert light into chemical energy through photosynthesis. Consequently, light is a key parameter for microalgal culture. Thus, a design principle for PBRs is to maximise the S/V ratio. Light saturation and photoinhibition may occur when light intensity exceeds a critical level. Photoinhibition can be reversible or irreversible, depending on the light stress and the length of time the microalgae are exposed to stress. Light inhibition should be avoided as much as possible.

Light spectral quality is another important factor to consider in PBR design as cultures grow differently when exposed to different colours of light. Although sunlight covers a wide spectral range, only the light within the range of 400 and 700 nm is photosynthetically active radiation, accounting for approximately 50% of sunlight (Suh and Lee 2003). Red light matches perfectly with the requirements of the first excited state of pigments present in the light-harvesting antenna complexes central to photosynthesis in green algae (Matthijs et al. 1996).

L/D cycle is another important factor, showing remarkable effects on the overall efficiency of solar energy capturing. Given the lack of light energy, microalgae must

undergo respiration during the night to support metabolism to maintain cell viability. As a result, up to 42% of biomass produced during the daytime can be lost throughout the night (Jacob-Lopes et al. 2009; Tredici et al. 1991). Respiration during night time should thus be minimised.

The combination of factors, such as the length of L/D cycles and light intensity, results in the overall light regime in a PBR. Light regime strongly influences photo-acclimation, which describes the physiological responses of cells to rapid changes in light intensity. An example of a common response to light intensity alteration is the change in chlorophyll pigment content. However, a sudden surge of light can be fatal for numerous algal species. Thus, considering light regime and photo-acclimation when designing a reactor is important to particularly maximise photosynthetic efficiency (Kunjapur and Eldridge 2010).

Currently, the common materials used for PBRs include glass, plexiglass, polyvinyl chloride (PVC), acrylic PVC and, the most common, polyethylene. These materials satisfy the transparency requirement and are mechanically sufficient for PBR construction. However, they all feature advantages and disadvantages, which must be evaluated case by case for use in the construction of specific PBRs. For instance, glass is a strong, transparent and an excellent material to construct small-scale PBRs. However, glass requires many connection parts to construct large-scale PBRs. This process can also be costly. The capability of the material surface to prevent biofilm formation is another important feature to consider. Biofilms are not only difficult to clean but can also drastically reduce light transmission through PBRs.

Several parameters that can affect light distribution include light scattering by algal cells in solution and absorption by intracellular pigments. In high-density microalgal cultures, mutual shading between different cells becomes the main factor influencing light distribution among cells. In an under-agitated culture, an undesirable scenario can occur, wherein certain cells (i.e. those on the light-receiving surface) are exposed to overdose of light, whereas other cells (i.e. those at regions at distant from the surface) receive less or zero light. To this end, limiting the length of light path (e.g. thin PF-PBR or small-diameter TPBR) and improving mixing are the most commonly adopted strategies to improve light distribution.

3.2 CO_2/O_2 Balance and Gas Exchange

Carbon dioxide is necessary for photosynthesis (1.8 g of CO_2 is required to produce 1 g of biomass). However, excess CO_2 can also be detrimental to photosynthesis and cell growth as high CO_2 partial pressures (pCO_2) can cause declining growth rates of *Chlorella* cells (Lee and Tay 1991). Feeding CO_2 is dissolved in a medium, forming carbonic acid which is used by microalgae during photosynthesis. As mentioned above, added CO_2 also plays an important role for pH control. The low mass transfer coefficient is the main limitation of CO_2 transference from the gaseous to liquid phase (Fernandez et al. 2013).

Microalgal cell growth involves three competing cellular processes: photosynthesis, photorespiration and (dark) respiration. Photosynthesis of microalgae utilises light energy to fix CO_2 and releases O_2 as by-product. High-concentration DO can be harmful for microalgae (photo-oxidative damage). Moreover, high O_2 concentration promotes the activity of oxygenase enzymes, leading to high uptake preference for O_2 rather than CO_2 and consequent loss of fixed carbon and reduction of biomass productivity. Thus, DO should be maintained below 400% of the air saturation value (corresponding to 30 mg L^{-1}, assuming the 7.5 mg L^{-1} equilibrium solubility of O_2 at 30 °C) (Pires et al. 2017).

The measures to maintain an optimal balance between dCO_2 and DO include the following: (1) a dedicated space for gas exchange usually included in the PBR;, (2) a mixing mechanism as an effective means to promote turbulence and therefore mass transfer between gas and liquid phases inside the PBR, (3) CO_2 enrichment and/or O_2 stripping that can also significantly improve the dCO_2/DO balance in algal culture; and (4) addition of an air stream containing a proper O_2/CO_2 concentration ratio.

3.3 Temperature

Temperature is also an important variable to control microalgal cultures because it directly influences metabolic activities, enzymatic activities and conformation of vital structures (Goncalves et al. 2016). The temperatures experienced by algae grown outdoors can vary as much as the extreme outdoor temperatures characteristic to the geographic region of cultivation. Although algae may grow at varying temperatures, optimal growth is limited to a narrow range specific to each strain. Without temperature control, the temperature in a closed PBR can reach a level of 10–30 °C higher than the ambient temperature. Devising cost-effective and reliable temperature control mechanisms is therefore a significant challenge in PBR design. These mechanisms include submersing the entire culture in a water pool, spraying with water and shading or incorporating a heat exchanger with PBR for cooling.

3.4 pH

The pH value significantly affects microalgal culture with regard to the availability and assimilation of the nutrients dissolved in the medium. Each strain of algae also features a narrow optimal pH range. The optimal pH of most cultured algal species is in the range of 7–9. The dCO_2 concentration may be the dominant factor that determines the culture pH (García Sánchez et al. 2003). pH affects the liquid chemistry of polar compounds and the availability of nutrients, such as iron, organic acids and CO_2 (Lee and Pirt 2010). The pH control mechanism should be integrated

with the aeration system as adding basic solution to the culture, which is a standard practice in conventional bioreactors, cannot control culture pH effectively.

3.5 Mixing

The level of mixing in a reactor strongly contributes to the growth of microalgae, especially in high cell-density culture. This condition may result in drastically reduced transmission of light and increased rates of dCO_2 consumption and DO accumulation and rapid increase in culture temperature. Mixing affects growth in two primary ways: by increasing the frequency of cell exposure to L/D volumes of the reactor and by increasing mass transfer between the nutrients and cells (Kunjapur and Eldridge 2010). Specifically, mixing of microalgal cultures is necessary to (1) prevent sedimentation of algal cells, (2) ensure that all cells of the population undergo uniform average exposure to light and nutrients, (3) facilitate heat transfer and avoid thermal stratification and (4) improve gas exchange between the culture medium and the air phase.

Depending on the scale and choice of cultivation system, mixing can be accomplished by aeration, pumping, mechanical agitation (e.g. rotation wheels and static mixer) or a combination of these means. Notably, not all algal species can tolerate vigorous mixing. The mixing level must be optimised meticulously as high levels will result in cell death from shear stress. Mechanical agitation and bubble break-up often lead to hydrodynamic stress, resulting in restriction to algal growth and metabolic activity (Gordana Vunjaknovakovic et al. 2005; Suh and Lee 2003).

3.6 Other Considerations

3.6.1 Sterility (Species Control) and Clean Ability

Certain extent of impurity in microalgal cultures must be tolerated when the processes are designed for low-value objectives, such as biofuel production and CO_2 sequestration. Nevertheless, cautions must be implemented to avoid excessive contamination. Fortunately, contamination by heterotrophic microorganisms in autotrophic microalgal farming facilities usually causes no significant concern due to the lack of organic carbon sources in the system. However, the control of exotic and invasive algal species and predators is critical for stable and continuous operations and the stable quality of products. Species control can be particularly difficult for cultivation of relatively slow-growing microalgal species.

Clean capability is of critical importance to a PBR due to the following reasons: (1) preventing biofilm formation on the wall and therefore maintaining high light transmission and (2) minimising the chance of contamination. To increase the clean capability, the following principles should be observed: (1) smooth internal surface

of a PBR, (2) minimum number of internals and bends and (3) sufficiently large internal dimensions of a PBR to allow convenient cleaning.

3.6.2 Material Selection

Most studies focused on the modification of the design and geometry of PBRs to enhance biomass productivity. However, the light-capturing capacity of the material used in PBRs is the most important design that should be considered. Other significant factors include the transparency of materials and the ratio of illuminated S/V ratio. The common materials currently used in PBRs include glass, plexiglass, PVC, acrylic PVC and polyethylene, which is the most commonly used material (Table 3.4). These materials feature extremely high rate of transparency, durability, sunlight-harnessing capacity and mechanical strength. The above materials benefit PBRs because they avoid the formation of additional biofilm.

The materials used in PBR construction must be nontoxic and inexpensive and should show high transparency and mechanical strength, durability and chemical stability. Considering these factors, the most commonly used materials include acrylic, polycarbonate, LDPE and crystal PVC. PVC presents the advantage of lower cost, long-term stability and flame resistance compared with other commodity plastics, and its mechanical properties may be controlled by varying the amount of plasticiser (D'Aquino et al. 2012).

4 CFD of Open Pond and Closed Bioreactors

4.1 General Description

CFD is a discipline based on the theory of fluid dynamics. CFD provides a qualitative prediction of fluid flows using the following: (i) mathematical modelling (Navier–Stokes transport equations), (ii) numerical methods and (iii) software tools (solvers and pre- and post-processing utilities). Process engineers and scientists use CFD models to study complex and integrated systems without the need for extensive experiences. In recent years, CFD has been applied to model bioreactors. Regarding other optimisation methods, CFD presents several advantages: (i) low cost, (ii) reduced workload and (iii) short design period.

4.2 Bioreactor Modelling

Bioreactor design should consider lighting, mixing, water and CO_2 consumption, O_2 removal, nutrient supply and temperature maintenance. A complex design is versatile but expensive to construct and operate. Thus, design selection depends on

Table 3.4 Characteristics of common materials for CPBRs reported in literature (Ahmad et al. 2017)

Material	Material energy content (mg/kg)	Material life (year)	Energy content (MJm^{-2})	Life span (year)	Advantages	Disadvantages
Glass	25–28	18–20	310	15.5–17	T-PBRs display significantly higher net energy ratio than rigid polymers, such as polymethyl methacrylate (PMMA) (acrylic)	Glass tubes are naturally delicate and require more connection fittings as they are only a few metres long; otherwise, they are difficult to transport and assemble
					Requires low energy content	
Low-density polyethylene (LDPE)	78–80	3–5	40.5	40–42	LDPE PBRs can vary in thickness depending on the algal species used	Environmental factors, such as temperature, solar radiation and pollution, affect the life span of the film
High-density polyethylene (HDPE)	131–135	20–22	1456	73–75	Opacity of HDPE prevents photo-oxidative damage or photoinhibition and biofouling	Welding the material presents difficulty and hence shows less tensile strength
Rigid acrylic (PMMA)	–	–	–	–	Biomass productivity is increased, and control over algal culture is enhanced	Installation costs contribute to the expensiveness of the system
PVC	–	–	–	–	The use of specially clear PVC piping is beneficial in utilisation of PBRs, and CPBR clarity allows algae to grow and feed. Other advantages include corrosion resistance, non-conductivity and light-weight construction	UV rays cause discoloration of the surface of conventional PVC pipe, preventing or limiting light from penetrating the medium

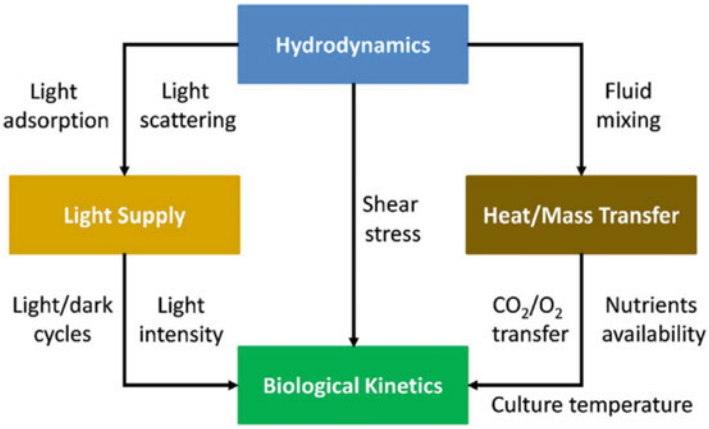

Fig. 3.8 Interactions between microalgal cultures. (Pires et al. 2017)

several factors: (i) microalgal growth characteristics, (ii) chemical composition of medium and (iii) commercial value of the final product. For instance, for strict quality control products (human food, cosmetics and pharmaceutics), high variability of culture variables should be avoided to maintain the qualitative composition of microalgal biomass. Meanwhile, RPs should be applied for low-cost applications of microalgal biomass (biofuel production).

PBR geometry should maximise the biomass areal productivity. Figure 3.8 shows the main interactions between hydrodynamics, light supply, mass and heat transfer and biological kinetics. These interactions determine the yield of microalgal culture. Only the model considering all these phenomena will correctly describe the microalgal growth (Acién Fernández et al. 2017). Table 3.5 presents the main CFD studies for PBR modelling. Fluent and CFX are the most applied software codes in the studies of PBR design. The majority of the studies only focused on fluid dynamics due to the complexity of the studied phenomena.

In hydrodynamics, evaluating the spatial distribution of fluid velocities is important to reduce dead zones (stagnation regions within the flow). RP geometry influences energy consumption of the paddle wheel power, representing a high percentage of operational production costs. CFD was applied to calculate the power consumption for mixing the studied RPs. High energy savings can be achieved if flow velocity is reduced with well-designed bluff bodies in water flow. CFD was also applied to evaluate head losses in RP. As expected, the majority of head losses are located at the bend (Chiaramonti et al. 2013). CFD was also applied to simulate the hydrodynamic parameters of T-PBR (turbulence kinetic energy of downcomers, duration of downcomer period, cycle time and dead zones). This condition influences microalgal growth, and the results show the potential of CFD for the optimisation and scale-up of PBRs. Light supply is an important variable for autotrophic cultures. The optimisation of light intensity and L/D cycles may con-

Table 3.5 Recent CFD studies focusing on different PBRs (Pires et al. 2017).

Photobioreactor	CFD code	Modelled phenomena	References
RP	EFDC	FD; MT; LS, Steel's equation; BK	James and Boriah (2010)
	CFX	FD: k-ε model	Sompech et al. (2012)
	CFX 12	FD: k-ε model	Liffman et al. (2013)
	COMSOL 4.4	FD: k-ε model; particle tracking model	Ali et al. (2015)
	FLUENT 14.5	FD, standard k-ε model and k-ω model; LS, Beer–Lambert's law; MT, Henry law; BK, Monod model; EV, 120 m^3 outdoor RP	Park and Li (2015)
	CFX 12.1	FD, standard k-ε model; EV, PIV with 168 L RP	Huang et al. (2015c)
	CFX 12.0	FD, LES and k-ε models; EV, PIV with 3 m^3 RP	Zeng et al. (2016)
Internal airlift	CFX 4.2	FD, k-ε model; EV, two configurations of PBR	Baten et al. (2003)
Airlift FP	CFX 5	FD, k-ε model; EV, 15 L and 300 L PBRs	Yu et al. (2009)
	FLUENT 6.3	FD, k-ε model; EV, 12.8 L PBR	Massart et al. (2014)
	CFX 12.1	FD, standard k-ε model; particle tracking model; LS, Cornet model; EV, 15 L PBR	Huang et al. (2015a)
FP	CFX 12.1	FD, standard k-ε model; particle tracking model; LS, Cornet model; EV, three types of 15 L PBR	Huang et al. (2015b)
Tubular	FLUENT 6.0	FD: standard k-ε model; particle tracking model; LS	Pernernochta and Posten (2007)
	FLUENT 12.1	FD, realisable k-ε model; particle tracking model; LS	Moberg et al. (2012)
	FLUENT	FD: standard k-ε model	Wongluang et al. (2013)
	COMSOL	FD: k-ε model; particle tracking model	Gómez-Pérez et al. (2015)
Flooded bed	FLUENT 6.3	FD, k-ε model; MT, Akita and Yoshida equation; BK, Monod model	Smith et al. (2013)

FD fluid dynamics, *EV* experimental validation, *MT* mass transfer, *BK* biological kinetics, *LS* light supply

tribute to the increased biomass productivities. Thus, predicting the L/D cycle frequencies for PBR geometries is important. Prediction can be performed by simulating individual cell trajectories. The temporal and spatial variability of light patterns and radial velocity in PBRs were studied with CFD modelling (Huang et al. 2015b; Moberg et al. 2012). The application of CFD to describe biological processes is less explored. The simulation of microalgal growth is the final step to fully

characterise PBRs. Consequently, the effect of physical and chemical variables on the process of microalgal growth can be estimated. James and Boriah (2010) presented the first study that integrates hydrodynamics, heat and mass transfer, light supply and microalgal growth kinetics. The Environmental Fluid Dynamics Code from the US Environmental Protection Agency and Water Quality Code from US Army Corps of Engineers were used to simulate the growth of *Phaeodactylum tricornutum* in an RP.

5 Process Management

Given that the reactor is the hardware part of the process, only sophisticated operations make the equipment viable. Process management offers several options to further improve the performance of PBR systems and decrease energy demands.

5.1 Temperature Control

The effect of temperature control on the energy balance of the process is highly dependent on the applied reactor system, algal strain and most of all the operating region of the plant. At warm, highly irradiated sites, such as the Southern USA or Australia, cooling of the cultures is possibly a critical parameter of the process. Whether this problem is tackled by direct evaporation or a closed cooling system, excess heat must be actively removed from the system, adding to the energy demand of the process. Spraying the outer wall of the reactor with water is a means, but it requires the availability of cooling water.

Avoidance of infrared (IR) radiation is one way to reduce the heating problem. This part of the sunlight spectrum accounts for 40% of the total energy without being used by algae. IR-reflecting glass or plastic is already available (Holland and Siddall 1958) and is used to reduce heat in parked cars or to reduce heat radiation from light bulbs.

Heating in spring is another option discussed especially in Central Europe, wherein sun irradiance is already at a remarkable level. However, outdoor temperatures are extremely low for sufficient cell growth. Here, low-temperature heat, such as that observed with cooling water from power plants, is in principle available and can be used for heating cultivations. In the ‘water bed reactors’ mentioned above, temperature fluctuations between day and night can be compensated by the amount of water around the growth chambers, exceeding the normal values in open ponds. This concept can be sharpened by employing the so-called phase change materials. These materials are commercially produced in wallpapers for flats to control the room temperature at the given value of phase transition. For PBRs, this phenomenon implies that temperature control is not only at the day/night average but also at an adjustable value.

5.2 Feeding Strategy

PBRs are usually operated in batch or sequential batch (semicontinuous), wherein harvesting is conducted preferably in the afternoon. Maximum biomass concentration with the highest mutual shading is reached with the highest irradiation during daytime. Meanwhile, the lowest biomass concentration in the night leads to the lowest biomass loss by respiration. The total productivity is given by plant carbon expenditure (PCE) value and local irradiation during continuous cultivation, leading to low dilution rates for high cell concentrations ($PCE \cdot I \sim D \cdot cX$). This phenomenon affects strain selection because not only high maximum growth rates are required, but strains with high PCE at low growth rates can also be successfully cultivated.

Sunlight is only available at daytime at broadly varying intensities. An option to reduce energy intake is to couple gassing and mixing with the photosynthesis rate (Buehner et al. 2009) as CO_2 demand is proportional to the photosynthetic activity. However, no photosynthesis occurs at night. Consequently, the energy input for gassing can be reduced to the absolute minimum necessary for the oxygen supply necessary for respiration.

The minimum requirements for the medium composition arise from the elemental balances, e.g. for nitrogen or phosphate. An intentional low nitrogen level prohibits the formation of proteins and nucleic acids, forcing certain microalgal strains to store CO_2 and light energy as lipids. Remarkably, the usual mineral media for laboratory use are unsuitable to reach high biomass concentrations. This condition leads to extremely high salt concentrations that are subject to precipitation or growth inhibition. These cases require a fed-batch-like additional dosing of single-medium compounds during growth. However, to these points, insufficient quantitative scientific data or practical experiences are published.

5.3 Measurement and Control

PBRs must provide ideal conditions for microalgal cells with respect to a desired physiological state under the constraints of incoming light or other given external parameters. This condition can be conducted by measurement of physical conditions inside the medium and controlling technical variables, such as gas supply. Two pO_2, pH and pCO_2 sensors along the main reactor axis—signifying the strongest mass transfer gradient—should be mandatory. However, this topic is slightly neglected in current installations. Given that cells are the only reasons for maintaining the process, online measurement of optical density and fluorescence pulse amplitude-modulated fluorometry can aid in the assessment of the physiological state and reaction with online optimisation of mixing, gassing or diluting.

6 Maintenance and Cleaning of Pond/PBRs

Various cultivation systems are employed at the pilot and commercial-scale levels for microalgal cultivation. The culturing systems vary in terms of contamination chances, control of growth parameters, biomass productivity and operation and capital costs (John et al. 2011). Maintenance of PBRs or RPs causes heavy burden on the economy as approximately 25 billion US dollars is required annually for the maintenance and process operations (Christenson and Sims 2011).

In general, industrial-scale facilities prefer RPs due to their easy maintenance, cleaning and low cost. Weekly monitoring of biomass and nutrients is sufficient to maintain biomass cultivation (Singh et al. 2011). High growth rates are achieved using this technique as a maximum of 10 g L^{-1} of *Chlorella* is produced in RP (Brennan and Owende 2010) although this value is not as high as that in PBR. The major problem associated with RPs is high contamination chances from other biological predators. Maintenance of extreme environmental conditions (pH, nutrients, salinity and light) is one possible solution to maintain microalgal monocultures (John et al. 2011). Media sterilisation is another possible option to reduce contamination chances. However, this method significantly increases the energy requirements and processing cost. Covered RPs were also used for this purpose but achieved no notable success. Sand filter is used to remove protozoa from water, therefore reducing their contamination chances. Moreover, the selection of biopreservative (strains which can dominate the environment by outcompeting invading pathogens) algal strains and/or algal strains adapted to high or low pH can be employed in open ponds; these strains feature the potential to outcompete the contaminating microbes. To reduce fungal contamination chances, fungicides are used, but the favourable and environment-friendly option is the cultivation of fungal infection-resistant algal species. Another possibility is 'crop rotation' at the time of infection (Lam et al. 2018). Toxic weed or marine algal species considerably threaten the commercial applications of microalgae. Thus, the proportion of nontoxic algal species should be maintained by monitoring culture conditions, especially flow rate, nitrogen–phosphorous ratio and population density. Open ponds are high targets of bacterial contamination, which can be prevented by chemical disinfectants; however, the feasible option is the elimination of harmful bacteria by maintaining a suitable ration of good bacteria that can control the previously mentioned bacterial species (Lam et al. 2018). Recently, ultrasonication was applied to remove contaminants (protozoa, fungi, amoebae and ciliates) from mass cultures of *Chlorella* cultivated in open RPs. Application of ultrasonication waves with 100% amplitude with 495 W power every hour on this system was effective for removal of contaminants (Wang et al. 2018). In another study, sodium dodecyl sulphate (SDS) was added as a surfactant to facilitate foam floatation as a possible approach for ciliate removal. This result suggested a 96.3% ciliate removal efficiency when applied on ciliate–microalgae culture by reusing SDS and employing multistage floatation (Umar et al. 2018). An alternative approach for mass cultivation of microalgae was proposed using HCO_3^- (bicarbonate) instead of supplying CO_2; its effect on

microalgae was evaluated. Approximately 50% of biomass productivity with 90% reduction in energy consumption and 55% reduction in cultivation cost was reported. This technique was found to show promise in reducing contamination risks in the open pond system (Hanifzadeh et al. 2018). A 16% production cost reduction due to energy reduction by the use of baffled flow and solar-powered pumping system for mixing and pumping, respectively, in the ARID culturing system was achieved (Moreno-Garcia et al. 2017).

PBRs provide control over environmental conditions, resulting in PBR operational cost that is 100 times higher than that of RPs; therefore, the biomass produced from the bioreactor is twice expensive than that of RPs (Ali and Park 2017). T-PBRs face the challenges of biofouling, overheating, toxin accumulation and high maintenance cost (Christenson and Sims 2011).

The same case is observed in MPBRs, wherein membrane fouling is the major issue hindering commercial-scale applications. Chemicals are used to treat this problem, but they cause heavy burden on the process economy. Membrane fouling also reduces the productivity and thus increases energy demand. Routine cleaning is required to maintain PBR functionality by increasing permeability. This condition shortens the membrane's life, therefore increasing the membrane replacement cost. To ensure long-term and cost-effective PBR operations, the problem of membrane fouling should be mitigated. Possible strategies include (i) membrane cleaning by chemicals, (ii) enhancing backwashing by chemicals, (iii) using chemically modified medium or membrane surfaces and (iv) aerating with bubbles to enhance membrane scouring (Krzeminski et al. 2017). Integration of electrocoagulation and advanced oxidation processes with MPBR is effective for membrane fouling reduction because this method removes recalcitrant compounds (Neoh et al. 2016). Thick layers of biomass on the surface hinder light penetration and reduce biomass productivity. This condition ensures the potential of the S/V ratio by biomass removal to solve this issue. To avoid algal sedimentation, airlift devices or mechanical pumps are applied to induce turbulent flow of algae (Płaczek et al. 2017).

7 Advances in the PBR System and Manufacturers

Remarkable advancement has been achieved in manufacturing PBR with advanced and required facilities for monitoring culture conditions and harvesting. Industrial Plankton Inc. produces a PBR system from a 100 L research reactor to a 1250 L turnkey PBRs for on-site algal production in research institutions and commercial biotechnological facilities. These bioreactors feature highly automated operation steps. The PBRs can add filtered and UV-sterilised water and nutrients and automatically inoculate from the respective source without coming in contact with the external environment. These PBRs feature automatic light, temperature and pH control system to allow continuous algal growth. The PBRs can be run in batch or continuous mode and can be harvested from at any time by inputting the volume to harvest on the touchscreen. In batch mode, the operator can decide when the algal

Fig. 3.9 Research reactor (100 L) (left); industry reactor (1250 L) (right). Photos and information are used with permission from the Industrial Plankton Inc.

density is sufficient for harvest. In continuous mode, the PBR harvests a set volume each time the PBR reaches the set density. Then, new water and nutrients are automatically added to replace the harvested volume, allowing this cycle to continue automatically for the next harvest. The touchscreen controls give operators complete control over critical parameters from adding water to cleaning until harvesting of cells. In real time, the software logs and graphs of culture allow the user to fine-tune and maximise algal growth. Although the reactor is automated, operators can also manually control it, allowing for simple and rapid harvesting and scaling-up (Fig 3.9).

Phenometrics produces PBR and software (Algal Command™) for algal research and industries. Its *PBR101* line of products is currently the standard platform for algal research worldwide. Researchers can study specific algal strains by creating the same conditions outside of a pond, but the process must be performed from inside the laboratory instead of using the proprietary software Algal Command™. The PBR101 includes a bright light-emitting diode (LED) that simulates sunrise and sunset every day. In addition, the temperature can be set to increase and decrease during the day, similar to a real outdoor pond. Numerous laboratories use a matrix of PBRs to simultaneously test multiple algal strains. Using the *Algal Command*™ software, the PBR101 can be programmed by itself or in a matrix of up to 12 for comparative analysis or to evaluate different algal strains simultaneously. This condition compresses research time into days or weeks but not months nor years. The *Algal Command*™ computer software provides the following:

- Centralised command from one control computer that controls multiple bioreactors over a standard Ethernet network from any location on the network, such as an office. The computer does not have to reside in the lab next to the PBR101 matrix.

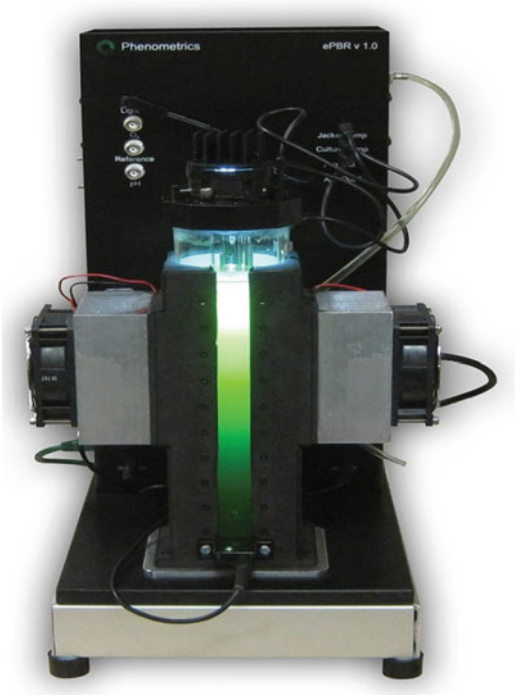

Fig. 3.10 PBR101 produced by Phenometrics Inc. Photos and information are used with permission from Phenometrics Inc.

- The Algal Command™ software displays high-throughput data that show in vivo and real-time measurements of algal density, pH probes and temperature probes (Fig. 3.10).

Algenuity possesses a flexible laboratory-scale algae culturing system (Algem) with precisely calibrated, independently controlled red, white and blue light, light intensity and light cycle controller. The equipment also contains pH, temperature and mixing and aeration control systems. All the parameters are connected to a computer and controlled by Algem software. The company also produces another laboratory-scale computer-controlled PBR (Algem HT24) for microalgal research; this PBR can run 24 (50 mL vessel) independent single experiments or tight replicates with independently controlled white LED with a uniform temperature system and can record optical density (740 nm) continually. This system is ideal for strain identification in laboratory.

Shanghai Guangyu Biotech Co., LTD, China (with brand name leadingtec), produces various types of PBRs, such as columnar PBRs, runway-pool PBRs, plastic thin-film PBRs, T-PBRs and plate PBRs for large-scale cultures. All these PBR systems can solve the DO discharge, mix evenly, reduce the sticky wall and reduce energy consumption but not destroy the algal cells (Fig. 3.11).

Fig. 3.11 Low-cost columnar PBR (left) and T-PBR (right) of leadingtec. Photos and information are used with permission from leadingtec (Guangyu Biotech Ltd., China)

8 Conclusion: Challenges and Future Prospects

Despite the considerable work conducted on developing PBRs for algal cultures, further efforts are still required to improve PBR technologies and know-how of algal cultures. None of the single bioreactor fulfils all the requirements. Hybrid reactors have been proven useful in mass production of algae compared with single bioreactors. Efforts may be exerted to combine different types of bioreactors to develop suitable bioreactors for mass algal culture.

The major issue in the design of efficient PBRs should be their capacity to maximise outdoor solar radiation. Large-scale outdoor PBRs should feature large volume and occupy less land space. In addition, they must possess transparent surfaces, high illumination surfaces and mass transfer rates and should give high biomass yields. Furthermore, the design and construction of any PBR should depend on the type of strain, target product, geographical location and overall cost of production (Ugwu et al. 2008).

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Chapter 4
Standard Techniques and Methods for Isolating, Selecting and Monitoring the Growth of Microalgal Strain

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Abstract The characterisation of microalgae is based on features, such as morphology, cell ultrastructure, pigments, photosynthetic products, reproduction, growth patterns, biomass and cellular proximate composition. These features are essential in identification, isolation, selection and cultivation of various microalgae for nutrition and as renewable resources, such as biofuels and biochemicals for human and animals. Although various methods have been used to isolate, select and monitor the growth of microalgal strain as described in the literature, few methods have limitations and not appropriately presented to users. Reviewing the standardised and validated methods for isolating and evaluating the characteristics of microalgae and providing a complete and simple report for the end users are necessary. This study aims to provide a complete and easily accessible guideline with all necessary

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standards and validated laboratory methods related to applied phycology, which can be used as reference by students and researchers who handle microalgae. In this chapter, major standard techniques for isolation and selection and calculation methods for monitoring microalgal growth are discussed with substantial number of flow charts and diagrams as the working manual in the field of applied phycology. The information provided in this chapter will be helpful for any users from the laboratory for the biomass production of commercial scale microalgae.

1 Introduction

Microalgae are photosynthetic microorganisms available from most water resources. They have been used as feedstock to produce biofuel, biopharmaceuticals, high-value chemicals and food additives (Alam et al. 2017). The isolation and identification of microalgae is vital for their application prior to studying their basic characteristics in culture, such as growth trend and biomass production. Most species breed asexually in nature, thereby establishing new order of strains in culture that is unique in character and relatively straightforward compared with wild species. The isolation and identification of the characteristics of microalgae (such as growth, biomass and proximate composition) is essential for culture collection units, which are important repositories of living cyanobacteria and eukaryotic microalgae. Within the framework of this chapter, strain is a genetically homogenous clone of algal cell propagated from a distinct eukaryotic microalga. In essence, distinct microalgal cells are isolated from particular geographical locations and placed in an appropriate environment for growth and accumulation of desired product into the cell with directed evolution and genetic or metabolic engineering to achieve best performance. The most traditional and practicable method to identify macroalgal strain is through light microscopy for imaging and subsequently using molecular biology for genotyping identification. Most recently, some modern technologies, such as optofluidic or microfluidic technology, introduce a rapid and accurate approach to identify different species. Various reports indicate that 7000–800,000 microalgal species exist in the world. However, only an extremely small number of species have been investigated. Historically, algal cultures have been isolated, identified and grown on a small scale in research laboratories and universities. Most commercial growers have cultured a limited range of species principally as dietary supplements, for production of pigments or as food for aquaculture. Commercial production of microalgae, including *Chlorella*, *Dunaliella*, *Spirulina*, *Haematococcus* and additional species for aquaculture (Xin et al. 2009), is still not large in volume (Paper et al. 2009).

Prior to the detailed study of commercial cultivation of microalgae, a clear idea of the justification for culturing parameters of microalgae must be established using strain information. To address taxonomic issues, laboratory cultures may be essential for comprehensive morphological evaluation. In addition, physiological questions on life cycle stages, including ‘cysts’, may require cultivated species. Detailed

analyses of bioactive compounds or pigments also necessitate the production of a large biomass of microalgal species, from which these compounds can be isolated. Isolating the strain from its local environment is favourable because it easily tolerates biotic and abiotic stresses.

2 Strain Isolation and Screening Technique

Strain isolation acquires single pure species of viable culture. Screening processes are conducted in examining biodiversity to establish the finest microalgal strain for a definite purpose. Microalgae can be found in various natural geographical environments all around the world, such as water resources, rocks and mountain, soil and desert. Their major inhabitation includes freshwater resources, brackish and marine water environment. Upon isolation of new strains from natural habitations, conventional cultivation procedures can be used, such as plating and growth in enriched media cultures (main source of nutrient phosphate and nitrogen) that contain exact and known qualities that support the growth of an expected algal strain whilst resisting the growth of others (using antibiotics in the medium at appropriate amount). Single-cell isolation using conventional technique from the sample is time-consuming and involves sterilised cultivation media and equipment. However, the result of this procedure allows us to obtain a pure culture to identify the strain easily. Some algal strains take several weeks to months to obtain pure culture and perform identification after being isolated by conventional techniques. As a result, wide range sampling and isolation attempts have been designed as high-throughput automated isolation techniques based on fluorescence-activated cell sorting and microfluidic devices (Sieracki et al. 2005). This microalgal cell sorting technique is extensively used for different microalgal strains from water. Most microalgal species have the ability to survive in various multitudes of environmental conditions and life cycles. Planktonic microalgae can be used in suspended mass cultures, whereas attached or benthic microalgae may be application in biofilm-based production.

2.1 Screening Criteria and Methods

A principle investigation technique ensures three main directions, as follows: (1) physiology associated with growth and reproduction, (2) production of desired metabolite and (3) strain robustness. The 'growth physiology' of microalgae refers to determining the growth factors, such as maximum specific growth rate, ample cell density and adaptability and/or acceptance to environmental variables (such as light, temperature, pH, salinity, CO_2 levels and O_2 levels,), photosynthetic efficiency and nutrient uptake capability. For effective results and standardisation of screening methods, various parameters, such as composition of culture media, light intensity

and sources and sampling time, should be considered. Furthermore, production of metabolites can be confirmed by recording the composition of protein, lipids, carbohydrates and other stress metabolites, and the output of the organism is quantified in terms of metabolite production of biofuels or other targeted products. Moreover, some strains produce metabolites secreted into the growth medium. Few metabolites work as important co-products to protect the strain from other organisms. However, product-specific applications are required to develop screening methods of secreted extracellular materials. Strain robustness is important for large-scale culture setup to ensure culture stability and manage abiotic stress and susceptibility to pathogens and predators; however, the situation is not consistently precise outdoor (Sheehan 1998). Therefore, microalgae should be cultivated in small quantities initially prior to the inoculation of the raceway for optimised growth. High-throughput methods employ iodine staining to monitor microalgal strains with modified starch metabolism cultured in a large pond (Black et al. 2013). A flow cytometry approach with lipid-staining dyes is considered an innovative new screening apparatus for isolating single cells with high lipid contents (Doan and Obbard 2011; Hyka et al. 2013; Xie et al. 2015). Moreover, near-infrared spectroscopy and Fourier transform infrared are used and have been receiving attention from researchers for the characterisation of microalgal lipids; they simultaneously determine the content of proteins, lipids and carbohydrates as a regular method for high-throughput lipid assay tool (Dean et al. 2010; Laurens and Wolfrum 2013; Mayers et al. 2013; Wagner et al. 2010).

2.2 *Sample Collection*

The best method for collecting microalgae sampling varies with habitat types and study objectives. Most collection sites contain different types of microalgae at different concentrations, and most types of microalgae grow only in certain seasons. The necessary details of sample collection, including time, date, temperature and depth, must be recorded in an information sheet. In coastal and oceanic sites, plankton and algae are generally sampled with a plankton net with various mesh sizes, such as 200 μm and 600 μm, for 10 min. Special water sampling bottles consist of cylindrical tube with stoppers at each end, and a closing device activated by a messenger is generally used. Kemmerer, Van Dorn, Niskin and Fjarlie bottles are mostly used for collecting samples of known volume. However, any plastic pet bottle can also be used. Nutrient-rich waters have thousands of planktonic (suspended/floating) microalgal cells per mL of water. Microalgal species can also be collected from soil, rock pools or the shoreline. Safe sampling and rapid transfer are necessary to prevent failure of viable culture of microalgae. Collected samples should be maintained at low temperature and should be protected from light whilst transporting to the laboratory for further culture, screening and identification. Glass microfiber filtered (GF/C) water should be used to dilute samples of the same salinity.

2.3 Single-Cell Isolation by Micropipette

Single-cell isolation is a frequently used technique (Parvin et al. 2007) that can be performed with a micropipette (pasture pipette). Traditionally, a fine glass pasture pipette is used for isolating single cells. The main purpose of this pipette is to select a single cell from the sample and deposit it, without any damage, into a sterile droplet of medium. The single cells can be prepared as a curved or straight tip. A curved tip is useful for inoculation and cell transfer, whereas a straight tip is used for dispensing isolate cells into sterile droplet. Forceps are replaced to the thin area, where the weight of the tip turns downward. The end section is removed and disposed by a slight pull and bending movement. If the pipette breaks or fails, then another pipette is used. The purpose of micropipette isolation is to select a cell from the specimen, the cell is carefully dropped into a sterile droplet and the cell is selected again and shifted to another sterile droplet. This practice is continued until a single algal cell, free from all other protists, can be successfully placed into a culture medium. This procedure prevents cell damage and unnecessary handling and promotes safe isolation of a single cell. The diameter of the micropipette opening should be at least twice that of the cell and regularly several times the cell size. To isolate filaments, chains of cells or lengthy single cells (e.g. certain pennate diatoms), the micropipette should be at one end of the filament, chain or cell. The micropipette should be held at a position, such that the filament or cell slips into the micropipette tip without severe bending. Given that microscopic cell identification is important, the target species are placed in a glass or plastic dish in a multi-well plate on a microscope slide.

2.4 Single-Cell Isolation via Streak Plate Technique

Single-cell isolation using a streak plate is ideal for many coccoid algae and most soil algae because axenic cultures can often be established without additional handling (Parvin et al. 2007). *Chlamydomonas*, *Pavlova*, *Synura* and *Tetraselmis* are flagellate algae that grow well on agar; however, some algae, such as flagellates (including *Heterosigma*, *Pelagomonas* and *Peridinium*), do not grow effectively. Coccoid, cryptophyte and chlorarachniophyte cells can also grow normally on agar. Some algae require trace agar between 0.3% and 0.6%; however, algae prefer to grow on liquid state rather than on solid. Chances of bacterial and fungal contamination frequently exist on agar medium. Unialgal growth is possible when agar medium is streaked appropriately. Microalgae culture can be established by streaking sample on agar surface. A loop is loaded with sample and spread over the agar surface by using different streaking patterns. After streaking, agar plates are incubated until colonies develop. The incubation time varies from species to species. When colonies appear, an algal colony is transferred to a new agar plate, agar slant or liquid medium for pure culture using micropipettes. Parvin et al. reported single-cell isolation techniques by micropipette and streak plate. Both techniques are easily understandable and reliable and can be applied at laboratory scale.

2.5 Dilution Techniques

Dilution techniques have been used for a long time, and their main purpose is to place only one cell in a test tube or a multi-well plate for single-cell isolation. Such a technique is established by repeated serial diluting of 1:10 volume for five to six times. Generally, dilution technique is performed when selecting random algal species from samples. Dilution should be conducted with distilled water or culture medium. Full-strength culture medium is used for fastidious organisms. Previously, this technique has been used in chemistry laboratory experiments to obtain appropriate molar concentrations of chemicals. This technique can also be applied in reducing dense culture of the algal cells to isolate and divide individual colonies. The serial dilution apparatus must be sterilised to remove contaminations. Ten test tubes are prepared and filled with 9 mL distilled water. Then, 1 mL of centrifuged microalgae stock sample is transferred into the first test tube. The volume is mixed by gentle shaking, and 1 mL mixture is transferred into the second test tube and then into the third, fourth and so on, as depicted in Fig. 4.1. After serial dilution, three random test tubes are selected, and the sample is inoculated on separate agar culture plates and liquid medium.

2.6 Gravity Separation: Centrifugation and Settling

Gravity separation splits heavy and light particles and cells from each other and can be applicable to settling and centrifugation. Moderate centrifugation for a short period brings large dinoflagellates and diatoms to a free pellet, and the smaller cells can be poured out until the cells remain suspended; otherwise, the process is repeated. Extreme centrifugation can damage cells. The time and centrifugation force are variable with the type of organism (3000 rpm for 15 min) and rotated at 1000–1500 rpm up to homogeneous suspension. To separate mixed cultures, gradient density has been used and can appear into separate sharp bands (Price et al. 1974). Settling is efficient for non-swimming large or heavy cells. The mixed sample can be placed in a tube or a flask and allowed to settle. The smaller cells remain suspended in the medium, the larger cells remain at the bottom and the supernatant can be removed. However, at present, these methods are no longer commonly used because of the low chances of pure culture.

3 Monitoring of Microalgal Growth

The growth of microalga culture can be determined by measuring the abundance of algal cells in the shape of dry weight (DW) or chlorophyll content. Optical density can also measure the algae abundance. Caution is necessary when calibrating optical

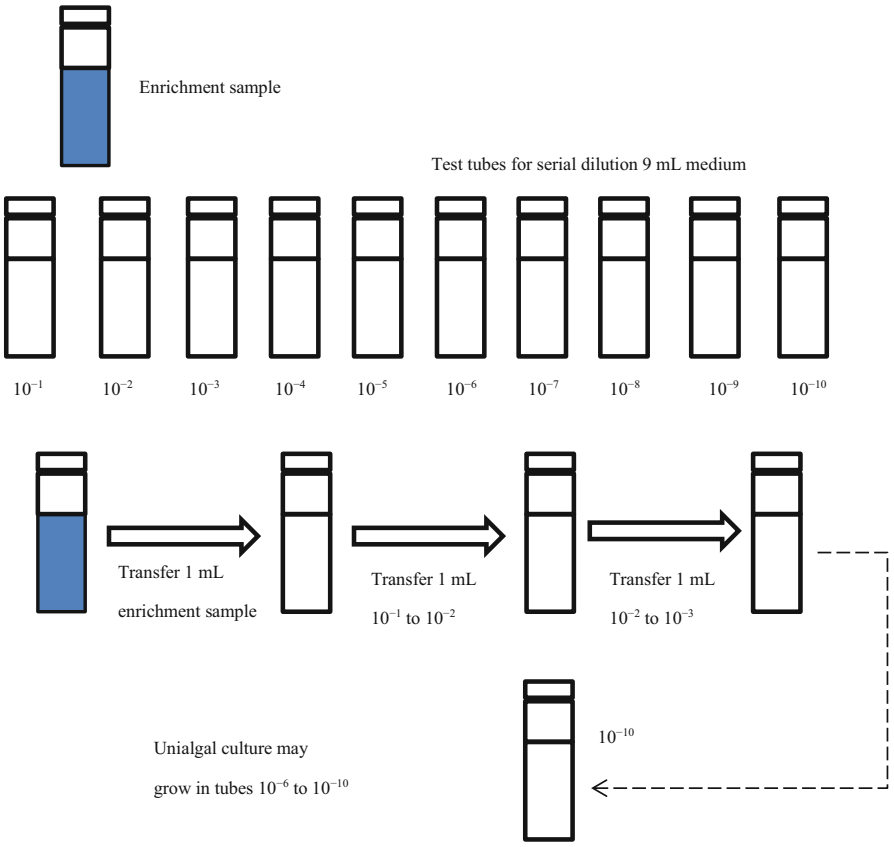


Fig. 4.1 Schematic of the serial dilution method

density or direct cell counts. Various protocols for measurement of algae abundance are discussed in the following section.

3.1 Cell Counting by Microscope

Counting the number of cells in a given volume is essential to determine cell concentration. Several methods are available for counting algal cells. The method may be direct or indirect and range from simple microscope-counting chambers to sophisticated electronic devices. The most accurate is to see and count cells with a microscope. The selection of equipment depends on several factors, such as culture density, size and shape of the cells.

3.1.1 Haemocytometer

Haemocytometer and Sedgewick Rafter chamber are suitable devices to measure the size of less than approximately 100 μm in diameter. If the cell density is higher than 10^5 mL^{-1}, then Sedgewick Rafter is a suitable counting device. The haemocytometer has two chambers, each with 9 mm squares and several sub-layer divisions. The most common ruling is the improved 'Neubauer', which is made of 3×3 mm grid distributed into nine 1 mm^2 areas with 0.1 mm depth. Another chamber is the 'Fuchs-Rosenthal', which has a total rule of 9 mm^2, whereas a depth of 0.2 mm is used for larger cells. Although the depth is 0.2 mm, the size of the cells can be counted accurately and limited at approximately 70 μm given that larger cells seldom form an even distribution in the chamber. Motile cells must be fixed when using this method. The cover slip and chamber must be cleaned by using ethanol or methanol. The cover slip is tightly fixed to the chamber by cohesion, as shown by the presence of diffraction rings. The cover slip must be symmetrically placed over the double rulings to prevent non-random cell distribution. The cell suspension is transferred to the chamber using a fine pipette. The chamber must be filled using a single application, without overflowing into the channels and must not contain bubbles. Prior to counting, the cells must be prioritised for settling.

For cells touching the centre line of the triple ruling, only two sides of the square must be included in the count, and the cells touching the centre line of the other two sides are excluded. To maintain an acceptable degree of error, at least 50 cells should be counted. Cell density is calculated as follows:

$$\text{Cells per } \mu L = \text{number of cells counted} \times 1/\text{area} \times 1/\text{depth} \times \text{dilution}.$$

The procedure for using haemocytometer is shown in Method 4.1 (Fig. 4.2).

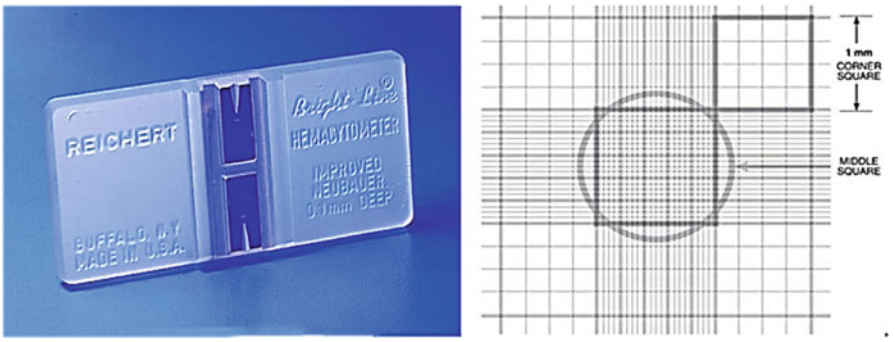


Fig. 4.2 Haemocytometer (left) and haemocytometer image at 100× microscopic magnification (right)

Method 4.1: Cell Counting by Haemocytometer

Clean the haemocytometer and cover the cover slip with 70% (v/v) ethanol and wipe dry with lens.

↓

Place the cover slip over the grid on the haemocytometer.

↓

Shake the suspension properly to ensure homogeneity of the sample.

↓

Shake the suspension in a standard manner to ensure homogeneity of the sample.

↓

Place a small drop of the suspension, which contains the algae, at the edge of the cover slip.

↓

Wait for few minutes for the cells to settle down.

↓

Select the suitable size square on the haemocytometer grid and count the number of cells in the grid for at least three separate squares. Count at least 100 cells for accuracy. To achieve this numbers of cells, count more than three separate squares. Record the number of cells counted and the number of squares counted.

For the Neubauer haemocytometer, the volume over one of the 1 mm squares is 0.0001 mL; therefore, the cell number per mL can be calculated as follows:

$$Cell\ Number\ (cells\ mL^{-1}) = \frac{Total\ number\ of\ cell\ counted}{Number\ of\ squares\ counted} \times 1000$$

3.1.2 Sedgewick Rafter Counting Cell

The chamber is rectangular (50 × 20 mm) with depth of 1 mm, area of 1000 mm^2 grid engraved on the bottom and lines 1 mm apart, as shown in Fig. 4.3. If the sample is accurately calibrated and filled, then each grid covers 1.0 mL volume. Glass and plastic versions are available; although expensive, glass is better than plastic. Glass is best for analysing large cells (20–500 μm) with cell densities ranging from ~30 mL

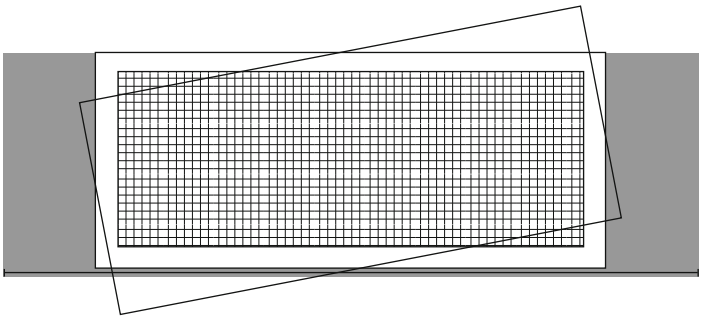


Fig. 4.3 Sedgewick Rafter counting cell

to approximately 10^5 per mL. This method does not require an overnight settling period; it is rapid and can provide immediate assessment. The setup cost is low due to the use of a compound microscope. An extremely thin microscope cover slip is required to cover the cells. A chamber must be free from air bubbles. Cells in each cubic millimetre can be counted using an inverted or low-power compound microscope. When cells lie on the lines, only the organisms on the two sides of the square are counted. The chamber and cover slip must be perfectly cleaned by ethanol or methanol. Fixation is necessary for motile cells and is commonly performed using Lugol's iodine or formaldehyde-based fixative. For Lugol's iodine, 10 g potassium iodide, 5 g crystalline iodine and 100 mL distilled water are combined. Once dissolved, 1 drop Lugol's iodine (~0.05 mL) is added per 5 mL of algal sample.

3.1.3 Palmer–Maloney Slide

The counting chamber has no rulings with diameter of 17.9 mm, depth of 0.4 mm and volume of 0.1 mL. The chamber is provided with two charging channels at 2 mm (width) × 5 mm (length). To use the Palmer–Maloney counting chamber, a cover slip is placed on the edge of the chamber, and the sample is introduced as the sample fills the chamber; it draws the cover slip over the chamber. The Palmer–Maloney is useful for organisms with a size range of 5–150 μ.

3.2 Coulter Counter

The machine has one or more microchannels that separate two chambers with electrolyte solutions. The culture medium contains the algal cells that pass through the microchannel. Each algal cell causes a brief change to the electrical resistance of the liquid. The counter detects these changes in electrical resistance and then counts each cell. The benefits of using a Coulter counter are accuracy and relative simplicity, thereby enabling large number of samples to be counted efficiently. However,

the method does not discriminate between algal cells and other particles; a dense culture must be diluted to obtain an accurate count, and it is relatively expensive. Particle counters, such as Coulter counter, have been available for a long time and can be convenient. However, they can only be used for single cells and may also be inaccurate for cells that easily deform, such as *Dunaliella* cells.

3.3 Optical Density of Culture

This method is rapid and is widely used to measure the growth rate of algae culture; its wavelengths are 550 nm or 750 nm. This method determines the estimation of culture cell density and is not suggested for cultures of *Porphyridium* and some diatoms. During absorbance, developing the correlation among the cell count and absorbance and how reliable this relationship is under different culture condition is important. This relationship changes with the size of the cells, thereby varying the growth rate. In addition, other factors affect the absorbance. This method is unreliable when measuring the cell density of various types of algae. Griffiths et al. (2011) reported that the changes in the cell pigment content during growth can also increase the error of using absorbance as a growth measurement method. Spectrophotometer, which provides an indirect method that correlates the algal density with light absorbance at specific wavelengths, can be used to measure cell concentration. Spectrophotometers use microplates that enable cell density to be measured in a wide range of volumes. Spectrophotometry is reliable and can be easily set up for automatic monitoring systems (Rodrigues et al. 2011). The major drawback is the limited accuracy because the cells are not counted directly as the machine measures the light and not the cells. This method, coupled with the stochastic nature of liquid cultures, is the only means to estimate cell numbers. Through spectrophotometry, chlorophyll concentration in culture can be identified. An indicator of algal biomass is approximately 1–2% (DW). Although the chlorophyll content in an algal cell is not constant and varies with the nutritional state of the cell, this content evidently affects the accuracy of the derived cell density estimates. The procedure for using spectrophotometry to determine optical density of cultures is shown in Method 4.2.

Method 4.2: Optical Density of Cultures

Purpose

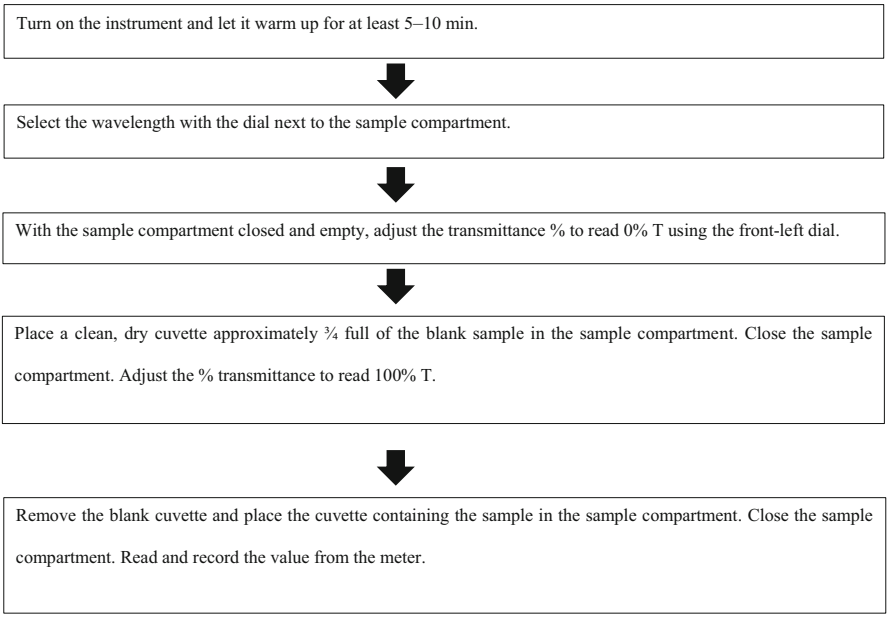
- Spectrophotometric protocol for qualitatively assessing the growth rate of microalgae cultures

Equipment, materials and reagent

- Spectrometer cuvettes, 1 mL Gilson pipette, spectrophotometer capable of reading 600 and 750 at 1–4 nm range and laboratory RO or DI water

Precautions

- Wear a laboratory coat, goggles and gloves whilst handling biological material in the laboratory.
- Read the MSDS information or consult with local health and safety personnel in case of direct solvent contact.



Note: Every time the wavelength of light is changed, the instrument must be recalibrated to 0% T and 100% T with blank. Repeat Steps 2–5.

3.4 Flow Cytometry

A new and recently available instrument, which is a type of digital image analyser, may be a good alternative (Steinberg et al. 2012) but has yet to be analysed widely. Such a device can also measure cell density (Collier 2000; Marie et al. 2005). It is sophisticated and expensive for counting cells whilst using flow cytometer. The culture containing the algal cells flows in a narrow stream in front of a laser beam, and a light sensor detects the light reflected from the cells as they hit the beam. Flow cytometers can analyse the shape of cells and their internal and external structures, as well as measuring specific cell components. The expenses and complexity of flow

cytometers indicate that they are rarely used for the sole purpose of counting cells. Flow cytometers that incorporate cell sorter may also be useful for rapid single isolation. The main advantage of this device is that it not only can measure the number of cells but also simultaneously measure a range of different parameters, such as fluorescence yields (Sosik et al. 2010), lipid content (Cooksey et al. 1987) and lipid composition (Mendoza Guzmán et al. 2010).

3.5 DW Measurement of Biomass

The cells are separated via centrifugation or filtration before measuring the DW, ash free dry weight (AFDW) and extraction step for analysis of the proximate composition. Filtration is the fastest method. Algal biomass can be measured by using DW method. Method 4.3 shows the procedure for identifying the DW of fresh algae. Meanwhile, the DW of marine algae is affected by the amount of salts absorbed on the cell surface. The most common technique, which is employed to cell mass, is washing. However, the use of various washing agents is inaccurate for obtaining desired values. Some researchers did not mention washing in their DW or AFDW determination (Ben-Amotz et al. 1985; Chu et al. 1994), whereas others used isotonic solutions of ammonium formate (Brown et al. 1993; Wikfors et al. 1992), sodium chloride (Zhu and Lee 1997) and isotonic ammonium (Zhu and Lee 1997). An alternative method for determining the DW and AFDW is summarised in Method 4.4.

Method 4.3: DW Determination of Fresh Water Algae

Purpose

To explain the protocol for obtaining DW of microalgae culture

Precautions

- Wear a laboratory coat, goggles and gloves.
- Use the oven carefully.

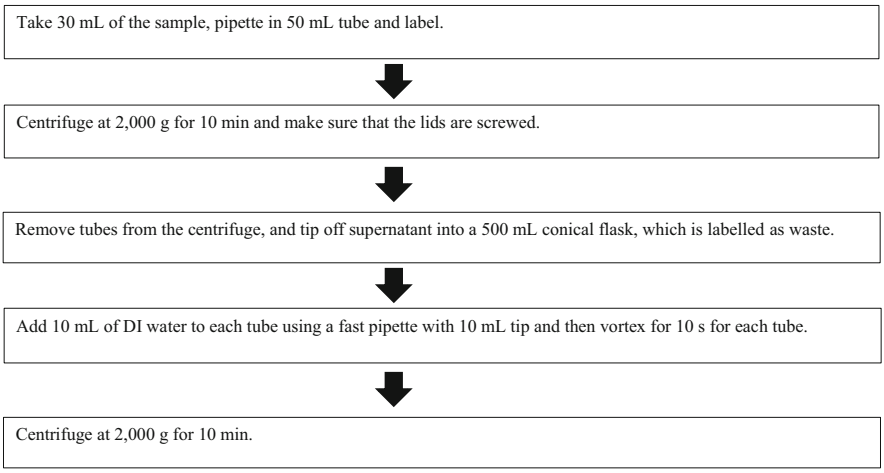
Requirements

- Whatman GF/C 45 μm, glass or plastic funnel, 100 mL flask, 50 mL plastic falcon tubes, ultrapure H_2O, drying oven and analytical balance

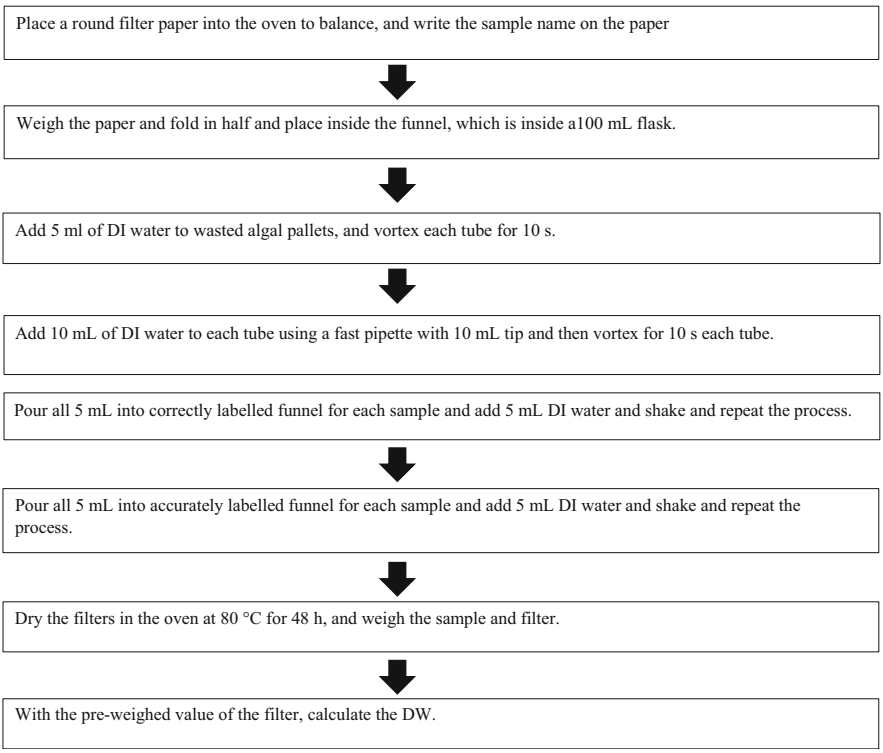
Procedures

- Add 500 DI water into a beaker.
- Place a filter paper in the oven at least two days prior to use.

a. Preparation of standards and sample



b. Preparation of standards and sample



Calculation:

$$\text{Dry weight} = \frac{(\text{filter} + \text{dry sample wt}) - \text{filter wt}}{\text{Volume of filtered}}$$

Method 4.4: DW and ADFW Determination

GF/C Filter Pre-treatment

The glass fibre filters used for concentrating the algae must be pre-combusted to determine the DW and ADFW.

- Pre-combust Whatman GF/C (2.5 cm in diameter) filters at 100 °C for 1 h.
- Store filter in a vacuum desiccator over KM_nO_4 crystal until use.

Precautions

Dried algae can be highly hygroscopic; caution must be observed whilst weighing. If the weight increases slightly during weighing, then the sample is dried again in the desiccator for 24 h over fresh KOH.

Carefully weight pre-combusted filters.

↓

Place the filters in the filter unit, and filter culture until filter appears completely dry.

↓

Wash the filter with 10 mL distilled water or isotonic ammonium formate for saline species.

↓

Remove the filter from the filter unit, dry at 100 °C for 1 h and then place in a vacuum desiccator overnight.

↓

Weigh the dried filter that contains algae to four decimal places using a five-digit balance.

DW = (weight of filter plus algae) - (weight of filter)

Determining AFDW

Take filters from Step 5 of DW determination and ash at 450 °C in a muffle furnace for 5 h.

Cool filters in a vacuum desiccator over desiccant.

Rapidly and carefully weigh the filter.

AFDW = DW _ Weight after ashing

3.6 Calculation of Growth Rate

Growth rate (estimation of population) can be monitored by establishing the growth curve of the exponential phase that provides details of cell division per day. Growth rate can be obtained from batch and continuous cultures. Other measurements of growth, such as fluorescence, chlorophyll concentration, DW of biomass and optical density, should be applied before calculating the growth rate to correlate the growth rate criteria. Growth rate is expressed by log number of cells calculated against the number of days.

Growth rate (K values) can be calculated as follows:

$$K = \frac{\log_{10} N_1 - \log_{10} N_2}{0.301 \times t},$$

where K is the number of generations per day, N_1 is the initial cell concentration, N_2 is the cell concentration after time t and t is the number of days.

The algal culture can also be cultivated in continuous batch mode. In this type of culturing method, fresh medium is continuously added at the same rate at which it is withdrawn. The cell concentration in continuous culture can be determined by using the dilution rate, as shown as follows:

$$D = \frac{\text{Medium flow rate}}{\text{Volume of culture}} = \frac{F}{V},$$

where F is the medium flow rate to and from the culture vessel, V is the volume of the culture vessel and D is the dilution rate.

4 Microfluidic System for Selection, Identification and Development of Microalgal Strain

Identifying microalgal cells and sorting them based on their properties (such as viability, size, lipid content and tolerance to environmental stress) are highly interesting for the microalgae industry. Cell sorting is naturally performed to separate cells with desired qualities from a large population of cells. Mainstream techniques used in microalgal strain isolation, selection and development based on conventional methods (light microscopy for imaging and molecular biology for genotyping) are bulky, labour-intensive and time-consuming. However, microfluidic lab-on-a-chip systems can offer time-efficient and advanced alternatives for isolation, selection and development of microalgal strain in an entirely automated approach (Kim et al. 2018). Microfluidic cell cytometer developed by Benazzi et al. (2007) is the first in this area. It can analyse and differentiate microalgal cells based on chlorophyll autofluorescence of cells that flows through the optical detection zone in a microfluidic channel. The upgraded version of this microsystem constructed by Hashemi et al. (2011) can differentiate four diverse species of microalgae that have a wide size range (1–50 μm) and various amounts of inherent chlorophyll and phycoerythrin in the cells at the same time. Photodetector signals obtained from nine different microalgal species show unique and different signatures from one another and aid separate each species using an optofluidic device developed by Schaap et al. (Allison et al. 2012). The main component of all microfluidic cell cytometers includes laser diode or microscopic light and quadrant-cell photodetector, where microalgal cells inside a microchannel are illuminated by laser light and then directed through a curved waveguide or chlorophyll emission spectra by upwards and downwards positions next to the microfluidic channel. Characteristic wavelets associated with microalgal cell geometry and size are detected. Another type of microfluidic flow cytometer measures photosynthetic characteristics and lipid accumulation of microalgal cells. Deng et al. (2013) proposed a microfluidic device that can distinguish microalgal cells that have different lipid contents by dielectrophoresis (DEP). DEP is an electric field-based, label-free cell manipulation technique, the force of which is dependent on the dielectric properties of cells and their surrounding medium. By using a combined electrode array in a microchannel, a non-uniform alternating current (AC) electric field can be exposed in microalgal cells, which reveal cells to positive or negative DEP force depending on their lipid content, thereby moving the cells either towards the electrodes (positive DEP) or away from the electrodes (negative DEP). For example, *Chlorella vulgaris* cells with 11% and 45% lipid contents have been successfully separated using this device.

5 Conclusion

The available methods for laboratory- or large-scale practice continuously evolve, and advanced techniques and equipment are developed rapidly. The proposed methods are the most suitable and convenient for performing laboratory-scale analysis. However, these methods are not the only available approaches in the literature. At present, many groups are focusing on automation to accelerate research efficiency. Methods for those modern devices depend on the manufacturer's guideline.

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Chapter 5
Stress Response of Microalgae and Its Manipulation for Development of Robust Strains

Chun Wan, Bai-Ling Chen, Xin-Qing Zhao, and Feng-Wu Bai

Abstract Microalgae are promising producers of lipids and various valuable chemicals. Improved production titers and productivities of the desired products will reduce the production cost and subsequently benefit industrialization of microalgal biorefinery. Accumulation of lipids and valuable products in microalgae is commonly triggered by various stress factors. However, microalgal growth is compromised under stress conditions. Therefore, understanding stress response of microalgae and manipulation of stress conditions may enable development of robust microalgae strains that efficiently produce target molecules. In this chapter, recent advances in manipulating environmental stresses for lipids and pigment accumulation in microalgae are summarized, and development of superior microalgal strains as the efficient microbial cell factories based on omics approaches are highlighted.

Keywords Microalgae · Biorefinery · Stress response · Omics analysis · Adaptive laboratory evolution

1 Introduction

Microalgae are widely distributed in diverse ecological habitats including freshwater, marine, and hyper-saline (Gimpel et al. 2015; Hu et al. 2008). The ability of microalgae to adapt and proliferate in diverse environments has made microalgae a rich source of valuable chemicals, such as biofuels (derived from lipid) and bioactive compounds (i.e., carotenoids and fatty acids) (Gimpel et al. 2015). Biofuels are an alternative to fossil fuel with the advantages of being sustainable and lower emission of greenhouse gas (GHG). Biofuels production from food and oil crops is constrained by the competition with either food production or arable land,

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emphasizing the importance of using microalgae for biofuels production (Chia et al. 2018; Chisti 2007; Wijffels and Barbosa 2010).

Microalgae offer many advantages over traditional oil crops to produce biofuels and bioactive chemicals, such as high photosynthetic efficiencies and lipid productivities, no competition with food or arable land, rapid fixation of environmental carbon thereby reducing emissions of a major GHG, year-round cultivation, and robust environmental adaptability (Chia et al. 2018; Hu et al. 2008). So far the microalgae-based biofuels contributed to less than 5% in the total energy pool which includes fossil fuels and solar and wind energy (Chia et al. 2018; Su et al. 2017). Moreover, profits in commercialization of microalgal biofuels is still limited (Su et al. 2017). Therefore, enormous efforts on improving the desirable properties of microalgae to facilitate the economics of microalgal biofuels production process are required.

Accumulation of lipids and valuable products in microalgae is commonly triggered by stress factors. Therefore, manipulation of stress responses by altering nutrient concentration, pH, salinity, and light benefits accumulation of lipid and valuable compounds (e.g., carotenoids) (Chen et al. 2017; Markou and Nerantzis 2013; Wan et al. 2013). Specifically, lipid productivity in *Nannochloropsis* (Wan et al. 2013) and *Neochloris oleoaboundans* HK-129 (Sun et al. 2014) reached 30 and 52 mg L^{-1} day^{-1}, respectively, under nitrogen limitation. Nevertheless, stressful conditions often significantly inhibit the microalgal cell growth, leading to decreased yield of desired products (i.e., lipid). It has been calculated that twofold increase in biomass yield contributes to more than 40% decrease of biodiesel price. Therefore, it is of great significance to develop microalgae strains that can rapidly accumulate both biomass and desired molecules (Nagarajan et al. 2013). Unfortunately, so far most studies focus on the unilateral strategy, including nitrogen limitation salinity to trigger lipid accumulation in laboratory, there is a need to combine multiple stresses and microalgal strain development, as well as process integration, for economical and industrial production of microalgal biofuels.

On the other hand, due to the tremendous genetic and physiologic diversity of microalgae, a great challenge remains in applying established robust strain development strategy into new species. Thus, it is necessary to comprehensively understand microalgae evolution via omics technology, such as genomics, transcriptomics, proteomics, and metabolomics (Gimpel et al. 2015; Guarnieri and Pienkos 2015). Namely, omics information from various stress responses benefits the development of robust microalgal strains with optimal carbon and nitrogen flux for improving the yield of desired biochemicals (Bajhaiya et al. 2017; Guarnieri and Pienkos 2015). Besides, several microalgae, such as *Chlamydomonas reinhardtii* and *Dunaliella salina*, have been improved in lipid and carotenoid biosynthesis via adaptive laboratory evolution (ALE), a widely practiced strategy for economically selecting and engineering valuable and inheritable traits (Fu et al. 2013; Perrineau et al. 2014). The ALE is commonly started with environmental stress change; however, few studies integrate omics and stress responses in the ALE during robust microalgal strain development.

In this chapter, recent advances in manipulating environmental stresses of microalgae for lipid production, as well as bioactive compounds, were reviewed, and robust strain development based on integration of omics and the ALE was highlighted.

2 Improvement of Lipid Production Via Manipulating Stresses

Tremendous species diversity enables microalgae the characteristics of various growth rate and lipid yield, and recent studies have presented a common trend that microalgae could accumulate more biomass but generate little amount of lipids under the environmental conditions favorable for the growth and that lipids usually start to be accumulated under stressful conditions (Chen et al. 2017; Ho et al. 2014). Therefore, various stresses, such as nutrient stress, salinity stress, and oxidative stress, have been used to improve lipids yields (Ho et al. 2014; Pancha et al. 2015). The effects of different stresses on lipid production are described in detail below.

2.1 Nutrient Stress

Nitrogen, one of the most important nutrients in microalgae growth, have been intensively studied to increase lipid content in microalgae, especially nitrogen depletion (Chokshi et al. 2017; Hu et al. 2008; Rodolfi et al. 2009). Specifically, nitrogen depletion contributed to the biosynthesis of more neutral lipids (mainly triacylglycerides, TAG) due to the decrease of polar lipids in *Nannochloropsis* sp. (Martin et al. 2014). Nitrogen limitation further doubled the lipid content of *Nannochloropsis* sp. F&M-M24 and reached 60% by microalgal dry cell weight (DCW) phototrophically (Rodolfi et al. 2009), while fatty acid methyl esters in *Chlorella vulgaris* NIES-227 reached 89% by DCW heterotrophically (Shen et al. 2015). Other than lipid content, lipid productivity, an important factor in evaluating the potential of microalgae in biofuels production, is closely linked with nitrogen availability and varies in diverse species. For instance, the lipid productivity of *Nannochloropsis* sp. F&M-M24 reached 204 mg L^{-1} day^{-1} under nitrogen limitation (Rodolfi et al. 2009), higher than that of *N. oceanica* DUT01 (31 mg L^{-1} day^{-1}) (Wan et al. 2013). Most microalgae tend to alter the carbon flux from protein to lipid under nitrogen-deprived conditions, and the released nitrogen from degradation of protein will alleviate the nitrogen crisis in microalgal cells (Chen et al. 2017). Moreover, genes involved in nitrogen assimilation, such as *NRT2*, *NAR1*, *GLN2*, *GSN1*, and *AAT2*, are significantly upregulated under nitrogen starvation (Wu et al. 2015), while cellular enzymatic antioxidants, including superoxide dismutase (SOD) and catalase (CAT), and nonenzymatic scavengers proline and polyphenols are

remarkably higher as responding to the nitrogen starvation-induced oxidative stress (Chokshi et al. 2017). Interestingly, the highest lipid productivity in *Isochrysis zhangjiangensis* reached 136 mg L^{-1} day^{-1} with 3 g L^{-1} nitrate feeding (Feng et al. 2011). Together, the preference of nitrogen conditions in different microalgal strains has enlightened that it should be careful to play with nitrogen concentrations to increase lipid productivity and that the importance of robust microalgae strain selection and development with higher lipid yield under designated nitrogen conditions.

Phosphorus is another essential nutrient for microalgal cell growth and usually combined with nitrogen stress to improve lipid production in microalgae. Phosphorus assimilation was proposed to maintain the pool of enzymes involved in the biosynthesis of lipid, DNA, ATP, and other metabolites, and sufficient phosphorus was necessary for achieving the highest lipid productivity of *C. vulgaris* under nitrogen-limiting condition (Chu et al. 2013). Similar to nitrogen, effects of phosphorus on lipid production exhibit species depended, since phosphorus showed no significant influence on lipid production in *C. vulgaris* NIES-227 (Shen et al. 2015) or *C. reinhardtii* (Kamalanathan et al. 2015). The observation that phosphorus shows less effects on lipid production in microalgae when compared to nitrogen is probably due to the different mechanism beneath the nitrogen and phosphorus stress response, as microalgal cells reduce numbers of ribosomes to rescue the protein synthesis or storage of polyphosphates under phosphorus limited conditions (Kamalanathan et al. 2015).

Carbon dioxide (CO_2), another major nutrient for the optimal phototrophic growth of microalgae, has been used to improve lipid productivity, yet its optimal concentration varies in different species. It was reported that the lipid productivities of *N. oculata* NCTU-3 were 142 and 82 mg L^{-1} day^{-1} with 2% and 15% (v) CO_2 aeration at 0.25 vvm (volume gas per volume broth per min), respectively (Chiu et al. 2009). On the other hand, 1% (v) of CO_2 addition promoted the growth in *C. vulgaris*, *Dunaliella*, and *Scenedesmus quadricauda*, and the biomass and lipid productivities ranged from 16.3 to 35.1 and from 2.1 to 5.3 mg L^{-1} h^{-1}, respectively (Eloka-Eboka and Inambao 2017). Also, a mutant of *C. vulgaris* was able to accumulate higher lipid at 15% (v) CO_2, which might be attributed to the excess carbon resource causing nitrogen deficiency in the medium and thus improving the lipid content (Cheng et al. 2016).

Some minerals, such as sulfur, magnesium, cadmium, and copper, have been used to improve lipid accumulation in microalgae. For instance, increase in lipid content of *C. reinhardtii* CC-124 and CC-125 were 258% and 302% in response to sulfur starvation, respectively (Cakmak et al. 2012). On the other hand, addition of 100 μM Mg^{2+} contributed to higher lipid content (59.8%) and productivity (65.9 mg L^{-1} day^{-1}) in *Monoraphidium* sp. FXY-10 with no significance effect on fatty acids composition (Huang et al. 2013). Also, optimal Fe^{3+}, Mg^{2+}, and Ca^{2+} concentrations at 1.2, 7.3, and 0.98 mg L^{-1} enabled *Scenedesmus* sp. reach maximum lipid productivity (275.7 mg L^{-1} day^{-1}) (Ren et al. 2014), while *C. minutissima* UTEX2341 accumulated more lipid by 21% and 94% with the addition of 1.0 mM copper and 0.4 mM cadmium, respectively, during wastewater

treatment (Yang et al. 2014). Therefore, it is advisable to integrate bioremediation and CO_2 fixation with minerals stress for efficient lipid production.

2.2 Salinity Stress

Similar to nutrients stress, salinity stress has also been applied into improving the accumulation of lipids in microalgae. More precisely, *Chlorella* sp. CG12 and *Desmodesmus* sp. GS12 exhibited the highest lipid productivities at 52.8 and 55.2 mg L^{-1} day^{-1} in the presence of 25 mM $CaCl_2$, respectively, yet with the retarded growth (Srivastava et al. 2017). It has been reported that NaCl at a concentration of 30 g L^{-1} triggers the generation of reactive oxygen species (ROS) in *C. protothecoides* heterotrophically, and thus ROS-mediated signal transduction process alters carbohydrate to lipid with increased content of Acetyl-CoA carboxylase, while the fatty acids show no significant difference (Wang et al. 2016b). A recent study shows that *Chlamydomonas* sp. JSC4 switches the accumulation of starch to lipid under the 2% salt (NaCl) stressful condition and the flux that from pyruvate to Acetyl-CoA is identified as the metabolic rate-limiting step for lipid overproduction (Ho et al. 2017). *N. salina* cultivated at open systems showed increased lipid content and the lowest density of invading organisms (such as cyanobacteria and rotifer) when the salinity was 22 practical salinity units (PSU) (Bartley et al. 2013), indicating the possibility of using salinity and nutrient stresses for lipid production in large scale.

2.3 Other Stress Factors

Other than nutrients and salinity stresses, physical factors, including light, temperature, and pH, considerably affect the biosynthetic pathway of lipid in microalgae. Light is one of the key elements driving the photosynthesis, and high density at 400 μmol photon m^{-2} s^{-1} shifts carbon to neutral lipid (NL) in *Chlorella* sp. L1 and the NL productivity reached 51.4 mg L^{-1} day^{-1} accompanied with the significant decrease of membrane lipids (He et al. 2015), while low density at 60 μmol photon m^{-2} s^{-1} contributes to the highest TAG yield on light at 112 mg mol^{-1}_{ph} in *Phaeodactylum tricornutum* (Remmers et al. 2017). Similar observations are found in the temperature factor. For instance, *Chlamydomonas* sp. ICE-L accumulates 78.5% polyunsaturated fatty acids (PUFAs) in the lipid at 0 °C (An et al. 2013), while a mixed microalgae culture exhibits fivefold increase of NL at 30 °C in wastewater treatment (Subhash et al. 2014). The optimal pH of microalgae for lipid production is species-specific; *Ettlia* sp. YC001 reaches the highest lipid productivity (34 mg L^{-1} day^{-1}) at pH 8.5 (Yoo et al. 2015), while *Chlorella* sp. OS4-2 exhibits the highest TAG yield (61.1 mg L^{-1} day^{-1}) under incremental pH stress (pH ~10) (Skrupski et al. 2012). Moreover, combination among

aforementioned stresses have been applied for enhanced lipids production in microalgae, such as high light density (300 μmol photon m^{-2} s^{-1}) and nitrogen starvation (Ho et al. 2015), high light density (300 μmol photon m^{-2} s^{-1}) and low temperature (20 °C) (Nogueira et al. 2014), alkaline (pH 10.0), salinity stresses (420 mM salts), and nitrogen depletion (Santos et al. 2012). It has also been reported that *C. reinhardtii* and *C. desiccata* yield more TAG with addition of brefeldin A (75 μg mL^{-1}) and sodium azide (20 μM), respectively (Kim et al. 2013a; Zalogin and Pick 2014). Similar observations have been found when *Chlorella* sp. KR-1 and *Anabaena variabilis* are submitted to the mid pressure (10–15 bar, 2 h) and ultrasonic stresses (200 W, 5 min), respectively (Han et al. 2016; Praveenkumar et al. 2016), providing alternative methodology to improve lipid accumulation in microalgae.

Nitrogen limitation seems to be a universal strategy to improve lipid yield in microalgae, yet still microalgae exhibit species-specific responses to various stresses, convoluting the transplant of optimal stressful condition among promising microalgae species for biofuels production. Therefore, microalgae biorefinery has been proposed to fully valorize the microalgal biomass via coping multiple high-value bioactive compounds with lipid production.

3 Overproduction of High-Value Molecules via Manipulating Stresses

Microalgae are mainly consisted of carbohydrate, proteins, and lipids, and many efforts have been made to alter carbon flux to lipids, the feedstocks for biofuels production. Nevertheless, microalgae also contain a certain amount of high-value biochemicals, such as PUFAs and pigments. The PUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exert protection effects against various cardiac disorders and are being served as food and healthcare additives, while the pigments, such as β-carotenes, astaxanthin, and xanthophyll, are also promising candidates in food additives, cosmetics, and healthcare with great market potential (Chew et al. 2017). It has been reported that *D. tertiolecta* accumulates carotenoid at 40 mg L^{-1} under high-intensity light (300 μE m^{-2} s^{-1}) and nitrogen starvation (Kim et al. 2013b), while *Desmodesmus* sp. F51 maintains lutein at 3.6 mg L^{-1} day^{-1} under very-high-intensity light (600 μmol photons m^{-2} s^{-1}), sufficient nitrogen, and high temperature (35 °C) (Xie et al. 2013). High light intensity is also critical for maintaining the productivity of β-carotene at 2.5 mg m^{-2} day^{-1} during a long period (>47 days) cultivation of *D. salina* (Hejazi et al. 2004). Manipulating stresses to promote the accumulation of high-value molecules in microalgae are summarized in Table 5.1. These examples have indicated that it is a feasible strategy to enhance the pigment production from manipulating light stress, since pigments directly participate in photosynthesis as light antenna.

Table 5.1 Overproduction of high-value molecules in microalgae via manipulating stresses

Microalgae	Stress condition	Products	Effects	References
C. zofingiensis CCAP 211/14	Multi-stresses	Lutein and astaxanthin	The yield for lutein or astaxanthin maintained 3–6 mg g DCW^{-1} under stresses of 0.2 M NaCl, and 960 μmol photons m^{-2} s^{-1}	Del Campo et al. (2004)
Coelastrella sp. KGU-Y002	Multi-stresses	Carotenoid	The maximal content was ~10 mg g DCW^{-1} under stresses of 0.15 M $MgCl_2$, nitrogen deficient, and 200 μmol photons m^{-2} s^{-1}	Saeki et al. (2016)
Crypthecodinium cohnii	Multi-stresses	DHA	The low concentration of Cl^- and high K^+, with pH less than 5, contributed to 0.04 g DHA per 10^9 cells	Lippmeier et al.(2010
D. salina	High-intensity light	β-carotene	The maximal productivity reached 30 pg $cell^{-1}$ day^{-1} with a decrease in the degree of fatty acids	Lamers et al. (2010)
H. pluvialis	Light shifting	Astaxanthin	The concentration reached ~35 mg L^{-1} when the light shifted from red to blue with increase from 50 to 150 μmol photons m^{-2} s^{-1}	Xi et al. (2016)
I. zhangjiangensis	Sufficient nitrate	DHA	The DHA consisted of ~13% of total fatty acids with 3 g L^{-1} nitrate feeding at an interval of 24 h	Feng et al. (2011)
N. oceanica CY2	High-concentration salts	EPA	The EPA yield was ~10 mg L^{-1} day^{-1} when salt stress was combined with sufficient nitrate	Chen et al. (2018)
P. tricornutum	Nitrogen starvation	EPA	The highest concentration was ~30 mg L^{-1} after 17-day cultivation, partially from photosynthetic membrane lipids	Remmers et al. (2017)
	Low-intensity light	Fucoxanthin	The optimal bioreactor productivity was 2.3 mg L^{-1} day^{-1} with nitrate enriched medium	McClure et al. (2018)
	Low-intensity light	Xanthophyll	A larger pool of xanthophyll cycle pigments was obtained under blue light (~469 nm) than those from white and red light	Costa et al. (2013)
Spirulina sp.	Combined stresses	Phycocyanin	Nitrogen starvation and 0.2 M NaCl improved the content of phycocyanin to 18–22% by DCW	El-Baky (2003)

DCW dry cell weight, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid

Multi-stress combination has also been applied for lipid and high-value biochemicals production. For instance, the maximal productivities of TAGs and astaxanthin reached 297 and 3.3 mg L^{-1} day^{-1}, respectively, when *C. zofingiensis* was cultured under high-intensity light (300 μmol photons m^{-2} s^{-1}) and nitrogen depletion (Liu et al. 2016). Similarly, simultaneous accumulation of TAGs and β-carotene was obtained in *D. salina* under nitrogen replete and moderate nitrogen limitation, while continuous nitrogen starvation could uncouple this relationship (Bonnefond et al. 2017). Thus, it should be meticulous to program various stresses for concurrent production of multi-biomolecules. Furthermore, compared with the efforts devoted in strain development for lipid production, less have been made for high-value products, neither for the integration of production of lipid and high-value bioactive molecules. Recent advances in improving the lipid or high-value biochemical production have revealed a phenomenon that the improved production of targeted molecules in microalgae via manipulating stresses is likely species-specific, baffling the application of referential experience among species (Minhas et al. 2016). Therefore, not only the comprehensive understanding of environmental factors but also does the in-depth investigation of intrinsically genetic factors come to the priority on the development of robust microalgal strains with improved lipid and high-value compound production.

4 Development of Robust Microalgal Strains

Microalgae strains are temporally improved for desired molecule (i.e., lipid) production under stressful conditions, yet the growth could be constrained, and the trait of high productivity is hardly inheritable. Therefore, screening the promising targets (e.g., stress-responsive genes) by uncovering microalgal responses to various stresses based on omics approaches is becoming increasingly imperative on the development of robust microalgal strains with inheritable traits. Genetic engineering of these targets will circumvent the controversy between growth and valuable compounds accumulation in microalgae under stresses and further provides a shortcut for robust microalgal strain development via adaptive evolution.

4.1 Omics Approaches for Uncovering Stress Responses

Omics technology includes genomics, transcriptomics, proteomics, lipidomics, and metabolomics and is being applied to uncover the mechanisms underneath stress responses (Guarnieri and Pienkos 2015). Specifically, genomics analysis has indicated that multiple genome pooling, horizontal genetic exchange, selective inheritance of lipid synthesis genes, and species-specific gene loss lead to an enormous genetic apparatus for oleaginousness and wide genomic divergence among *Nannochloropsis* (Vieler et al. 2012; Wang et al. 2014), which is also plausible in

explaining the species-specific observation of lipid and high-value compounds production under stressful conditions. Transcriptome analysis of *Coccomyxa subellipoidea* C-169 has showed that more carbon is assimilated to maintain carbon/nitrogen balance upon elevated CO_2, and significant downregulation of fatty acid degradation genes, as well as upregulation of fatty acid synthesis genes, might contribute to the rapid lipid accumulation at 222 mg L^{-1} day^{-1} (Peng et al. 2016). The *KBT/CRTO* genes, encoding the rate-limiting synthases converting beta-carotene to astaxanthin in *H. pluvialis*, were upregulated more than threefold under salicylic and jasmonic acid (25 mg L^{-1}) stressful conditions as revealed by RNA-seq analysis (Gao et al. 2015). Another transcriptome analysis of *D. parva* under nitrogen limitation had suggested that upregulation of genes encoding beta-ketoacyl-ACP synthase II (KAS II), triacylglycerol lipase (TAGL), and 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) was responsible for the increased lipid accumulation and that expression of *WRI1*, induced by nitrogen limitation, could regulate the biosynthesis of TAG (Shang et al. 2016). Further proteomics analysis of *D. parva* showed that increased levels of proteins related to TAG biosynthesis (AGPAT) and decreased levels of proteins related to fatty acids degradation were the main reason for improved lipid content under nitrogen-limited condition (Shang et al. 2017). The level of nitrate reductase decreases when microalgae, such as *D. parva* (Shang et al. 2017), *P. tricornutum* (Longworth et al. 2016), and *T. pseudonana* (Hockin et al. 2012), are cultivated under nitrogen limitation stress, while increase of glutamine and glutamate synthase serves as an important role in nitrogen supply when peripheral nitrogen is in paucity, and carbon flows into lipid biosynthesis due to the downregulation of ADP-glucose pyrophosporylase (Shang et al. 2017). Nevertheless, the discrepancy between transcriptomics and proteomics data indicated the critical role of posttranscriptional regulation for the proteins involved in lipid metabolism, such as KSA II (Shang et al. 2016, 2017), highlighting the importance of omics technology in excavating the intriguing mechanism of lipid production under stressful condition. Moreover, the mutual corroboration of mRNA and protein data has distinguished the β-subunit of methylcrotonyl-CoA carboxylase (MCC2) as a negative regulating gene during TAG accumulation in *P. tricornutum* under nitrogen depletion (Ge et al. 2014).

A change in the ratio of polar lipids, like phosphor-, glycol-, and betaine lipids, was reported to be dependent on the culture medium pH, since extreme low pH (1.0) and high temperature (39 °C) led to the replacement of cellular phospholipids by betaine lipids in *Galdieria sulphuraria* as illuminated by lipidomics analysis (Vítová et al. 2016). Similar observation was reported in a snow alga *C. nivalis* cultivated under phosphate-limited condition, where the contents of phospholipids reduced by 68–84%, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI), and those of non-phosphorous lipids increased more than 76%, such as digalactosydiacylglycerol (DGDG) and 1,2-diacylglycerol-3-O-4′-(N, N, N-trimethyl)homoserine (DGTS) (Lu et al. 2013). Metabolic profiling and transcription analysis of *Chlamydomonas* sp. JSC4 cultivated under salinity stress had suggested that accelerating the metabolic reaction from pyruvate to Acetyl-CoA could further improve the lipid biosynthesis, and upregulation of

genes involved in starch degradation (e.g., starch phosphorylases) and lipid biosynthesis (Acetyl-CoA carboxylase and synthesis) mainly contributed to the rapid lipid production (Ho et al. 2017).

The omics technologies zoom in the lipid biosynthesis of different microalgae under various stresses, especially nitrogen limitation, and several stress-responsive genes, such as *DpLip* (Shang et al. 2017), *MCC2* (Ge et al. 2014), *PSR1* (Bajhaiya et al. 2016a), and *WRI1* (Shang et al. 2016), have been discovered and could be served as the targets for genetic engineering of robust microalgal strain development, as well as the genes encoding proteins and other metabolites identified via omics technologies. However, omics analysis on improved production of high-value molecules, such as carotenoid, is still too few when compared with lipid production. Thus, further research should pay more attention to explore the mechanism of high-value bioactive production via omics rather than lipids.

4.2 Genetic Engineering Using Stress-Responsive Genes

Genetic engineering of metabolic pathways is cumulatively applied in improving targeted molecule yield based on omics analysis (Bajhaiya et al. 2017; Banerjee et al. 2017; Chen et al. 2017), and the advances in enhanced lipid production and bioactive proteins via manipulating the genes involved in lipid synthesis and via rewiring the heterologous genes, respectively, have been thoroughly reviewed in previous articles (Goncalves et al. 2016; Guihéneuf et al. 2016; Jinkerson and Jonikas 2015). Similarly, it is practicable to engineer stress-responsive promoters in microalgae to enhance lipid production. Precisely, *P. tricornutum* with the expression of *H. pluvialis* oil globule protein (HOGP) regulated by a N starvation-inducible promoter (Acyl-CoA: diacylglycerol acyltransferase, *DGAT* promoter) possessed 20–30% higher lipid productivity than in wild type (Shemesh et al. 2016), while *Chlamydomonas* exhibited ~2.5 times higher of TAG content (~13 pg cell^{-1}) when a P starvation-inducible promoter (sulphoquinovosyl-diacylglycerol 2, *SQD2* promoter) was used to drive the expression of type 2 diacylglycerol Acyl-CoA acyltransferase (DGTT4) (Iwai et al. 2014), similar results were obtained in *Nannochloropsis* sp. NIES-2145 when cultivated under P starvation (Iwai et al. 2015). High light and sodium acetate inducible promoter of β-carotene ketolase gene (*bkt*) from *H. pluvialis* is functional in *C. reinhardtii*, even though such gene was used to drive the expression of bleomycin resistance protein (Wang et al. 2016a). Together with the untested stress-responsive genes described above (i.e., *Dplip* and *PSR1*), expanded library of inducible promoters are expected to be used for high-value chemical production.

Recent studies have demonstrated that transcriptional engineering of metabolic pathways can also improve the lipid production in microalgae. For example, overexpression of a stress-responsive transcription factor (TF) encoding gene *NsbHLH2* (basic helix-loop-helix TF from *Nannochloropsis*) improved the content of fatty acid methyl esters (FAME) more than 30% in *N. salina* CCMP1776 under

nitrogen-limited or nitrogen-unlimited condition, as well as the growth rate and nutrient uptake (Kang et al. 2015). Overexpression of PSR1 (Pi starvation response1) increased TAG content without compromising cell growth in *C. reinhardtii* (Ngan et al. 2015), while boosted starch accumulation with reduced neutral lipid content was obtained in *C. reinhardtii* CC125 (Bajhaiya et al. 2016b). Another case also showed that mutating CHT7 (Compromised Hydrolysis of Triacylglycerols 7), a TF functionally switching between quiescence (during starvation) and proliferation (during nutrient repletion), promoted nutrient-starvation-induced TAG accumulation without limiting cell growth in *C. reinhardtii* (Tsai et al. 2014). Updating studies have discovered several stress-responsive TFs involved in lipids and carotenoids production, such as MYB (Gao et al. 2015), PHR1 (Rubio et al. 2001), RGQ1 (Matthijs et al. 2016), and WRKY (Liang and Jiang 2017). Genetic engineering of these TFs, as well as being identified other TFs, is a viable methodology to manipulate the overproduction of specific valuable metabolites.

Microalgae cultivated under stressful conditions will over-accumulate reactive oxygen species (ROS), thus hampering the cell growth and membrane fluidity, while deoxidization via superoxide dismutase (SOD) and catalase (CAT) contributes to improved lipid production (Chokshi et al. 2017), indicating that genetically enhancing the contents of antioxidants is an alternative way to improve the microalgal cell vitality and subsequently to increase the productivity of desired compounds.

More successful reports may emerge about genetically engineering stress-responsive genes and TFs for desired purposes based on the increasing knowledge of metabolic regulation in microalgae. The CRISPR/Cas9 system, a precise genome editing methodology, has been demonstrated functionally in the model microalgae (Naduthodi et al. 2018), e.g., *C. reinhardtii*, *Nannochloropsis* sp., and *P. tricornutum*, and offers many possibilities for microalgae-based biochemical production. However, it is of great importance to point out that stress properties, species diversity, biosafety of transgenic elements, and stability of mutants must be taken into consideration while engineering the metabolic pathway for improved performance. Further studies are required to expand TFs and CRISPR/Cas9 engineering in different lineages of microalgae other than model microalgae and in different catalogs of products other than lipid.

4.3 Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE) has been widely used for developing novel biological and phenotypic function in robust strain construction, and it is commonly performed by sequential serial passages or chemostat to achieve increased fitness and mutations, including single nucleotide polymorphisms and fragment insertions and deletions (Dragosits and Mattanovich 2013). Nutrients and environmental stresses are popular in the ALE for microalgae strain development. For instance, evolved *Chlorella* sp. AE10, obtained after 31 cycles (97 days) of the ALE under 10% CO_2, exhibited maximal biomass concentration at 3.7 g L^{-1}, 2.9 times higher

than control, when cultivated under 30% CO_2, and cells tended to alter carbon flux to carbohydrate rather than lipid (Li et al. 2015). Similar observation was obtained in *C. vulgaris* via the ALE under light stress (255 μE m^{-2} s^{-1}) (Fu et al. 2012). The ALE under 170 μE m^{-2} s^{-1} light stress yielded an evolved *D. salina* strain with doubled content of β-carotene, hitchhiking the increased accumulation of chlorophyll *b* (Fu et al. 2013). The model microalga *C. reinhardtii* exhibited increased lipid production via the ALE under 200 mM NaCl or 3–7% salts stresses, and further omics analysis had showed that the evolved strain exhibited downregulation of stress-responsive genes in transcription/translation, as well as inactive of starch-to-lipid biosynthesis shift (Kato et al. 2017; Perrineau et al. 2014). Interestingly, The ALE with sufficient nutrient also generated a robust *C. reinhardtii* strain with double increment in lipid content, and slight growth impairment was observed under nitrogen starvation stress (Yu et al. 2013).

It should be pointed out that a typical ALE usually takes several weeks to several months; thus the most increased performance and the least trade-offs in other stressful conditions should come to the preference for robust microalgal strain development. Fortunately, results from nutrients, slats, and other stresses used for lipids and high-value molecule production indeed provide the guideline for the ALE operation due to fruitful stress-responsive information, and directly pre-evolution via genetic engineering of specific genes in microalgae will certainly shorten the ALE period. Therefore, successful application of genetic engineering and the ALE on more robust microalgae strain development with improved performance is expected. And stable microalgae strains with robust productivity and adaptability from anteriorly discussed schemes will advance the commercialization and industrialization of microalgae-based biofuel and other value-added bioactive production in large scale (Fig. 5.1).

5 Challenges and Perspectives

Numerous efforts on manipulating stresses and subsequent genetic engineering for improving the yield of desired compounds have expedited the microalgae-based bioproduction. However, there are still some challenges constraining its commercialization, such as culture growth conditions, relative low productivity, and limited market share (Chia et al. 2018). The cost of biofuel production alone from microalgae remains higher than traditional fuel sources, calling for integrated lipid production with high-value products (e.g., carotenoids and PUFAs), as well as bioremediation, to reduce the production costs. Also, the robust microalgae strains with high biomass and lipid/value-added product yields will certainly further compress the expense during industrialization. Nevertheless, prolonged stress condition will lower the biomass production, subsequently constraining the lipid productivity, while doubled biomass productivity contributes to nearly 50% off in biodiesel price (Nagarajan et al. 2013). A breakthrough on increasing lipid content without affecting the biomass production has been achieved, where genetically engineered

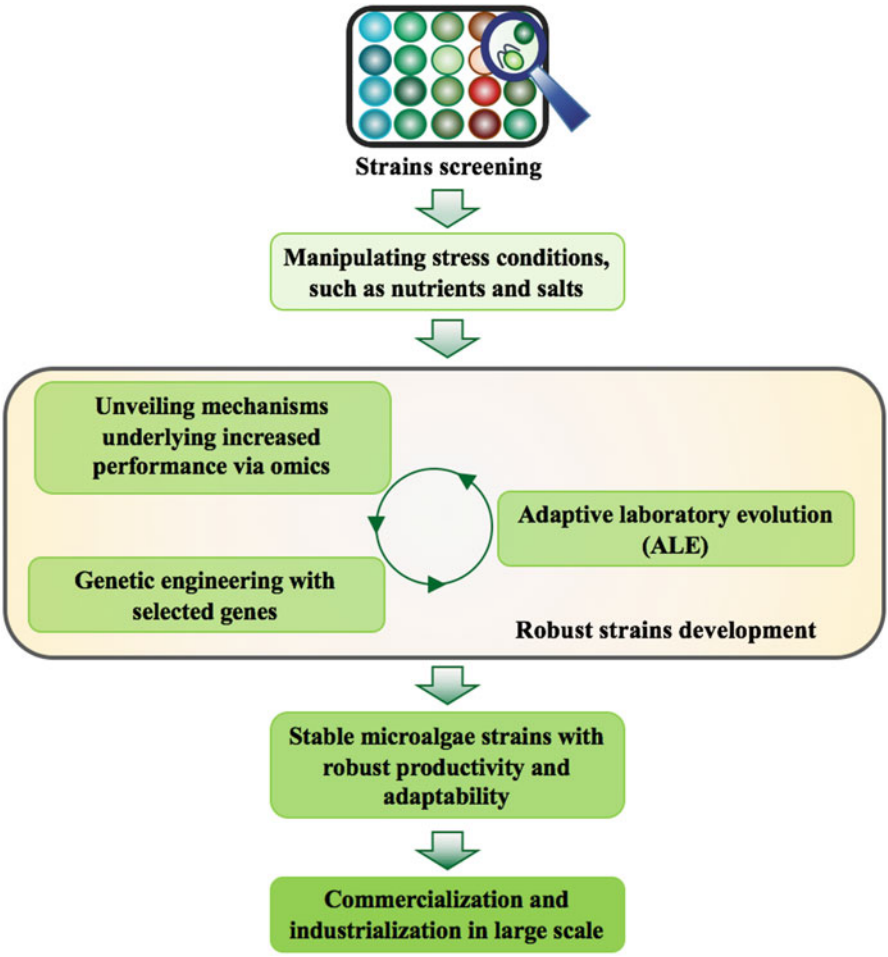

Fig. 5.1 Development of robust microalgae strains via omics technology based on manipulating stresses

T. pseudonana, a lipase/phospholipase/acyltransferase knockdown mutant, exhibits 3.2–4.1-fold higher lipid content (16–22 pg cell^{-1}) under silica starvation while the cell growth rate remains unaffected (Trentacoste et al. 2013). Parallelly, attenuation of $Zn(II)_2Cys_6$ expression, a transcriptional regulator, yields a *N. gaditana* strain producing twice lipid (~5.0 g m^{-2} day^{-1}) with little influence on cell growth (Ajjawi et al. 2017), similar to an evolved *C. reinhardtii* strain from the ALE (Yu et al. 2013). Therefore, it is possible to simply outwit the contradictory between compromised biomass production and increased lipid content under stressful conditions via genetic engineering. Also, more successful cases may embrace other microalgae species, expanding the strain pool for industrialization.

Genetic engineering of microalgae for enhanced biosynthesis of targeted compounds has been demonstrated in model microalgae, such as *C. reinhardtii* and *Nannochloropsis*, yet more models for each phylum are needed in order to provide meaningful guideline for corresponding groups. Further, species diversity and environmental niche-specific differences are veiling the interactions of microalgae with their environment, including the mode of stress sensing, signal transduction, and cross-talk among transduction pathways. Also, the insufficient utilization of microalgal biomass is challenging the economically viable coproduction of bioenergy, since the development of microalgae strains commonly focuses on a unilateral aspect or single targeted product, such as lipid production on nitrogen stress from one microalga. Therefore, comprehensive understanding of intra and inter cellular cross-talks under stressful conditions based on multi-omics technology is imperative for the development of robust microalgae strains with concomitant production of lipids and value-added bioactives. Despite axenic culture, mixture culture of different microalgae or microalgae with bacteria provides an alternative option to yield multi-products. Programed microalgae strains with the characteristics of easy harvest, autolysis and product secretory systems, and wastewater treatment will facilitate the commercialization of microalgae-based biorefinery. However, the challenge remains that whether the developed robust microalgae strains in the laboratory via genetic engineering and the ALE still exhibit superior performance in scale-up. Namely, it is urgent to evaluate the microalgae-based production in large scale with the robust and stable stains developed based on aforementioned strategies.

6 Conclusion

This chapter provides summary of current progresses of lipid and value-added compound production in microalgae via manipulating various stresses (i.e., nutrients and salts), and microalgal strain construction through genetic engineering and the ALE is emphasized. Advances in omics offer plentiful possibilities in robust microalgal strain development via genetic engineering of stress-responsive genes and the ALE. Stable microalgal strains with superior productivity of multilateral high-value products and robust adaptability will accelerate the commercialization of microalgae-based bioproduction of fuels and bioactive compounds.

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Chapter 6
Somatic Hybridization for Microalgae Domestication

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Abstract Microalgae biotechnology represents a new era for investigation in the human future concerning bioenergy, food, and environment relief. This emerging technology is still under investigation to suit the expected beneficial outcomes conditional to selection of high-yielding strains through domestication as major food crops we know today that are best exemplified by the long selective breeding walk from teosinte to modern high-yielding seed corn. Somatic hybridization has a potential to generate thousands of new combinations of genetic variations and new contexts for gene expression, thanks to its ability to overcome the incompatibility barriers between unrelated species and its nature to allow both nuclei and cytoplasmic genome exchanges. Application of somatic hybridization to microalgae biotechnology would certainly revive this old forgotten approach, biased by modern recombinant DNA technology, as a natural and powerful mean for asexual breeding.

1 Introduction

Microalgae industry offers good opportunities for researchers, investors, and industrials to develop the skills necessary to meet the strong demand concerning bioenergy, food, and environmental remediation processes which may play a major role in the global socioeconomical enhancement in the near horizon.

Indeed, the microalgae biomass exploitation has not yet reached to date sufficient levels of technological maturity, called "emerging technology" for biomass production and processing. The challenge depends on the cost-effectiveness of the whole process (Alam et al. 2017; Chisti 2013). Even though each step needs to be developed the way that will contribute in cost reduction, the production of robust microalgae owing good rates of proliferation with acceptable level of desired

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biomolecules could represent the founding block for the feasibility of this green sustainable industry. Today, the major microalgae investigation is based on main strategies including the selection processes through simple screening of wild types for desired traits and the improvement of target traits through synthetic biology or conventional breeding. However, drawbacks and limitations of these strategies are not scarce and may impede the whole process through the following: (i) selected wild-type strains for desired traits cannot reach the required industrial expectations; (ii) synthetic biology approaches for metabolic pathway engineering are more exploited today, but, besides their expensiveness, they are sometimes controversial which may limit the acceptance of modified strains across global opinion, especially if they are designed for direct use such as food and feed; and finally (iii) conventional breeding may suffer from incompatibility between species, preventing the release of the desired hybrids as in plants.

Microalgae domestication through natural breeding represents the context for which global opinion is favorable. More precisely, the approach can attract major stakeholders who are keen on the development of natural and green sustainable bio-products and environmental bioprocesses, in a cost-effective way, that are more appreciated today. The prevailing way for microalgae proliferation is asexual reproduction, but it can reproduce sexually under unfavorable growth conditions which make possible breeding between different species. Nevertheless, for the reasons listed above, sexual incompatibility and infertility problems may raise which may impede the feasibility of desired traits crossing as in crop plants. To overcome these interspecific barriers, somatic hybridization represents an alternative and attractive tool for breeding of distantly related species and genera where conventional methods of genetic manipulations are not applicable by transferring massive assortment of genetic information (Eeckhaut et al. 2013; Grosser et al. 2000; Johnson and Veilleux 2001; Liu et al. 2005; Tiwari et al. 2018; Waara and Glimelius 1995). During the last century, the technique of somatic hybridization, called also protoplast technology, became a popular method for the introduction of novel traits in commercial crops (Brown and Thorpe 1995), but limited advancement have been achieved in macroalgae strain development (Table 6.1). The principle is based on the potency

Table 6.1 Examples of somatic hybridization successfully performed in various microalgae species

Microalgae strain	Identified traits and fusion events	References
Chlamydomonas reinhardtii	Mutants with new osmoregulation features	Matagne et al. (1979)
Dunaliella spp. and *Porphyridium cruentum*	Salinity tolerance and antibiotic resistance	Lee and Tan (1988)
Porphyridium sp., *red* microalgae	Phycoerythrin complementation	Sivan et al. (1995)
	Herbicide resistance	Sigeno and Arad (1998)
Ochromonas danica and *Haematococcus pluvialis*	Successful recombination events between the two species	Abomohra et al. (2016)

of protoplasts that can be released from cell walls using appropriate enzymatic digestion technologies in the presence of osmotic stabilizer. These protoplasts can fuse together in appropriate media giving rise to fusion progenies called somatic hybrids that can be classified into roughly three types (Guo et al. 2004b; Singh et al. 2015). These include (i) symmetric somatic hybrids which represent a combination of nuclear and cytoplasmic genomes, i.e., chloroplast and mitochondria genomes of both parents; (ii) asymmetric somatic hybrids which consist of transferring parts or whole nuclear genome from the donor parent to the intact genome of the recipient parent; and finally (iii) cytoplasmic hybrids, called also cybrids, that harbor only one parental nuclear genome and either the cytoplasmic genome of the nonnuclear parent or a combination of both parental cytoplasms.

Somatic hybrids are very attractive for natural crop improvements because after adding one or more traits through somatic hybridization, the cultivar integrity is maintained. This is reminiscent of genetic transformation but without artificial gene manipulation and allows the transfer of multiple uncloned genes. Furthermore, the technology is very cost-effective, and the generated crops are not subjected to the same legal as transgenic lines (Grosser and Gmitter 2011). The cybrids are novel germplasms representing a novel nucleocytoplasmic combination with a mixture of DNA-containing organelles (chloroplast, nucleus, and mitochondria) from both fusion parents, permitting the broadening of cytoplasmic diversity within crops. It has been extensively exploited for crop improvement including plants and seaweeds (Gleba and Sytnik 1984; Reedy et al. 1994).

In plants many important traits have been successfully improved through somatic hybridization including the enhancement of high-valued secondary metabolites (Jiang et al. 2012; Wang et al. 2011; Yu et al. 2012; Zhang et al. 2011), abiotic and biotic stress resistance (Xiang et al. 2004; Xiao et al. 2009; Sigeno et al. 2009; Taski-Ajdukovic et al. 2006), and herbicide and antibiotic resistance (Yemets et al. 2000; Xia et al. 1992; Jourdan et al. 1989; Gleba and Shlumukov 1990; Glimelius et al. 1991; Galun 1995). Overall, somatic hybridization has evolved the CMS-based hybrid seed technology which represented a major contribution to world food supply by maintaining hybrids and heterosis in the main crops including rice, sorghum, canola, petunia, sunflower, bean, and sugar beet with more than 50% increase since the last century (Chen and Liu 2014; Islam et al. 2015; Bohra et al. 2016). In nature, evidences have been reported for spontaneous occurrence of somatic hybridization such as in fungi. Many somatic hybrids have been identified on the basis of pathogenicity and virulence characters transferred by hyphal fusion among genetically different individuals (Park and Wellings 2011).

The fact that this dormant biotechnology long time slowed down far behind modern recombinant DNA technology did not halt arguing its potentialities as a reliable tool for gene transfer (Holmes 2018). Besides its proofs for genetic transfer in various macro- and microorganisms, somatic hybridization is natural and environmentally responsible, a context where microalgae should be exploited because of their nature as aquatic species. It would represent a valuable strategy for cellular and genetic manipulation of microalgae to achieve commercial phases with promising agronomic value.

2 Context of Somatic Hybridization for Microalgae

The application of somatic hybridization in microalgae is scarce. It lags far behind that of conventional agricultural crops because the exploitation of this micro-crop for valuable economic traits is new. The challenge requires the production of viable protoplasts, capable to merge into stable fusion products.

Early studies evidenced the feasibility of protoplast production, fusion, and hybridization in the unicellular *Chlamydomonas* cell wall mutants (Matagne et al. 1979). Thereafter, the protoplasts of the halophytic green algae *Dunaliella* and *Porphyridium* species were successfully released and fused together using polyethylene glycol treatment (Jourdan et al. 1989; Lee and Tan 1988). The regenerated fusion progenies on selection media showed variability, salinity tolerance, and antibiotic resistance, evidencing genetic transfer between species. This feasibility of somatic hybrid production was further extended to red microalgae exploited for their economic potential (Sivan et al. 1995) with successful transfer of herbicide-resistant trait into stable progeny, doubly resistant to siulfometuron and diuron (Sigeno and Arad 1998).

The limitation that may arose for the applicability of this technology concerns the release of viable protoplasts from microalgae. The standard protocols available for digestion of cell walls are well documented in plant cells which represent the basis for protoplast releases. Microalgae cell walls contain the main components as found in plant cell wall including cellulose, hemicelluloses, and pectin. However, complexity in this solid component may arise and possibly will require preliminary analysis of the cell wall constituents in order to target appropriate degrading enzymes. Strategies for microalgae cell wall degradation can also take advantage from many available methods used for microalgae cell wall hydrolysis to extract fermentable sugars and to weaken the cells for the improvement of oil extractions (Fu et al. 2010; Rodrigues and da Silva Bon 2011).

To be optimistic, the feasibility of protoplast release and fusion has been reported to succeed in wide ranges of unrelated genera, species, and tough organs with intense complex cell wall such as pollen (Bhojwani and Cocking 1972), bacteria, and fungi that were exploited commercially for somatic hybrid production (Ferenczy et al. 1974; Gokhale et al. 1993; Wei et al. 2001). As examples, in fungi, interspecific protoplasting and fusion into hybrids have been achieved between auxotrophic mutants of *Aspergillus* species (Kevei & Peberdy, 1977). Protoplasting technology included the improvement of the beta-lactam antibiotics of hybrids issued from strains of *Acremonium* species (Peberdy, 1989) and the significant hypervirulent transfer in the entomopathogenic fungi *Beauveria* species (Viaud et al. 1998). Recently, initiatives of harnessing the feasibility of microalgal cell wall digestion and protoplast fusion start to emerge and put on the track as exemplified by somatic hybridization of unrelated microalgae, Chlorophyceae, and Chrysophyceae species (Abomohra et al. 2016), promising new era of microalgae domestication for new outcome potentials.

3 Methodology

Somatic hybridization and cybridization through protoplast fusion provides a process of combining diverse genomes of different genera and species through intra- and interspecific genome combination. Methods for protoplast isolation and fusion are similar to those documented in plants which represent the basic protocol that perhaps should be optimized for microalgae as it was performed for fungi and other microorganisms.

3.1 Viable and Totipotent Protoplast Obtention

Totipotency is the ability of a single cell to divide and proliferate indefinitely in appropriate media. In higher organisms, the totipotent cells possess all potentials to differentiate into a new identical organism which represent the basis of asexual reproduction in plants and related organisms (Fig. 6.1). For microalgae, the first step and conditions for somatic hybridization necessitate the production of viable and totipotent protoplasts. To meet these essential features, the physiological status of the cells prior to protoplasting must be critical, depending on the composition of media and growth status (Fig. 6.2). Harvesting cells from cultures supplemented with glycine and at the exponential phase of growth gave the best quality of the protoplasts as shown in many microorganisms (Okanishi et al. 1974; Shahin 1972). These conditions permit to meet large number of cells in good shape and viability.

The release of protoplasts from the cell wall involves many procedures including mechanical disruption which is not applicable for microalgae and the universal enzymatic digestion and puncturing that remain the best methods applicable for a wide range of macro- and microorganisms including fungi and bacteria. The digestion media must contain an osmotic stabilizer such as sucrose, sorbitol, or mannitol in appropriate concentration, generally 0.3 molar, to prevent protoplast membranes from rupturing upon release from cell walls. It was well documented that the addition of calcium chloride in the isolation media increases the protoplast stability and improves plasma membrane activity (Rose 1980).

In this new context of microalgae, the ability of successful viable protoplast release should involve at first stage basic enzymes or combinations that were found effective for other organisms such as pectinases and cellulases in plants, zymolyases in yeast, snail enzymes in *Neurospora crassa*, and related commercial cocktails. These should be manipulated empirically for their optimal activities with respect to enzyme concentrations, ionic strength of the medium, and time and temperature of incubation, depending on microalgae species. In some instances, treatment with lysozyme or related lytic enzymes and divalent cation chelating agents such as EDTA would be necessary to protoplast a wide range of species as

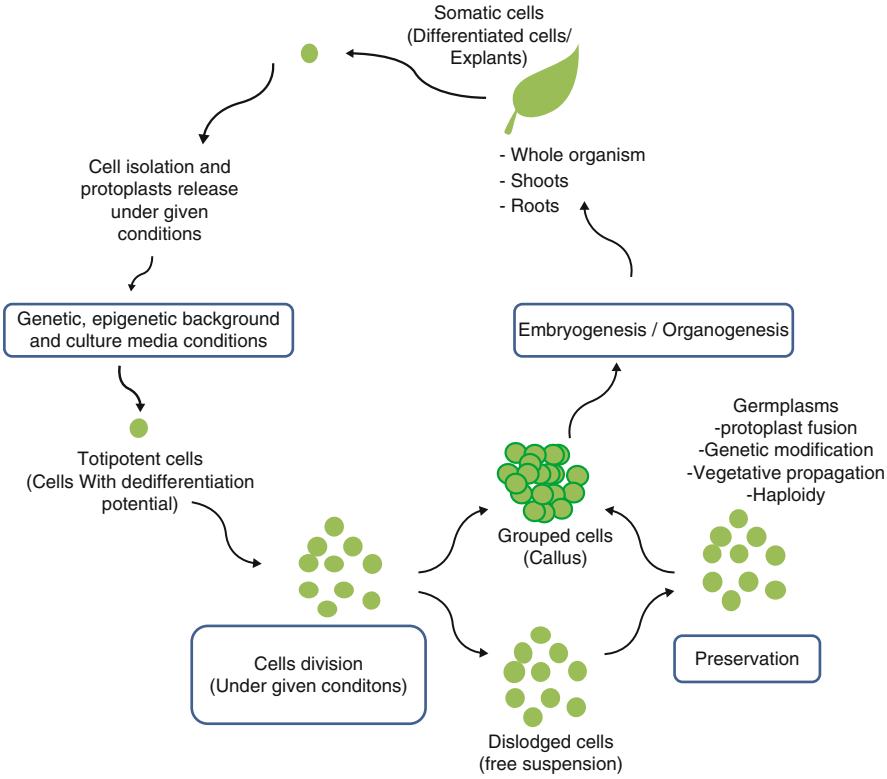

Fig. 6.1 Totipotency of somatic cells in green organisms. These cells have the potential to dedifferentiate and proliferate to give rise to specified organs or whole organism depending to their genetic background and the composition of culture media. They can also be maintained as germplasm for further traits improvement

reported in bacteria (Peberdy 1980). In case of non-digested cells, investigation of the cell wall composition would be required by means of biochemical analyses of its components prior to digestion in order to design specific conditions of digesting enzymes. However, numerous cell walls digesting enzyme cocktails are available commercially which suit various cases.

After digestion, protoplasts must be isolated from debris and enzyme media by subsequent steps of filtration through nylon mesh of 45–100 μ pore size and several wash cycles at low rate centrifugation (600 g, 5 min) in resuspension hypertonic media. The washed protoplasts must be purified afterward by centrifugation through continuous or discontinuous density gradients of sucrose, Ficoll, or Percoll where conditions are to be tested and optimized. This step is necessary since it permits to discriminate viable and dead protoplasts in terms of their differential density that is often not similar.

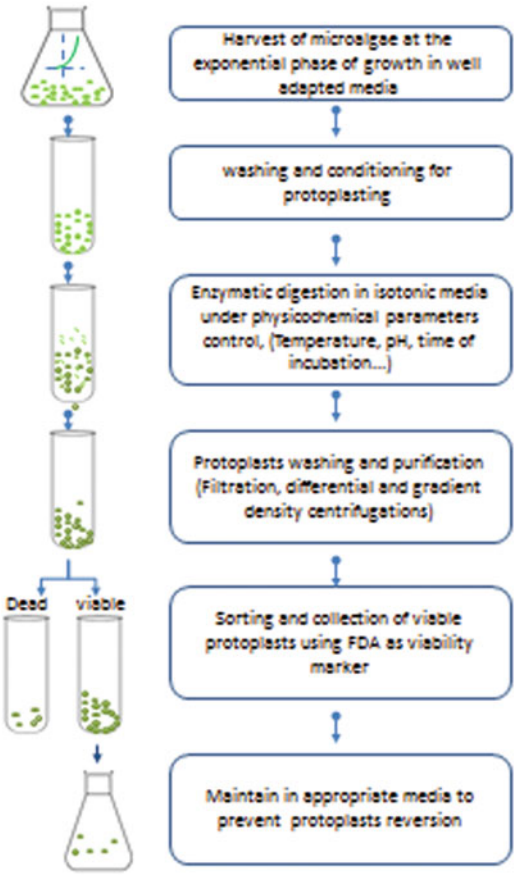

Fig. 6.2 Representative diagram of viable protoplast production through basic process involving enzyme digestions and protoplast purification

After these numerous steps of the process which must be quick, a non-negligible percentage of released protoplasts may be altered, injured, or completely dead; a viability test is therefore required to narrow the best fit for the subsequent steps of the process. Many approaches are available for protoplast viability assessment including fluorescent dyes. Aliquots of protoplasts are mixed with appropriate dye such as the popular fluorescein diacetate (FDA) to a low final concentration, i.e., 0.001% (w:v), and analyzed under epiflurescence microscopy. FDA molecule is not a fluorescent form; it diffuses into the cytoplasm where it will be hydrolyzed by esterase to release the fluorescein moiety which emits fluorescence. Dead protoplasts do not have this metabolic activity, and therefore only viable protoplasts will emit fluorescence, and the injured or dead protoplasts are invisible. Percentage of viable protoplasts is therefore obtained which will make the basis for technical optimizations. Discrimination of dead and viable protoplast could also be discriminated by flow cytometry equipped with cell sorter. The recovered viable protoplasts are tightly monitored and inspected to limit reversion to native form, i.e., regeneration of cell wall prior to fusion.

3.2 *Protoplast Fusion*

The methods for protoplast fusion should be chosen and optimized according to the selected species taking advantages from techniques performed on plants and micro-organisms such as fungi, yeast, and bacteria. Basically, after preparation of viable protoplasts from both parents, according to the procedures listed above, they are mixed in equal ratio (1:1) at a concentration of 10^6 cells per milliliter and neutralized to bring cells together before treatment with fusogen (Fig. 6.3). Initially, free protoplasts carry net negative charge causing cells to repel in solution. Several methods now exist for the fusion of protoplasts, but the two commonly used procedures are the chemo-fusion and electro-fusion techniques (Tomar and Dantu 2010). Chemo-fusion permits the protoplasts to adhere to each other, followed by fusion using

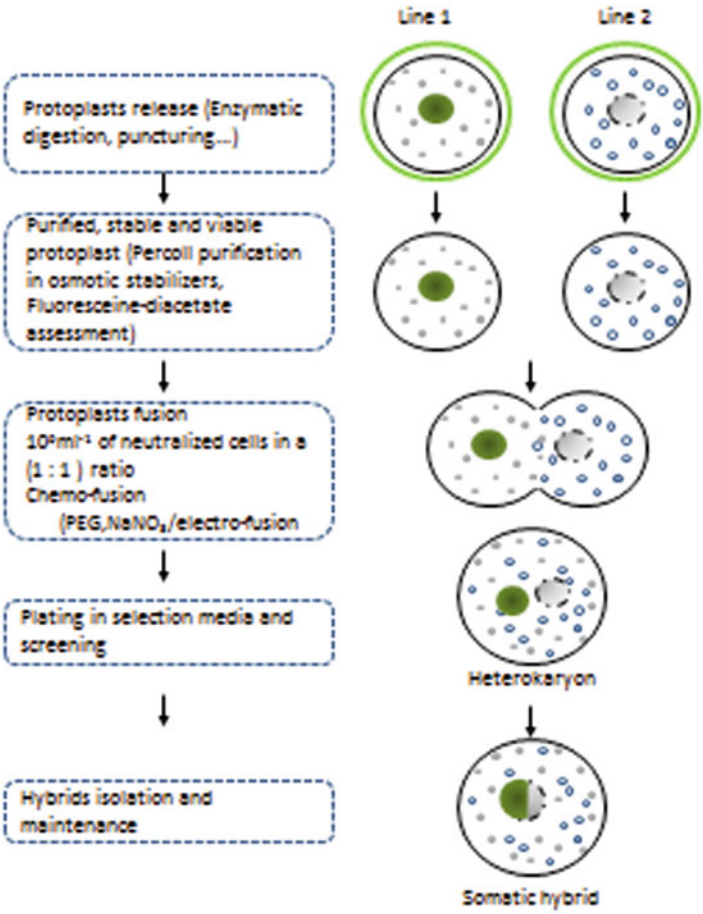

Fig. 6.3 Schematic representation of protoplast fusion and generation of somatic hybrid cell combining both nuclei and cytoplasm hereditary materials of both cell lines 1 and 2

chemicals. The electro-fusion is considered as more efficient and simple with less physical damage to the fused products. The chemo-fusion of protoplast could be assayed either by sodium nitrate ($NaNO_3$) or polyethylene-glycol (PEG) as universal fusogens. Technically, the isolated protoplasts are suspended and incubated in a fusogen solution containing 10% sucrose for 1 h. In general, 1 ml of the protoplast suspension is mixed to 1 ml of 56% PEG solution with mild shake for 5 min. The treated protoplasts are left to decant for 10 min and washed twice in the isolation medium, and the fused products are plated under selection media with respect to controls.

In the case of electro-fusion, the mixed protoplasts are subjected to mild electric stimulation (10 Kvm^{-1}). This leads to pearl-chain arrangement of protoplasts. Subsequent application of high strength of electric fields (100 kvm^{-1}) for some microseconds results in electric breakdown of membrane followed by fusion (Jogdand 2001).

To monitor the fusion efficiency, dual fluorescence labeling technique can be utilized. The donor parent and the recipient parent are labeled with rhodamine and fluorescein, respectively, prior to fusion (Durieu and Ochatt 2000; Pati et al. 2008). The fusion products will reveal a merge between the two dyes that could be visible under the epifluorescence microscope. The percentage of fusion products can be determined.

3.3 Protoplast Genome Manipulation for Cybrid Generation

Somatic hybrids can be obtained by direct protoplasts with integrated genomes; however, for cybrid generation, either nuclear or cytoplasmic genomes are inhibited prior to fusion.

Cybrids or cytoplasmic hybrids consist of a combination of two cytoplasms with one parental nucleus or the transfer of nuclei to a new cytoplasmic context for gene expression of unrelated species. In this case, utile genes can be either downregulated or overexpressed in their new environment, permitting the improvement of some useful traits. Cybrids permit the transfer of genes that are inherited maternally only, such as in CMS phenotype, through mitochondrial genome or conferring herbicide resistance through chloroplast genome (Glimelius et al. 1991).

Technically, the inhibition of nuclear genome can be fulfilled partially or completely by total enucleating (Fig. 6.4a). In this case, protoplasts undergoing this treatment are cytoplasm donor parents which consist of cytoplasts containing only mitochondrial and chloroplast genomes. Nuclear DNA inhibition can occur by several methods including X, gamma, and UV irradiation. The inactivation dosages should be monitored depending on the species, quality of radiations, and culture systems.

Alternatively, ultracentrifugation, 20,000–40,000 *g* for 45–90 min in a Percoll/mannitol gradient (Lörz et al. 2006), and cell permeation by the mycotoxin cytochalasin B treatment (Wallin et al. 1978) are also potential means for nuclei extrusion from intact cells.

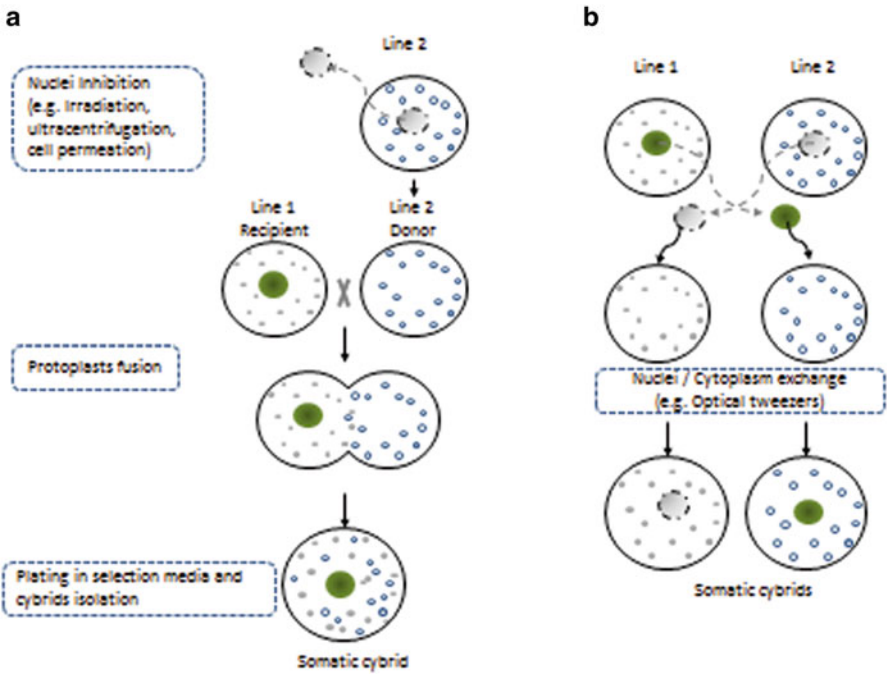

Fig. 6.4 Production of somatic cybrids via removal of nucleus through ultracentrifugation. Irradiation and cell permeation to produce donor lines. These cytoplasts are ready to fuse with recipient line which possess the nuclear background and produce somatic cybrids of combined cytoplasms (**a**). Cybrids can also be produced through nuclei exchange with conservation of one-type cytoplasm (**b**)

On the other hand, protoplasts where only cytoplasmic DNA is inhibited but contain intact nuclear genome are called recipient parents. The inhibition of cytoplasmic DNA involves chemicals such iodoacetate (Varotto et al. 2001). When fused with donor parents, the fusion product is a cybrid.

Alternatively, the exchange and transfer of nuclei from one species cytoplasm to another could be performed by laser microbeam and optical tweezer approach for micromanipulation of the cells (Fig. 6.4b) for cybrid production. This has proven its applicability for plant cells and wide range of organism (Greulich et al. 2000).

3.4 Identification and Selection of Somatic Hybrids

Successful protoplasting and fusion into stable somatic hybrids must be low as reported in many plant species (Glimelius et al. 1991) due to the lack of optimizations and standardized procedures for wide range of species. Basically, selection of desired somatic hybrids for particular characters employed many strategies of both

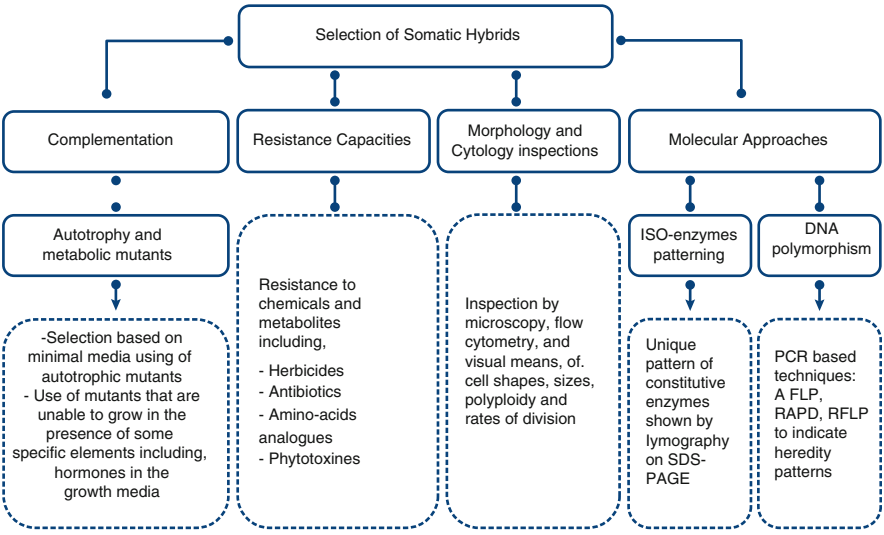

Fig. 6.5 Selection procedures of somatic hybrids at the level of molecular, physiological, and morphological basis to underline successful transfer of specific features evidencing hybridization in the fused products

significant and limited applicability which have been developed over the era of somatic hybridization technology (Fig. 6.5). These include:

Selection by complementation:

Auxotrophic complementation and resistance capacity as selectable marker, i.e., resistance to chemicals such as herbicides, antibiotics, amino-acid analogs, and concanavalin or concentration thresholds of various active organic compounds for metabolism regulation and growth, such as sucrose and hormones, would be the easiest way for hybrid selection. This is conditional upon the situation where only the fused products carry the necessary response properties required for survival on selective media due to genetic combination of the two parental protoplasts (Guo et al. 2004a; Schieder 1982). However, this strategy may be limited to the obtaining and availability of strains with natural desired dual auxotrophic characters linked to the targeted recombination traits of significant values. In this, context, introduction of selective marker gene by recombinant technology would be applicable where it is required.

Selection by morphological, cytological, and metabolic inspection:

To overcome the introduction of synthetic biology strategies, methods for the characterization of fusion progenies in terms of their morphological and physiological traits should be developed. These include rates of cell division, size of the cells, and metabolite contents in comparison to wild-type strains.

The morphology comparison is an attractive means to select the somatic hybrids in microalgae. In plants, this procedure is time-consuming because it needs the regeneration of the whole vegetative and reproductive tissues before comparison with the parental lines (Sundberg and Glimelius 1986; Waara et al. 1989). Microalgae are unicellular, and vigor of somatic hybrid cells including rate of division can be evidenced at earlier stages during regeneration and can be isolated mechanically. Flow cytometry mean equipped with suitable sorter can be efficiently used for these purposes including differential dye staining where the merge of dual staining is a characteristic of fusion products and chromosomes in case of the rise of polyploidy in the fusion progenies.

Selection by molecular analyses:

The molecular and biochemical techniques commonly used are well documented and used successfully, but the choice is based meanly on the experimental time required and costs. Somatic hybrids can be selected on their isoenzyme patterning (Gleba et al. 1984), sequence repeats analysis, and other markers that have been proven to be efficient methods for molecular polymorphism discrimination using modern PCR techniques for fingerprint DNA amplification. These include AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), and RFLP (restriction fragment length polymorphism) for DNA markers patterning. Stable progenies are defined, and their genome is mapped to identify the recombination events (Bauer-Weston et al. 1993; Hansen and Earle 1997; Sakomoto and Taguchi 1991; Xia et al. 2003; Zubko et al. 2002).

3.5 Culture and Propagation of Somatic Hybrid Cells

The isolated hybrids, selected against the desired economical values, should be maintained and cultured at low scale in close systems or bioreactors with regard to most favorable protocols defined for growth media and culture setting. These include the tight control of the major biological and physicochemical parameters such as pH, salt concentration, light intensities, and nutrients cocktails designed for optimal hybrid proliferation and preservation.

Cells optimized and tested in the laboratory do not behave in the same way when they are grown in commercial facilities such as wastewater treatment stations which operate mostly in open air (Sheehan et al. 1998).

For mass cultivation of microalgae in outdoor conditions, it is important to consider the robustness of the isolated hybrids in terms of their stability and consistency in response to the fluctuation of the prevailing environmental conditions and resistance against predators and contamination by local species.

In this context, the selected hybrids against the desired characteristics should be systematically tested at small-scale systems for their behavior in outdoor conditions

to determine the feasibility of their application such as wastewater treatments in local environmental conditions and to validate the selected candidate species. Outdoor conditions are also confronted to contaminants and predators which represent the major drawback of microalgae culture in outdoor conditions; however this issue could be easily monitored using a system based on the composition of the culture medium including the excess of ions and nutrient deficiencies to help hybrids to proliferate in a monoculture mode.

4 Optimization Issues

The feasibility of obtaining somatic hybrids used in breeding programs has been proven in plants for the transfer of useful genes and value characters between sexual incompatible species with generation of novel intergenic varieties. Beside gene transfer, the approach offers the possibility to combine nuclei into a novel cytoplasm context which may improve the expression of nuclear genes through the new interaction and cross talks between nuclear and cytoplasmic genomes.

In this young industry of microalgae exploitation, genetic improvement through somatic hybridization may raise several technical hurdles as in crop plants which require optimization to reach the final goals. Somatic hybrids production may be limited to few species, but the main challenge resides on viable protoplast isolation and increase in the percentage of fusion products and stability. Efficient methods for selection, identification, and isolation of fusion products through the process represent also another challenge due to the lack of standardized methods valid for all species. Furthermore, particular trait expression would not be reached certainly and may require time-consuming procedures to filter the desired character.

Indeed, all techniques used for the successful hybrids production through somatic hybridization are optimized in plant species, fungi, and bacteria. These basic techniques represent a start point to tackle the issues of this technology; therefore much efforts have to be employed to optimize the existent methods, developing new strategies for somatic hybridization in microalgae. Each step of the whole process should be optimized and adapted to single microalgae species. Several facets should be considered including (i) cell culture practices which involve media and the environments of cell growth, i.e., light intensity and period, temperatures, pH, agitation, and aeration. All these factors should be controlled individually not globally to fit the best endurance of cell quality suitable for digestion and fusion. Cell conditioning and pretreatment previous to cell wall digestion, protoplast release, and fusion may affect the resulting numbers of protoplast, their life span, their capability to fuse, and their stability of fusion products. The conditioning parameters could include periods of starvation, metal deficiency, cell density, dark regime, etc. In plants, pretreatment of leaf explants in the dark provided the best material for protoplast isolation and cell division (Sutiojono et al. 1998).

5 Outcomes for Social and Environmental Concerns

Microalgae biomass outcomes are numerous and well documented including the improvement of food, therapeutics, agriculture additive inputs, and water sanitation. All these matters are of global interest and top priorities for industrial actors to develop new strategies and economical sources to adequately reduce their growing deficit.

Proteins The world food consumption is expected to increase in the next few decades, and, especially, needs for proteins are actually outstripping supply due to many factors including global warming, continuous retreat of arable lands and desertification creeping due to lack of reasonable periods of precipitations, bad environmental management, and increase of population and income per capita. Large scale of microalgae farming represents the best opportunity for protein production which can be used directly for human food and also for livestock feed. In comparison to other vegetable sources of proteins such as soybeans, microalgae biomass cultures represent a valuable and sustainable source for proteins yet have a year-round harvest in well-established conditions without competition for arable lands and drinkable water. In these circumstances and societal practices, conveying the feedstock from improved algae will be very welcomed. Bringing this feed substitute into reality is not expected to imply high technologies other than select and breed robust algae strains for desired yields in a cost-effective manner.

Nutraceuticals Besides proteins, nutraceutical products such as omega-3 supplements of the long-chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are also promising outcomes of microalgae. These are essential for healthy metabolism in humans including retina protection, brain development, and heart disease preventions. This essential fatty acid is a perquisite element for healthier population. The main source of omega-3 is widely coming from fish oil. The latter raises many startling questions regarding its sustainability considering the fish stock depletion and the nutritional preferences in strict vegetarians not using omega-3 from fish oil. This reality urges for exploring new natural sources sustainable for the production of omega-3 or equivalent. Today, microalgae source is considered as the best candidate for its quality to contain DHA, and many companies are going successfully straightforward with commercial promising results. Microalgae exploitation therefore can represent an alternative pathway for the production of omega-3 with the same beneficial effects for human health as fish oil as suggested by several research studies (Bernstein et al. 2012). Currently, DHA from algae appear to fulfill the quasi-totality of health benefits as obtainable with omega-3s.

Therapeutics The genetically engineered therapeutics as biotechnological practices is mostly confronted to many hurdles to make them commercially viable. This is linked to the low yields and the expensive ways for their production. These hurdles are linked to many problems in the process of their production. These are

routinely produced in the prokaryotic bacteria and other eukaryotic cells including mammal and plant cells. The recombinant proteins are more often toxic for the host cells where they are produced. However, although in the case where they are not toxic, there reconstitution and folding into functional structure is always a challenging task. The folding of recombinant proteins and their restructuration into native forms necessitate well-established biochemical conditions which are not met in vitro. However, these stringent conditions could be met in foreign cells but the success is scarce. This pushes in some circumstances to use artificial cells for protein production and folding, but this way is not cost-effective. Recently, microalgae have been shown to be a good material that meets adequate cellular environment for producing therapeutic proteins in an inexpensive way (Tran et al. 2013). Therefore the exploitation of microalgae as host cells for heterologous expression of biomolecules, designed for human disease treatments, would be of great interest to insure their sustainability and cost effectiveness production.

Fertilizers Microalgae processing needs the cell to be alive for maintaining the good properties of the desired products including proteins, fats, and essential metabolites because cell death leads to the degradation of these compounds resulting in bad quality products. Maintaining cells alive during the process from harvesting to extraction could not be met sometimes and is a real challenge. In this case, algae biomass should be reemployed to other beneficial resources such as fertilizers as side products. The algal organic matter feeds microorganisms in the soil, improving its structure and fertility for plant growth. This is considered as organic fertilizer which increases also the capacity of water retention and nutrients for slow release to plant roots. Formulation of algae organic compounds with minerals would rise up good opportunities for fertilizer producers and related industries.

Environment Issues Microalgae cultivation represents a great potential for environmental preservation. They may allow the conversion of unwanted waste streams, like industrial effluents and agricultural runoff into biomass. Their ability to use nitrogenous and phosphorus residues, major pollutants in wastewater, represents therefore a convenient and cheap ingredient input to algal cultivation. This will allow an economical biomass production because nutrient and fertilizer costs are significant. In addition, microalgae also represent an unprecedented solution for the reduction of carbon dioxide emissions. This is a natural process of their capacity to fix ambient CO_2 and use it as sole source of carbon through photosynthesis bioprocess. Photosynthesis permits the conversion of sunlight energy into chemical energy and carbohydrates for lipid and metabolite biosynthesis in the presence of water, carbon dioxide, and minerals. Colocation of microalgae biomass producing stations and industrial facilities like sewage stations and CO_2-emitting sources such as power plants and cement factories will allow a wonderful synergistic solution that integrates microalgal feedstock production with industrial CO_2 mitigation and wastewater treatment that could be reused for other purposes such as agricultural sector.

6 Concluding Remarks

Even though somatic hybridization has contributed considerably to major agricultural crop improvement for various economical traits, with the potential of overcoming sexual incompatibility barriers, this technique sounds dumped during the last decades because of the emergence of synthetic technologies. Microalgae domestication initiatives could open new avenues to revive this natural approach for microalgae breeding programs.

As in plants and related kingdoms, the genetic recombination of microalgae species through intra- and interspecific somatic hybridization with the possibility of transfer of useful genes will certainly meet the success metric requirement for research dedicated to ecofriendly biomass exploitation for aquatic programs. The approach will lead to the development of hybrid varieties by widening their genetic base, with improved cell proliferation rates and high yield of target biomolecules. At large-scale exploitation, these hybrids of high biomass yielding will undoubtedly suit the release of bottleneck question for food and energy security demand as well as environmental remediation such as wastewater treatments and carbon mitigation. Thus, somatic hybridization has enormous potentialities and vision in future microalgae biotechnology for the exploitation and upgrading of species with high economic values. Working on a national and international partnership with a solid and multidisciplinary consortium will permit to jointly invest in a responsible future.

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Chapter 7
Towards the Genetic Manipulation of Microalgae to Improve the Carbon Dioxide Fixation and the Production of Biofuels: Present Status and Future Prospect

Encarnación Díaz-Santos

Abstract In recent years, interest in microalgae and its biotechnological use in the development of new technologies for the production of biofuels, high added-value compounds and the treatment of wastewater, among others, has increased considerably due to the fact that these microorganisms possess optimal growth properties, nutritional requirements and chemical composition making them a viable and natural alternative to the fossil fuels and the use of chemicals, which would reduce the greenhouse effect and the recent increment of the Earth average temperature. Nevertheless, in spite of all the optimal properties provided by the microalgae, the use of the genetic engineering, the synthetic biology and ultimately the manipulation of their genome is a challenge in development that would allow the improvement of microalgal strains, thus contributing to a greater efficiency and effectiveness in certain stages of the microalgae industry, as well as an enhancement in the yield and productivity, minimizing the economic costs derived from the total industrial process. Different approaches for the genetic manipulation of microalgae and its applications in biofuel production and carbon dioxide fixation, as well as its future implications for the biotechnological industry, are discussed and reviewed in the present chapter.

Keywords Microalgae · Biofuels · Carbon dioxide fixation · Productivity · Genetic manipulation

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1 Genetic Manipulation of Microalgae

1.1 General View

In the last years, the interest for microalgae and its biotechnological applications has considerably been increasing due to the fact that they are photosynthetic microorganisms, producers of a high variety of added-value compounds such as vitamins, carotenoids and secondary metabolites with antitumoral, anti-inflammatory, antifungal, antibacterial or antiviral properties (Michalak and Chojnacka 2015; Rasala and Mayfield 2015). Furthermore, microalgae are microorganisms, with high growth rates, capable of colonizing all the ecosystems on the Earth, using CO_2 as a source of carbon and solar energy, not competing with food or water resources for human life (Ahmad et al. 2011; Prasenjit et al. 2017; Thao et al. 2017). However, nowadays there are still certain barriers concerning the industrial process that have to be overcome so that microalgae can compete commercially with fossil fuel and other natural sources of bioactive compounds, such as plants or bacteria. The main limitations are found in the cultivation mechanisms and harvesting of microalgal biomass whose process cost increases considerably the market prices of the products from microalgae (Radakovits et al. 2010; Ng et al. 2017). In this aspect, the engineering of microalgae through their genetic manipulation, synthetic biology and metabolic engineering plays a significant role, and in consequence, new molecular tools are continuously emerging that would allow an improvement of this industry, to make it much more economically efficient. Despite of the new and powerful research advances, the genetic toolbox is still limited to a few microalgae species in comparison with the total of them. To date, since the development of the first genetic transformation techniques for *Chlamydomonas reinhardtii* in the 1980s (Boynton et al. 1988; Debuchy et al. 1989; Fernández et al. 1989), the most studied microalgae have been in addition to this, those with a special commercial interest belonging to the genus *Chlorella*, *Dunaliella*, *Nannochloropsis*, *Haematococcus* or *Scenedesmus* as well as some species of diatoms ; Walker et al. 2005; Radakovits et al. 2010). It is estimated that over 40 distinct species have been successfully genetically modified although with different efficiency and stability results (Gangl et al. 2015).

1.2 Strategies to Engineer Microalgae Genetically

Due to the wide range of different microalgae and their complex structural, chemical, physical, physiological and genomics properties, nowadays, a universal method for the genetic manipulation of all different microalgae with an important commercial interest has not been found yet. For this reason, many efforts are involved in the improvement of the traditional genetic toolbox, increasing mainly the efficiency and the stability of transgenes and their delivery into the microalgae cells. Moreover, the

more recent advances in the genome sequencing projects, the complete knowledge about nuclear, chloroplast and mitochondrial genomes and the possibility of targeting them, have greatly increased the development of new modern genome editing techniques. The most used mechanisms for the genetic manipulation of microalgae are briefly described in the following section.

1.2.1 Traditional Transformation Techniques

1.2.1.1 Glass Beads Agitation

It is considered the simplest and easiest method to deliver transgenes into the microalgae cells because any special or sophisticated equipment is not necessary. In that case, cells are exposed to the DNA of interest and agitated in presence of glass beads, normally of 0.5 mm, and the membrane fusion agent polyethylene glycol (PEG). It has been commonly used to introduce exogenous DNA into chloroplasts or for cell wall-less microalgae. The first report in microalgae was achieved in 1990 for the model microalgae *Chlamydomonas reinhardtii* (Kindle 1990).

1.2.1.2 Electroporation

In this transformation method, it is necessary to apply an electric field for the formation of transient pores in the cellular surface through which the entrance of transgenes is allowed. Although the first reports were found for cell wall-less mutants or protoplasts (Brown et al. 1991), more recently, this method has been successfully used in *Chlamydomonas* intact cells using a multi-pulses electroporation (Yamano et al. 2013) or in *Phaeodactylum tricornutum* non-deficient cell wall cells (Zhang and Hu 2014). Electroporation is considered one of the most efficient techniques of nuclear transformation although a specific instrumentation is required.

1.2.1.3 Biolistic

In biolistic system, the DNA is recovering gold or tungsten microparticles, and then, these are projected at high velocity into the cells using a microprojectile bombardment device. It is commonly achieved for the transformation of chloroplasts and intact cells due to the high penetration capacity of the particles surpassing the membrane and cell wall physical barriers. Although the necessary biolistic system is more expensive than others, the transformation efficiency rates are normally higher. This method was firstly reported for diatoms chloroplasts in 1996 by Apt and recently for *Dunaliella*, *Haematococcus* or *Tetraselmis* species (Purton et al. 2013; Doron et al. 2016).

1.2.1.4 Aminoclay Nanoparticle-Mediated Transformation

In this new and nontraditional transformation method, 3-aminopropyl-functionalized magnesium phyllosilicate nanoparticles (Mg-aminoclay) are used in a mix with the exogenous DNA for a nuclear transformation of microalgae intact cells. The size of the Mg-aminoclay nanoparticles, over 45 nm, is one of the most important advantages of this technique in comparison with the biolistic in which the gold or tungsten particles are around 1–2 um. To date, microalgae transformation using Mg-aminoclay nanoparticles has been reported for intact *Chlamydomonas reinhardtii* cells (Kim et al. 2014).

1.2.1.5 Agrobacterium tumefaciens-Mediated Transformation

It is a natural transfection in which a fragment of the Ti plasmid from *Agrobacterium tumefaciens* known as T-DNA is transferred into the microalgal cells and randomly integrated in the chromosomes. Usually described for plants, this nuclear transformation method was firstly reported in the marine red algae *Porphyra yeoenzis* (Cheney et al. 2001) and subsequently in other several microalgae such as *Chlamydomonas*, *Isochrysis* or *Haematococcus* species (Kumar et al. 2004; Pratheesh et al. 2014).

1.2.2 Genome Editing

In addition to the traditional transformation techniques for the genetic manipulation of microalgae, the new emerging technologies for editing genomes are starting to be used in microalgae in the current years. Genome editing uses recombinant nucleases engineered to recognize and cleave specific sequences in the genome, resulting in double-strand breaks which are repaired by homology-independent and error-prone DNA repair mechanism, called nonhomologous end joining, resulting in mutations at the cleavage site (Jeon et al. 2017). The different editing strategies include, among others, RNA interference, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, the most recent and popular, CRISPR/Cas9, clustered regularly interspaced palindromic sequences. These nucleases techniques could be an alternative to the traditional DNA delivery transformation methods in microalgae, improving the efficiency, the precision and the fidelity with which the microalgal DNA is modified. Although these new genetics technologies are still in progress and present some limitations and difficulties, especially in the reparation systems, the studies concerning the use in microalgae are more frequently reported during the last years. Most of them are focusing on the use of CRISPRs systems and were efficiently achieved for *Chlamydomonas reinhardtii*, the first microalgae in which CRISPR/Cas9 was successfully studied. Also, some reports are found for *Nannochloropsis oceanica*, *Nannochloropsis gaditana*, the marine diatom *Phaeodactylum tricornutum*, the prokaryotic microalgae *Synechococcus elongatus*

PCC7942, *Synechococcus elongatus* UTEX 2973, *Synechococcus* sp. PCC7002 and *Synechocystis* sp. PCC6803 (Ng et al. 2017). They are remarkably the studies in which genome editing using the CRISPR/Cas9 system is applied to enhance the lipid production in *C. reinhardtii* (Kao and Ng 2017) and to produce 1-butanol or ethylene in *Synechococcus* sp. (Johnson et al. 2016) and the TALEN strategy to increase the triacylglycerol accumulation in *P. tricornutum* (Daboussi et al. 2014).

1.3 Regulation of the Use of Genetically Modified Microalgae in Biorefinery

With the development of innovative technologies for the improvement of genetic manipulation systems in microalgae, as well as the arrival of new techniques, the use of genetically modified microalgae in the biofuels production industry, high added-value compounds and treatment of wastewater, has been growing, as well as the public and private economic contribution to develop an economically efficient and environmentally friendly industry. It is for this reason that the regulatory standards concerning genetically modified organisms (GMOs) and their potential risks, in particular for GM microalgae, are increasingly incorporated to the national and international biotechnological laws of the most developed countries. However, this biosafety legislation sometimes appears to be ambiguous and not defined depending on the territory spoken of. The main environmentally ecological risks for the use of the GM microalgae to produce biofuels or bioactive compounds are deviated mainly from their high-scale and open-pond cultivation, necessary to obtain large amounts of microalgae biomass. As it is well-described by Glass in 2015, the potential risks and impacts of genetically modified microorganisms (GMM) are considered due to serval factors: (a) the toxicity, infectivity or other risks inherent to the GMM itself or that might have been introduced by the genetic modifications, (b) the ability of the GMM to persist or become established in the environment, (c) the ability of the GMM to compete with or displace natural microflora at the release site, (d) the possibility that the GMM could spread or be dispersed from the release site and (e) the possibility that genes introduced into the GMM could themselves spread through horizontal gene transfer to be taken up by and expressed in different microbial species and more specific for GM algae, the possibility of these to form toxic and harmful algae blooms. Taking in account these concerns, the biosafety regulations start to include the effect deviated from the use of the GMMs and also their manufacturing and commercialization. In many industrialized countries around the world, from European Union, Canada, Australia or Japan, the biosafety standards follow the principles adopted in the international convection of Cartagena in 2000, "The Cartagena Protocol on Biosafety", in which living modified organisms (LMOs) are defined as any living organisms that possess a novel combination of genetic material obtained through the use of modern biotechnology, with "modern biotechnology" defined to include in vitro nucleic acid techniques as well as a "fusion of

cells beyond the taxonomic family" (Eggers and Mackenzie 2000; Glass 2015). In the USA, the Environment Protection Agency (EPA) and the US Department of Agriculture (USDA) are the governmental institutions which regulate the use of organisms for production of fuels or chemicals under the Toxic Substances Control Act (TSCA). On the contrary, in the European Union, although each member state has their own biosafety laws, the general regulations are determined by EU Directive 2001/18/EC on "Environmental Release" and EU "Contained Use" Directive 2009/41/EC (European Union 2001, 2009; Glass 2015).

Apart from the official governmental regulations, nowadays till exist some ecologically ethical barriers for the use and commercialization of transgenics organisms, mainly in the European Union, that restrict even more the research and development of the biodiesel industry using genetically modified microorganisms.

In the following sections, aspects related to the carbon dioxide fixation and the production of biofuels, using genetic engineering, are specifically detailed.

2 Metabolic Engineering: Carbon Dioxide Fixation and Biofuels Production

As was pointed in Sect. 1, the highly emerging of microalgae as potential cells factories, producers of an endless number of several bio-based chemicals, biofuels and bioactive compounds, has caused the increment of the studies about these photosynthetic microorganisms focused on the -omics disciplines, especially on genomics and ultimately on metabolic engineering through the DNA tools provided by the synthetic biology. Understanding the metabolic engineering as the alteration of the metabolic pathways of organisms, through the genetic modification of these either silencing or overexpressing genes intrinsic to the metabolic pathways or introducing new genes from other microorganisms in order to optimize and improve the production efficiency of the products resulting from their enzymatic reactions, their applicability to the optimization of photosynthetic reactions and lipid metabolism in microalgae, its discipline takes on a relevant importance.

2.1 CO_2 Fixation

The mitigation of CO_2 emissions, derived mainly from the use of fossil fuels and the incessant and growing industrial activity of recent years, has become one of the most important concerns of recent years as well as the subject of numerous environmental studies. In this sense, photosynthetic organisms, including phototrophic microalgae, play a key role due to their ability to use atmospheric CO_2 as source for the production of organic compounds, thus being a true eco-friendly and sustainable alternative for the bio-mitigation of these CO_2 emissions. Indeed, using microalgae

could couple the CO2 fixation to the biofuel production and wastewater treatment, highlighting the circular economy. These photosynthetic microorganisms use the Calvin cycle pathway, which take place in the chloroplasts, for the fixation of atmospheric CO_2. The Calvin cycle directs the inorganic carbon to the formation of carbohydrates precursors consuming energy stored in form of ATP and NADH, generated in the light-dependent phase, in a series of different metabolic steps, light-independent, including reactions of carboxylation, reduction and regeneration (Calvin and Benson 1948). The first enzyme involved in this enzymatic reaction cycle is the ribulose 1,5-bisphosphate carboxylase/oxygenase, RuBisCo, which incorporates the molecules of CO2 to the Calvin cycle catalysing the carboxylation of the ribulose 1,5-biphosphate to 3-phosphoglyceraldehido. The RuBisCo is considered the key enzyme of this cycle for the first position occupied, and hence, many researches have been focused on that for optimizing the photosynthetic global flux through their catalytic activity. Some approaches have been proposed concerning the genetic improvement of the selectivity and velocity of the RuBisCo or even the heterologously overexpression of some more efficient RuBisCo from different organisms as well as assembling the best of them (Ng et al. 2017). Nonetheless, in both, microalgae and plants and concerning the metabolic engineering, the studies successfully achieved are very limited (Yang et al. 2017; Raines 2011). On the other hand, there are some reports in which authors improve the fixation carbon rate engineering alternative enzymes as the aldolases. These enzymes are found in a central part of the Calvin cycle pathway catalysing the reversible conversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate to fructose 1,6-bisphosphate (FBP) and the reaction of DHAP and erythrose 4-phosphate to sedoheptulose 1,7-bisphosphate. Yang and co-workers (2017) introduced a cyanobacterial 1,6-biphosphate aldolase into the chloroplasts of *Chlorella vulgaris* improving in 1.2-fold the photosynthetic efficiency. Additionally, an increment of 41% for the carbon fixation rate has been reported for the cyanobacteria *Synechococcus elongatus* PCC7942, genetically modifying an enzyme involved in the accumulation of carbon pathway, the carbonic anhydrase (Chen et al. 2012). Other efforts are directed to modify the photorespiratory metabolism rerouting the phosphoglycolate to enhance thus the carbon fixation (Kebeish et al. 2007). The general control of the metabolic carbon flux into the microalgae cells improving the efficiency of the photosynthetic metabolic pathways could be directed to the accumulation of lipids and, hence, to the biofuels production.

2.2 Biofuels Production

In recent years, the search for sustainable and eco-friendly biofuels sources that could be a real efficient alternative to the fossil fuels has been the subject of numerous studies, investigating the production in plants, bacteria, yeasts and microalgae. Due to the higher lipid productivities, growth rates and biomass accumulation in smaller areas, not competing with human food resources, microalgae

have been postulated as a valuable solution. These photosynthetic microorganisms have the ability of producing numerous metabolic compounds which can be converted into different forms of biofuel such as biodiesel, biohydrogen, biomethane or bioethanol. The main potential is in the production of triacylglycerides, TAGs, the main component of biodiesel feedstocks through their transesterification into fatty acid methyl esters (FAMEs), obtained from the lipid synthesis metabolic pathway in microalgae (Radakovits et al. 2010, Jeon et al. 2017). Some microalgae species such as *Dunaliella salina* or *Botryococcus braunii* can accumulate up to 60% of triacylglycerides (Scott et al. 2010; Gangl et al. 2015). Among all enzymes involved in TAGs synthesis pathway, the diacylglycerol acyl-transferase, DGAT, is considered one of the most important enzymes because it catalyses the esterification of diacylglycerol into triacylglycerol in the last step of the lipid production pathway, assembling the final chemical structure of TAGs. The successful overexpression of this enzyme to increase the seed oil content in plants has been published (Lardizabal et al. 2008; Zheng et al. 2008). In microalgae, Chen and co-workers in 2016 reported the DGAT overexpression in *Scenedesmus obliquus* with an enhancement of 128% in the lipid content. Another enzyme which plays a key role in the TAGs biosynthesis and their overexpression that has been reported, although without high success, in plants, microalgae or bacteria, is the acetyl-CoA carboxylase that catalyses the carboxylation of acetyl-CoA to malonyl-CoA (Ng et al. 2017). Other strategies involved in the enhancement of the lipid accumulation in microalgae are aimed to block the metabolic pathways which can compete with the lipid productions, such as the starch, oxaloacetate or phospholipid biosynthetic metabolism. Some reports concerning these approximations have been achieved for *Chlamydomonas reinhardtii* for the deprivation of the starch synthesis, modifying the ADP-glucose pyrophosphorylase or isoamylase genes (Radakovits et al. 2010) or using the genomic editing technique CRISPRi to knock down the phosphoenolpyruvate carboxylase, enzyme that catalyses the production of oxaloacetate. Moreover, in *P. tricornutum*, the lipid metabolic productivity has been increased up 2.5-fold, engineering malic enzymes (Xue et al. 2015). In *Chlorella pyrenoidosa* have also been reported studies in which starch-deficient mutants increased the lipid content (Radakovits et al. 2010).

On the other hand, hydrogenases are enzymes which are involved in the hydrogen metabolic pathways and ultimately catalyse the reversible oxidation of molecular hydrogen through a proton-electron flux. In microalgae, these enzymes can be found, and the possibility of producing biohydrogen from them, as a real alternative to the fossil fuels, is increasing even more in recent times. Microalgae could produce biohydrogen by two different metabolic pathways, photobiologically using sunlight and water or by a fermentative pathway in determined anaerobic culture conditions and sulphur starvation, using organic carbon molecules as electron donor (Melis et al. 2000; Kumar et al. 2016). Since biohydrogen is considered one of the most environmental-friendly biofuels due to the avoidance of carbon dioxide gas emissions with a high energetic density (DOE 2016), many efforts are being done to improve the efficiency of the metabolic pathways for the production of biohydrogen by the engineering of hydrogenases. Some examples are found for the microalgae

Chlamydomonas reinhardtii in which the study of the hydrogen metabolism is being studied as a model organism. Melis et al. (2000) report a novel approach to produce photobiological hydrogen via the reversible hydrogenase pathway in *C. reinhardtii*, and in 2012, Scoma and co-workers attempted the outdoor production of biohydrogen in a sulphur-deprived culture of *Chlamydomonas*. Furthermore, as was described in Kumaraswamy et al. (2013), the metabolic engineering of the glycolytic pathway via genetic manipulation of the enzyme glyceraldehyde-3-phospate dehydrogenase in cyanobacteria can redirect the carbon flux to a more efficient carbohydrate catabolism and biohydrogen production.

3 Future Prospect

As it has been demonstrated that the biotechnological potential of microalgae increases year after year, with new technologies, new research possibilities and new advances in development and innovation, many future perspectives are opened for the exploitation of these photosynthetic microorganisms at an industrial level. The new *-omic* disciplines, the progress in bioinformatics and computational biology, the development of metabolic networks simulators and, above all, the advances in the technologies and software of gene annotation and, therefore, genome sequencing make the use of all of them widely useful to obtain a fully efficient microalgal industry, eco-friendly and above all competitive economically, especially in the aspect of biofuels derived from microalgae that currently have serious problems to be commercialized in a truly profitable way. Reducing the production costs, mainly in the phases of microalgae large-scale cultivation and microalgal biomass harvesting, is the concern in which science and research must be focused. More specifically at the level of genetic engineering, the new systems of genome editing and the extensive development that is undergoing metabolic engineering and synthetic biology will be of great help in the resolution of the current problems that arise in the genetic modification traditional, such as the stability of the transgenes, an efficient expression of these and especially the system for delivering the exogenous genetic material as well as the methodology for identification and selection of the new recombinant strains. Likewise, these advances would also deepen the need to create more ecologically efficient and eco-friendly systems, minimizing the risks that could arise from the use of genetically modified microorganisms and the ethical responsibilities that this fact still supposes nowadays.

4 Concluding Remarks

Microalgae are photosynthetic organisms with an endless potential for their use in industrial biotechnology because they are producers of numerous high added-value compounds with cosmetic, pharmaceutical, fungicide, herbicide and biofuel

properties, products that usually are chemically manufactured. Even so, today there is a real problem for marketing the microalgal bio-products because most of them cannot compete with market prices due to their high production costs. In this sense, the genetic manipulation of microalgae, through the development of metabolic engineering and the new techniques of DNA in synthetic and computational biology, could help to increase the production efficiency of these bioactive compounds. The new techniques of genetic manipulation related to the genome editing, mainly the CRISPR/Cas9 approach, are assuming a very important role so that the production of biofuels through the improvement of atmospheric carbon dioxide fixation is finally incorporated, economically efficient and environmentally friendly to the current industry.

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Chapter 8
Advanced Gene Technology and Synthetic Biology Approaches to Custom Design Microalgae for Biodiesel Production

Neha Arora, Shweta Tripathi, Krishna Mohan Poluri, and Vikas Pruthi

Abstract Photosynthetic microalgae are being recognized as propitious source for sustainable production of bio-based fuels particularly biodiesel. Oleaginous microalgae possess inherent capability to accumulate high amounts of lipids (mostly as triacylglycerols) under adverse physiological conditions, which can be transesterified to form biodiesel. Since the last decade, research is being focused on finding targets to increase the biomass and lipid productivity of microalgae contributing to large-scale cultivation feasibility. In this regard, algal omics plays a vital role in categorizing regulatory pathways responsible for increasing the lipid accumulation in microalgae leading to identification of suitable targets for genetic engineering. Metabolic engineering of microalgal strains improves the control over growth and lipid pathways resulting in more reproducible and predictable systems compared to the wild-type strains. The present chapter is a comprehensive catalogue of algal omics including transcriptomics, proteomics and metabolomics studies carried out for augmenting lipid accumulation in different microalgal strains under various physiological conditions. The chapter substantiates the rationale for transgenic microalgae and the requisite of integrated genome editing and synthetic biology approach for custom designing of lipid accumulation in microalgae for biodiesel production.

Keywords Microalgae · Lipid · Biodiesel · Omics · Genetic engineering

1 Introduction

Dependable supply of energy is one of the central necessities for economic prosperity for any nation's development. Currently, fossil fuels are the major contributors to the world's energy which are rapidly depleting due to drastic expansion in urbanization and industrialization (Sharma and Singh 2017). The international energy

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agency reported that approximately 63% of the fossil fuels are consumed by the transportation sector, therefore calling for an urgent replacement (Arenas et al. 2017). The burning of fossil fuels has also resulted in increase in the global carbon dioxide emissions reaching a new high of 37 Gt in 2035 along with a global temperature rise to ~0.17 °C per decade (Ho et al. 2014a; Shinde et al. 2018). Recently, Paris agreement established firmly to limit the earth's temperature increase to 2 °C, and this requires 90% coal reserves, 50% of gas and two-thirds fossil fuels reserves to be kept intact (Dhar et al. 2018; Shinde et al. 2018). Biofuels for transport are currently one of the most propitious alternatives which can replace the conventional fossil fuels.

Biofuels can be classified into four major categories based on the feedstock, namely, first generation, second generation, third generation and fourth generation. First and second generations are conventional biofuels which are derived from edible and nonedible terrestrial plants including corn, sugarcane bagasse, wheat starch, soya bean, rapeseed, canola, jatropha etc. (Doshi et al. 2016). However, the major drawback of these conventional fuels includes requirement of large area, water and nutrient supply for cultivation which direct competition with the agriculture food production (Maity et al. 2014). These disadvantages can be overcome by using third-generation biofuels, derived from biomass of various microorganisms including bacteria, yeast, fungi and microalgae which can be cultivated on smaller land areas along with high areal productivity (Maity et al. 2014). On the other hand, fourth-generation biofuels include genetically engineered microorganism for augmenting their biofuel potential (Lü et al. 2011). Among these, photosynthetic microalgae offer an edge over the other microorganisms due to their ability to utilize CO_2 and solar energy for generating biomass, thereby eliminating the need for organic carbon which entails hefty cost (Bajhaiya et al. 2017). Utilizing microalgal lipids for biodiesel production is advantageous due to its inherent ability to survive in sea, brackish water or wastewaters, thereby reducing the land and freshwater usage, rapid growth rate along with CO_2 and nutrient (nitrogen and phosphorous) mitigation from wastewaters and flue gases and all-year-round production (Brennan and Owende 2010).

The concept of cultivating microalgae for biofuel particularly biodiesel was introduced between 1978 and 1996 by the US Department of Energy (DOE), under the Aquatic Species Program (ASP), funded by Solar Energy Research Institute (SERI), which became National Renewable Energy Laboratory (NREL) in 1991 (Murphy and Allen 2011). ASP focused on production of biodiesel from oleaginous microalgae and documented their finding in "A look back at the U.S. Department of Energy's biodiesel from Algae" (Murphy and Allen 2011). Microalgae biomass majorly comprises of proteins, carbohydrates and lipids, and all the three components can be efficiently utilized for biofuel production. Microalgae can accumulate 40–70% of dry cell weight triacylglycerols (TAGs) under adverse conditions such as nutrient deprivation, light/pH/temperature fluctuations, heavy metal stress, etc. (Arora et al. 2017a, b). These TAGs serve as efficient raw materials for biodiesel production via a simple transesterification process (Ho et al. 2014b). Biodiesel is nontoxic, is biodegradable with negligible CO_2 emissions and can be directly or with minor modifications utilized in conventional diesel engines (Mehtani et al. 2017).

To this end, considerable effort has been made to cultivate microalgae on large scale in open ponds and closed photobioreactors (PBRs), but there is still a long road ahead for its commercial deployment. Economic evaluation of microalgae-derived biodiesel indicated selling price of $5–10.31/gallon, which is higher than the petroleum ($3.17/gallon) and conventional biodiesel ($4.21/gallon) (Zhang et al. 2017). To bridge this gap, improvements in the algal biomass productivity, oil content, cultivation cost and downstream production are quintessential. Further to harness the biodiesel production potential of microalgae, a thorough understanding of its metabolic pathways and genetic controls are imperative (Bajhaiya et al. 2017). Omics studies comprising of genomics, transcriptomics, proteomics and metabolomics can be helpful in underpinning the biomass and lipid augmenting pathways that could then be exploited as genetic engineering targets.

Keeping the above view in mind, the present chapter deals with the brief overview of lipid accumulation in microalgae and omics studies carried out in different microalgae under various physiological conditions for enhancing TAG accumulation. The chapter also catalogues the genetic engineering studies carried out using both conventional and emerging genome editing tools. Further, the chapter summarizes systemic approaches for integrating algal omics and genetic engineering for custom designing microalgae for biodiesel production.

2 Triacylglycerol Accumulation in Microalgae

Understanding the lipid synthesis mechanism in microalgae is one of the governing criteria for manipulating TAG accumulation. Whole-genome sequencing, de novo transcriptomics, proteomics and metabolomics of different microalgae have revealed a detailed mechanism of lipid metabolism under various growths and stress conditions (Lenka et al. 2016). Neutral lipid (TAG) synthesis in microalgae can be subdivided into two steps: de novo fatty acid synthesis occurring in the plastid and acyl-lipid assembly in endoplasmic reticulum as shown in Fig. 8.1**.** The first step de novo synthesis of fatty acid begins with the formation of glycerol 3-phosphate via Calvin cycle which is a photosynthetic product (autotrophic mode) and then its subsequent conversion to pyruvate in the plastid of microalgae (Radakovits et al. 2010). Pyruvate is then catalysed by pyruvate dehydrogenase complex (PDC) to acetyl-CoA, thereby initiating lipid synthesis (Fig. 8.1). The formation of acetyl-CoA is dependent on photosynthetic efficiency (PE) as ATP (energy), NADPH and NAPH (reducing power) which is provided by photosynthesis (Lenka et al. 2016). Acetyl-CoA then carboxylates to malonyl-CoA catalysed by plastid acetyl-CoA carboxylase (ACCase) which is the first rate-limiting step of lipid synthesis (Hu et al. 2008; Lenka et al. 2016). In the stroma malonyl-CoA is then transferred to an acyl carrier protein (ACP) with the help of fatty acid synthase (FAS) complex. The formation of malonyl-ACP starts a series of fatty acid elongation steps involving various intermediate products such as 3-keto-butyryl-ACP, 3-hydroxybutyrl-ACP, trans-Δ^3-butenoyl-ACP, butyryl-ACP and finally 3-ketoacyl-ACP which are catalysed by 3-ketoacyl-ACP reductase (KAR), 3-hydroxyacyl-ACP dehydrase

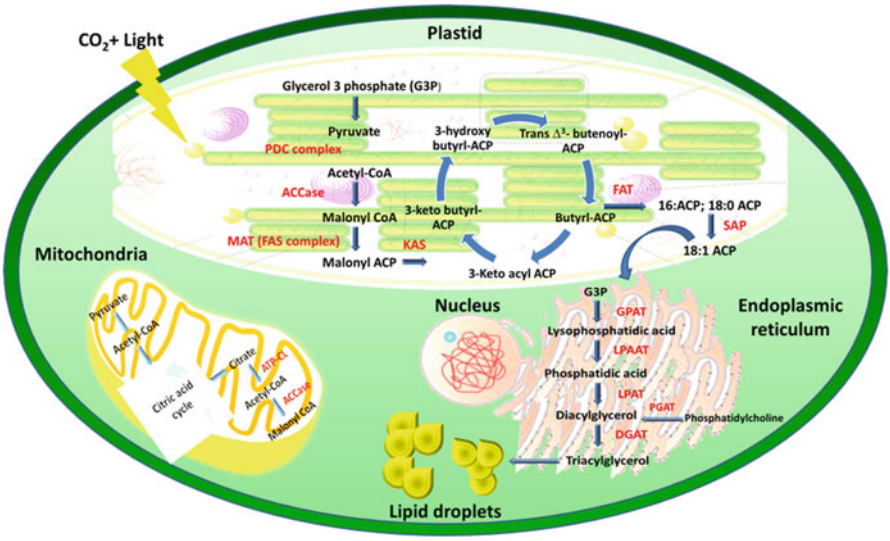

Fig. 8.1 Schematic diagram of TAG accumulation pathway in microalgae

(HD) and enoyl-ACP reductase (ENR), respectively (Hu et al. 2008). This cycle continues till saturated fatty acids (16:0 ACP and 18:0 ACP) are not formed after which the cycle is terminated by either removing the acyl group and then transferring it to glycerol 3-phosphate (G3P) in the cytosol catalysed by acyl-ACP thioesterase or by acyl transferases in the plastid (Hu et al. 2008; Radakovits et al. 2010). Further, to generate unsaturated fatty acid chains, double bond is introduced by a soluble enzyme stearoyl-ACP desaturase (SAP). The free fatty acids are transferred to the cytosol and then to endoplasmic reticulum (ER) for further processing and conversion to TAGs (Bellou et al. 2014; Lenka et al. 2016).

The second and last step of TAG synthesis: acyl lipid packaging also called the Kennedy pathway occurring in the ER results in formation of three major intermediates: lysophosphatidic acid, phosphatidic acid and diacylglycerol catalysed by glycerol phosphate acyl transferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and lysophosphatidylcholine acyltransferase (LPAT), respectively (Zienkiewicz et al. 2016). Diacylglycerol (DAG) is the precursor of triacylglycerol (TAG), and its conversion is catalysed by diacylglycerol acyltransferase (DAGAT) as shown in Fig. 8.1.

Nevertheless, there exists an alternative to Kennedy pathway, an acyl-independent pathway for TAG accumulation in microalgae involving phospholipid: diacylglycerol acyl transferase (PDAT). It is postulated that the free fatty acids that are incorporated as membrane lipids initially can be recycled back from the ER envelope and converted to TAGs. In brief, chloroplast membrane lipids including monogalactosyldiacylglycerol (MGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG) are converted to TAGs catalysed by PDAT.

3 Insights into Algal Omics for Augmenting Lipid Accumulation in Microalgae

The importance of omics technologies (genomic, transcriptomics, proteomics, metabolomics and lipidomics) has been long past realized for their eminent role in biological and biomedical applications by underpinning key regulators in disease progression and tailoring therapies. However, recently various omics technologies have been applied to understand algal biology and genome due to their prominent role as renewable energy sources. Algae system offers an advantage as the existing omics are well-developed for bacteria and single-celled eukaryotic systems such as *Saccharomyces cerevisiae* can be directly applied to it (Hannon et al. 2010). The detailed literature of omic components, transcriptomics, proteomics and metabolomics, along with related technologies pertaining to algal omics have been discussed in the sections below.

3.1 Transcriptomics

The complete collection of all the gene encoding RNA/messenger RNA (mRNA) isolated from a cell represents the transcriptome of a species. Transcriptomics is the study of these differentially expressed mRNA's (over/under expressed or new) under varying conditions to identify the underlying genes responsible and in turn shedding light on the pathway and regulation, thus offering a bigger picture than genomics (Jamers et al. 2009; Filiatrault 2011). Generation of expressed Tag sequences (EST) libraries is the most common tool for studying the transcriptome of any organism (Mclean 2013). EST are small (200–500 nucleotides) DNA sequences which are generated by reverse transcribing mRNA's into complementary DNA (cDNA) containing small portion identification sequence (Mclean 2013). Over the years various transcriptomics techniques have been developed including Northern blot hybridization, microarray, quantitative real-time polymerase chain reaction (RT-PCR), serial analysis of gene expression (SAGE) and next-generation sequencing (Shrager et al. 2003). Microarrays have been developed for model green alga: *C. reinhardtii* which contain around 10,000 oligonucleotides sequences, each representing a unique gene and covers nearly the entire genome (Shrager et al. 2003). However, with the rapid development of next-generation sequencing (NGS) technologies, researchers are now utilizing this technique to study the differential gene expression in various organisms. This technique is a multistep approach utilizing various software tools and starts by generation of raw sequence data, quality checking and filtering out low-quality and contaminant data (Fast QC) followed by assembling the genomes (ALLPATHS, Velvet, Abyss, EULER, Trinity, Oasis) and lastly annotation of the transcripts (BLAST2 GO: BLAST X, GO, ENZYME, CODE, KEGG) as shown in Fig. 8.2 (Lohse et al. 2012; Bradnam et al. 2013)

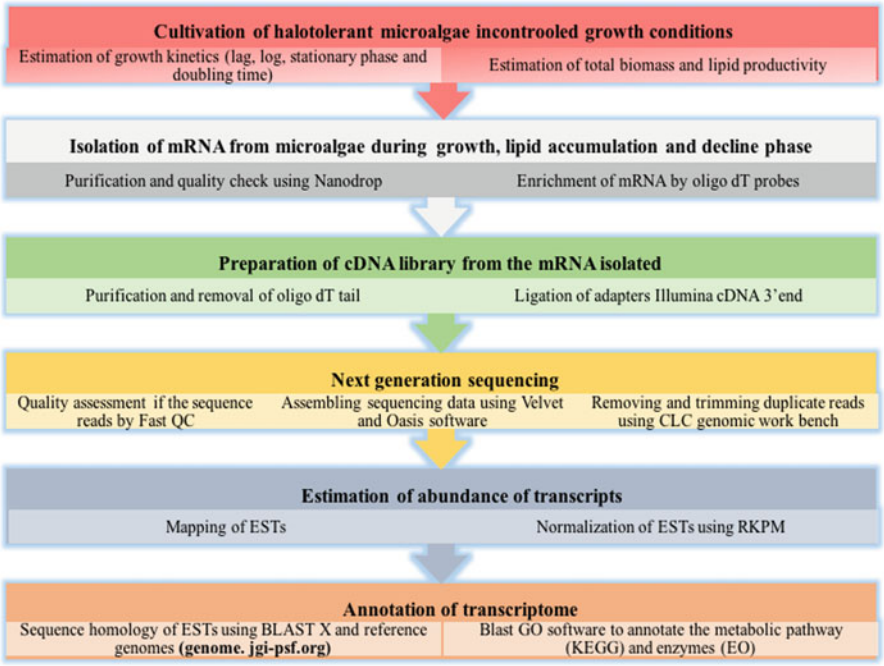

Fig. 8.2 Workflow of transcriptomics study for any microalgae

To date various transcriptomics studies have been carried out on different oleaginous microalgae cultivated under various stress conditions including nitrogen starvation, CO_2 deprivation, salinity stress and cold stress (Table 8.1). These transcriptomic studies revealed that in the face of stress, microalgae alter their metabolic pathways spanning protein synthesis, photosynthesis, carbohydrate metabolism, nutrient assimilation (nitrogen/phosphorous) energy generation and nucleotide biosynthesis. Under nitrogen starvation, an upregulation of lipid biosynthesis pathway genes such as acyl carrier protein (ACP) gene, diglyceride acyltransferase (DGAT) isoforms such as DGAT, biotin carboxylase, thioesterase genes, acyl-ACP desaturase (AAD), delta 15 saturase, and lipases was recorded (Msanne et al. 2012; Rismani-Yazdi et al. 2012; Sun et al. 2013; Yang et al. 2013; Li et al. 2014, 2016a; López García de Lomana et al. 2015). On the other hand, downregulation of ACCase (placidyl/chloroplastic) and malonyl transferase were recorded (Rismani-Yazdi et al. 2012; Valenzuela et al. 2012; Sun et al. 2013; López García de Lomana et al. 2015). Interestingly, an increase in the genes responsible for lipid recycling and fatty acid chain modification indicated that alternative pathway is playing a vital role in augmenting the overall TAG accumulation (Yang et al. 2013; Li et al. 2014; Tanaka et al. 2015).

Transcriptomics analysis of *C. pyrenoidosa* under CO_2 deprivation showed an increase in the levels of malic enzyme, carbonic anhydrase, pyruvate phosphate and acetyl-CoA enzyme A indicating carbon flux towards TAG synthesis (Fan et al.

Table 8.1 Summary of transcriptomics studies carried out on different microalgal strains cultivated under various stress conditions

Microalgae	Cultivation media	Method used for analysis	References
Nitrogen deprivation			
Botryococcus braunii 779	Bold's modified	NGS	Fang et al. (2015)
Botryosphaerella sudeticus	Bristol (BB)		Sun et al. (2013)
Botryococcus braunii UTEX 572	Chu-13	qRT-PCRs	Choi et al. (2011)
C. reinhardtii	TAP medium	NGS	Miller et al. (2010)
	Sueoka's high salt medium	Semi-quantitative RT-PCRs	Msanne et al. (2012)
	TAP medium	NGS	Boyle et al. (2012)
			Garcia de Lomana and Baliga (2010)
Chlorella sorokiniana	Kuhl medium	NGS	Li et al. (2016b)
Dunaliella tertiolecta	f/2		Shin et al. (2015)
Nannochloropsis		SoLiD	Corteggiani et al. (2014)
Neochloris oleoabundans	Modified Bold-3 N	NGS	Rismani-Yazdi et al. (2012)
Phaeodactylum tricornutum	ASPII		Valenzuela et al. (2012)
	F/2		Yang et al. (2014)
C. vulgaris var L3	Modified Bold's Basal Medium	Real-time PCR analysis	Ikaran et al. (2015)
Micractinium pusillum	High salt medium		Li et al. (2012)
Tisochrysis lutea (lipid mutant)	Modified Conway medium	NGS	Carrier et al. (2014)
Monoraphidium neglectum	ProF medium		Jaeger et al. (2017)
Tetraselmis M8	F/2		Lim et al. (2017)
Carbon dioxide deprivation (>5% CO_2)			
Chlorella pyrenoidosa	BBM	NGS	Fan et al. (2016)
Salinity stress (1 M NaCl)			
Picochlorum strain SENEW3	Artificial seawater-based F/2	NGS	Foflonker et al. (2016)
Dunaliella tertiolecta	0.5 M NaCl		Yao et al. (2015)
C. vulgaris	BBM		Sarayloo et al. (2017)
D. tertiolecta	F/2	NGS	Yao et al. (2017)
Cold stress			
C. reinhardtii (diploids-colcemid treated)	TAP	NGS	Kwak et al. (2017)
UV stress			
Chlorella sp. UMACC 237	BBM	NGS	Poong et al. (2017)

2016). An increase in the levels of proline, starch, nitrate and urea assimilation was recorded in *Picochlorum* strain SENEW3 when cultivated under salinity stress implying its halotolerance characteristics (Foflonker et al. 2016). Further, transcriptomics analysis of *Chlorella* sp. under UV stress showed a decrease in branched chain amino acid synthesis (valine, leucine and isoleucine) and antioxidant genes (superoxide dismutase and catalase) indicated damage of the microalga cellular machinery under UV stress generating stress which increases the lipid synthesis (Poong et al. 2017).

3.2 Proteomics

Expression of a gene can be correlated appropriately by quantifying the level of proteins as they not only provide the fundamental organization and pathways occurring inside a cell but also indicate the cell's state (healthy, stressed or apoptotic) (Mclean 2013). In-depth understanding of stress-induced TAG accumulation in microalgae requires integration of transcriptomics and proteomics. Quantitative algal proteomics identifies and quantifies the dynamics of protein abundance and its corresponding function both at translational and post-translational levels in response to any environmental stress leading to augmentation of TAG. There are various techniques to quantify the proteome including classical two-dimensional (2D/DIGE) gel electrophoresis and liquid chromatography (LC)-based methods such as isotopic labelling and label-free methods followed by mass spectroscopy (MS) (Wang et al. 2011). LC-based technologies have an edge over the conventional 2D as they can overcome the shortcomings of throughput and low coverage of extreme pH, hydrophobic, low-abundance proteins (Wang et al. 2011). With the advent of nano-HPLC systems combined with improved MS (high accuracy and resolving power), thousands of protein abundances can be identified and quantified (Xie et al. 2011). Stable isotope labelling techniques primarily tag the proteins with ^{13}C, ^{15}N and ^{18}O and then analyse using LC-MS or LC-MS/MS, thereby increasing the precision and accuracy of quantification (Xie et al. 2011). Recently, iTRAQ (isobaric tags for relative and absolute quantification) has been used to quantify proteomic changes in algal systems which are robust and easy to use (Longworth et al. 2016). Nevertheless, iTRAQ system has disadvantages such as underestimated ratios, expensive labelling and limited dynamic range (Wang et al. 2011). Label-free techniques, on the other hand, are inexpensive, rapid with wider dynamic range and broader proteomic coverage (Xie et al. 2011). The two major label-free quantification methods are spectral counting (number of MS/MS spectra) and MS ion intensity (peak area). A brief representation of proteomics workflow is depicted in Fig. 8.3.

The proteomics studies reported in the literature are listed in Table 8.2. The proteome of different microalgal species under nitrogen deprivation showed an upregulation of acyl carrier proteins (ACP), malonyl-CoA/ACP transacyclase, lipid droplet surface protein (LDSP), ACCase, MAT, enoyl-acyl carrier protein

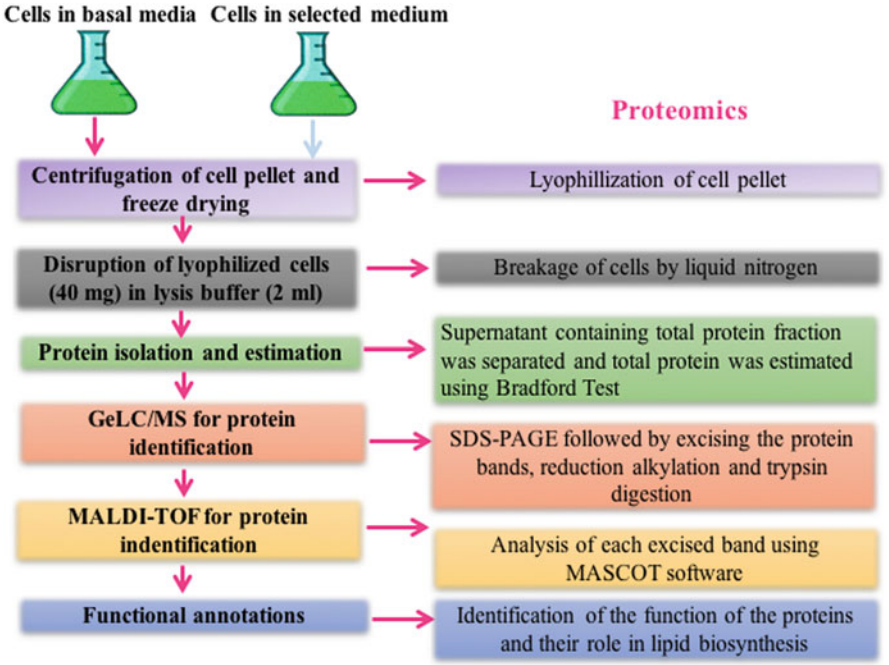

Fig. 8.3 Brief overview of proteomics steps

reductase (Fab I), trans-2-enoyl-CoA reductase and the four condensing enzymes involved during fatty acid synthesis (KAS, HD, ENR and DGAT). On the other hand, a decline in the levels of AMP-activated kinase (AMPK), fatty acid catabolism (acyl CoA dehydrogenase) and stearoyl-ACP desaturase was recorded. AMPK inhibits the ACCase activity by phosphorylating while stearoyl-ACP desaturase catalyses the formation of oleoyl ACP from stearoyl-ACP (Deng et al. 2013; Guarnieri et al. 2013; Li et al. 2013b; Song et al. 2013; Yang et al. 2014; Longworth et al. 2016; Tran et al. 2016; Shang et al. 2017). The proteomic studies also revealed decline in the proteins involved in the photosynthesis, chlorophyll and carotenoid synthesis, respectively (Deng et al. 2013; Li et al. 2013b; Song et al. 2013; Garnier et al. 2014; Yang et al. 2014; Longworth et al. 2016; Tran et al. 2016; Shang et al. 2017). However, an upregulation in the TCA cycle proteins, glycolysis enzymes, ATP synthase and nitrate reductase were observed (Dong et al. 2013; Song et al. 2013; Wase et al. 2014b; Yang et al. 2014). The above proteomic results indicated redirection of carbon and energy flux towards the TAG accumulation under nitrogen deplete conditions. Interestingly, the proteomic studies on the lipid mutants also indicated upregulation of glycolysis, TCA, ATP synthase and fatty acid biosynthesis proteins along with reduction of photosynthetic and nucleotide synthesis proteins (Wang et al. 2012; Choi et al. 2014).

Table 8.2 List of proteomics studies on different microalgal strains cultivated under various stress conditions

Microalgae	Media	References
Nitrogen depletion		
Chlorella vulgaris	BBM	Guarnieri et al. (2013)
		Guarnieri et al. (2011)
	Watanabe media + NaCl	Li et al. (2015)
C. protothecoides	Basal medium	Li et al. (2014)
	Watanabe medium	Li et al. (2013b)
Chlamydomonas reinhardtii	TAP	Wang et al. (2011)
		Longworth et al. (2012)
		Wase et al. (2014a)
Chlorella sp. *FC2IITG*	BG-11	Rai et al. (2017)
Phaeodactylum tricornutum	F/2 + Si medium	Longworth et al. (2016)
Nannochloropsis oculata	Artificial seawater enriched with f/2 medium	Tran et al. (2016)
Nannochloropsis oceanica IMET1	Artificial seawater medium	Dong et al. (2013)
Neochloris oleoabundans	BBM	Morales-Sánchez et al. (2016)
Phaeodactylum tricornutum	F/2 + Si medium	Yang et al. (2014)
Dunaliella parva	F/2	Shang et al. (2017)
Copper stress		
C. protothecoides	BCM + 10 g/L glucose	Li et al. (2013a)
Salinity		
C. reinhardtii	TAP	Mastrobuoni et al. (2012)
Dunaliella salina	1M NaCl	Tan and Lee (2017)
Lipid mutants		
Scenedesmus dimorphus	BBM	Choi et al. (2014)
Tisochrysis lutea	Walne's medium	Garnier et al. (2014)

3.3 Metabolomics

Metabolomics is the qualitative and quantitative measurement of low molecular weight compounds including amino acids, nucleotides, fatty acids, organic acids, osmolytes, sugars, etc. which are involved in cell's normal functioning (Dunn and Ellis 2005). The main advantage of metabolomics is that estimation/analyses of metabolites do not require prior knowledge of microalgal genome and thus can be an ideal tool for deciphering cellular response of non-model microalgae. Currently, metabolomics can be studied using various tools including capillary electrophoresis-mass spectroscopy (CE-MS), gas chromatography-mass spectroscopy (GC-MS), liquid chromatography-mass spectroscopy (LC-MS), nuclear-magnetic chromatography (NMR) and Fourier-transform ion cyclotron resonance mass spectroscopy (Dunn and Ellis 2005). Among these, MS combined with chromatography is the most widely used technique in

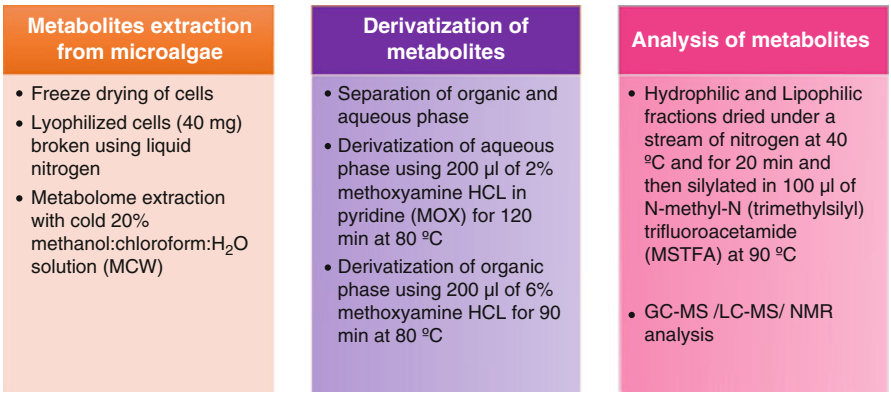

Fig. 8.4 Steps involved in metabolomics study

metabolomics as it is rapid, sensitive and selective, but it shows laboratory variation and requires expensive reagents and derivatization of samples. On the other hand, NMR is less sensitive than MS but at the same time is more robust and non-destructive along with high throughput and reproducibility and minimal sample preparation requirements (Dunn and Ellis 2005; Gupta et al. 2013) (Fig. 8.4).

A brief overview of the metabolomics studied carried out on various microalgae under different stress conditions such as nitrogen limitation, salinity and heavy metal stress are listed in Table 8.3. In the face of nitrogen depletion, an increase TCA intermediates and glycolysis intermediates while a decrease in amino acids (branched, aromatic) were recorded (Blaby et al. 2013; Ito et al. 2013; Wase et al. 2014a). These results were in line with the above reported transcriptomic and proteomics studies. The metabolomics studies also reported accumulation of osmolytes including proline and trehalose which are integral for reactive oxygen species scavenging and stabilization of membrane integrity under nitrogen stress (Wase et al. 2014a). Interestingly, on exposure to cadmium stress, an elevation in the levels of amino acids (proline, valine, isoleucine, sarcosine, phenylalanine and methionine) was reported (Chia et al. 2015). The metabolomics studies also revealed potential TAG accumulating biomarkers including ethanolamine, glycerol, glycerol 3-phosphate, acetyl-CoA, 3-phosphoglyceric acid, 2-ketoglutaric acid (Ho et al. 2014c, 2015; Sui et al. 2014).

4 Genetic Engineering of Microalgal Strains for Enhanced Lipid Accumulation

Genetic engineering of microalgal strains to augment the lipid accumulation can provide a leap towards the economic feasibility of algal-derived biodiesel. In the last decade, various genome editing tools have been established for microalgal systems

Table 8.3 Details of metabolomics studies carried out on different microalgae

Microalgae	Cultivation media	Limitation/mode	References
Chlamydomonas reinhardtii	TAP	Nitrogen depletion	Blaby et al. (2013)
			Wase et al. (2014a)
Chlamydomonas sp. JSC4	Modified Bold 3 N medium	Light + nitrogen depletion	Ho et al. (2015)
		Salinity + nitrogen depletion	Ho et al. (2014a)
C. reinhardtii	TAP	Salinity	Mastrobuoni et al. (2012)
Chlorella vulgaris	LC Oligo medium	Nitrogen depletion + cadmium stress	Chia et al. (2015)
C. sorokiniana	BG-11	Inoculum size	Lu et al. (2012)
Scenedesmus obliquus,		–	Cheng et al. (2012)
Synechocystis sp. PCC6803			
Anabaena sp. PCC7120			
Schizochytrium sp.	–	–	Mioso et al. (2014)
Pseudochoricystis ellipsoidea	A5	Nitrogen depletion	Ito et al. (2013)

including nuclear transformation (conventional technologies), adaptive laboratory evolution (ALE), RNA silencing (RNAi), transcriptional factor (TF) engineering, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR-associated nuclease 9) (Ghosh et al. 2016; Beacham et al. 2017). Each of the above-mentioned genome editing tools has been discussed individually in the sections below.

4.1 Conventional Genetic Engineering

Overexpression of specific lipid biosynthetic genes is one of the direct paths for boosting lipid yield in microalgal strains. The pioneer attempt towards genetically modifying the microalgal strain was overexpression of ACCase in diatom *Cyclotella cryptica* and then in *Navicula saprophila* by Dunahay et al. as a part of ASP program (Dunahay et al. 1996; Schuhmann et al. 2012). However, overexpression of ACCase resulted in only twofold increase in its transcript level, but overall no enhancement in total lipid content was recorded. On the other hand, overexpression of acyl-ACP (acyl carrier protein) esterase which is responsible for termination of chain elongation during fatty acid synthesis in *P. tricornutum* and *C. reinhardtii* resulted in increased C12–C14 fatty acid without affecting the overall lipid yield (Klok et al.

2014). In contrary, overexpression of fatty acid-ACP thioesterase in *C. reinhardtii* resulted in an increase in the total lipid by 14–15% (Wei et al. 2017). Heterologous overexpression of yeast-derived LPAT, PAP, GPAT, DGAT and G3PDH in *Chlorella minutissima* showed an increase in overall TAG accumulation (Klok et al. 2014). On a similar note, overexpression of DGAT-2 in *P. tricornutum* and *Nannochloropsis oceanica* increased its TAG accumulation by 35% and 62% as compared to wild type (Klok et al. 2014; Li et al. 2016b). Recently, Xue et al. reported overexpression of malic enzyme (isoform 1 and 2) in *P. tricornutum* increased the total TAG content (54.7% and 57.8% dry cell weight), being ~2.5-fold higher than wild type (Xue et al. 2015). Further, overexpression of acetyl-CoA synthetase (ACS) under nitrogen deprivation in *C. vulgaris* resulted in 1.5-fold increase in total lipid content, while 50% increase in lipid content when the nitrogen-deprived media was supplemented with acetate (50 mM), respectively (Rengel et al. 2018). Addition of acetate increased the pool of acetyl-CoA as ACS catalyses the conversion of acetate to acetyl-CoA, thereby increasing the flux towards lipid synthesis. Interestingly, heterogeneous overexpression of patatin-like phospholipase domain-containing protein 3 (PNPLA3) in *P. tricornutum* boosted its total lipid content to 59.8% as compared to wild type (Wang et al. 2018). PNPLA3 has been known to increase the hepatic lipid synthesis in humans as it exhibits transacylase activity which converts mono-/diacylglycerol to TAGs.

Alternative to modulating the lipid synthetic genes, a few of the researchers have overexpressed genes which provide precursors for TAG synthesis. For example, overexpression of glycerol kinase increases the intracellular pool of glycerol 3-phosphate which stimulated TAG accumulation (~41%) in engineered *Fistulifera solaris* JPCC as compared to wild type (~36%) (Muto et al. 2015). Further, alteration in the Calvin cycle, i.e. overexpression of aldolase in *Synechocystis* sp. PCC 6803, a key enzyme for the metabolism of dihydroxyacetone phosphate (DHAP) which serves as an intermediate for starch in sucrose synthesis resulted in enhanced photosynthetic capacity which increased the growth rate of the cyanobacteria (Yang et al. 2017). The increase in biomass along with lipid accumulation is crucial for increasing the overall lipid productivity – one of the most important factors for commercialization of algal biodiesel. Similarly, researchers have reported that a reduction in the antenna size can potentially prevent over absorption of sunlight, increasing the light penetration in dense algal cultures which overall enhances the productivity of microalgal cultures (Wobbe and Remacle 2014; Baek et al. 2016). Recently, a proof of concept to the above hypothesis was given by Sharon-Gojman et al., by overexpression of the nucleic acid-binding protein (NAB 1) and the low CO_2-inducible gene (Lci A) which decreased the chlorophyll content resulting in increased biomass and carotenoid production under nitrogen-deprived conditions in engineered strain for *Haematococcus pluvialis* (Sharon-Gojman et al. 2017). NAB 1 is the translational repressor of light harvesting complex II (LHC II) which reduces the antenna size in the microalga, while Lci A is a bicarbonate transporter which aids in increasing the biomass yield.

4.2 Adaptive Laboratory Evolution

Adaptive laboratory evolution is a scientific approach to generate single-nucleotide polymorphisms (SNPs), smaller insertions and deletions (indels) or larger deletions/insertions that alter the genetic blueprint of the microorganism leading to improved growth and biochemical properties (Dragosits and Mattanovich 2013). ALE is carried out in controlled laboratory settings with clear defined conditions for prolonged period of time (weeks or years) to obtain a strain with desired characteristics (Shin et al. 2017). Over the past few years, ALE has been utilized for developing novel biological and phenotypic characteristics in microalga strains. For example, adapted strain of *C. reinhardtii* CC-124H showed loss of mobility and flagella with increased palmelloid state which resulted in increase in the cell size, biomass productivity and total lipids (66% in normal media and 116% in nitrogen-deprived medium as compared to wild-type strain) (Shin et al. 2017). Further, the analysis of the adapted strain via whole-genome sequencing showed 44 CDS (coding DNA sequence) alterations in which 34 resulted from non-synonymous substitutions involving 336 genes which were mainly involved in cell cycle progression.

Additionally, adaptive strain of low-starch mutants of *C. reinhardtii* cc4326 and cc4334 showed augmentation in lipid accumulation (36.67% and 44.67%) as compared to wild type (13%) under nitrogen deprivation conditions (Yu et al. 2013). On a similar note, adaptive strain of starchless mutants of *C. reinhardtii* (sta6-1 cells) showed 175% increase in total lipid content as compared to wild type (50%) under nitrogen-deplete conditions (Velmurugan et al. 2014). Apart from increasing the lipid content, ALE has been deployed to increase resistance to phenol in *Chlorella* sp. (Wang et al. 2016), light in *C. reinhardtii* (Perrineau et al. 2014), salinity stress in *Chlamydomonas* sp. (Kato et al. 2017) and improved CO_2 tolerance in *Chlorella* sp. (Li et al. 2015).

4.3 Engineering Transcriptional Factors

Transcriptional factors (TFs) regulate expression profiles of multiple components of a metabolic pathway by binding to specific DNA motifs within cis elements capable (Bajhaiya et al. 2017). Engineering TFs alter the expression of multiple enzymes, thereby increasing the chances of successful genetic engineered strains. To this end, approximately 147 putative TFs and 87 putative transcriptional regulators (TRs) have been identified in *C. reinhardtii* (Manuelle et al. 2009). A few of the TFs have been validated in different microalgae including PSR1 (P_i starvation response), SNO3, GmDOF4 (Glycine maxDNA binding with one finger 4), CHT7 (compromised hydrolysis of triacylglycerols 7), WRI 1 (Wrinkled 1), bHLH (basic helix loop-helix), ROC40 (rhythm of chloroplast 40), NRR-1 (nitrogen response

regulator 1), TAR1 (TAG accumulation regulator) and Zn(II)2Cys6 ((ZnCys) homolog of fungal Zn(II)2Cys6-encoding genes) and have been reported, respectively (Boyle et al. 2012; Tsai et al. 2014; Zhang et al. 2014; Bajhaiya et al. 2015; Kajikawa et al. 2015; Kang et al. 2015; Shang et al. 2016; Ajjawi et al. 2017). PSR1 is essential for regulation of phosphorus acquisition via upregulation of phosphatases and P_i transporters (Bajhaiya et al. 2015). The mutants of *C. reinhardtii* lacking PSR1 showed inhibition of lipid and starch accumulation under phosphorus-deprived conditions, while overexpression of this TF resulted in enhanced starch accumulation but reduced neutral lipid content (Bajhaiya et al. 2015). Sapphire Energy Inc. recently filed a patent on mRNA encoding protein (SNO3) which is CREB-binding protein/P300 and related to TAZ zinc finger proteins (Yohn et al. 2016). Its overexpression in *C. reinhardtii* resulted in an increase in lipid accumulation under nitrogen deprivation without significantly impacting microalga's growth (Yohn et al. 2016). Similarly, mutants of NRR-1 accumulated half the amount of TAG compared to parental strain of *C. reinhardtii* under nitrogen-deprived conditions (Boyle et al. 2012).

On the other hand, overexpression of GmDOF4 from soya bean in *Chlorella ellipsoidea* increased the lipid content by 46–52% under mixotrophic conditions without affecting the growth rate (Zhang et al. 2014). GmDOF4 is involved in activating ACCase via directly binding to the cis-DNA elements present in the promoter region. It has been postulated that under nitrogen deprivation, microalga accumulates TAG as a stress response attaining cellular quiescence, but when the growth media is repleted with nitrogen, the condition is reversed with TAG degradation (Tsai et al. 2014). The TAG degradation was delayed following nitrogen repletion in *C. reinhardtii* mutants of CHT7 which showed a slow growth (Tsai et al. 2014). Recently, heterologous overexpression of WRI showed ~44% increase in the total lipid content as compared to non-transformed cells (Kang et al. 2017). Further, the overexpression of two bHLH TFs (bHLH1 and 2) in *Nannochloropsis salina* resulted in increased growth rate (60% higher) along with 24.5%, 46% and 32.5% increase in lipid content under normal, nitrogen-deplete and osmotic (50 g/L sea salts) conditions as compared to parental strains (21.7%, 42.3% and 30.1%), respectively (Kang et al. 2017). Moreover, TAR1 defective *C. reinhardtii* mutants resulted in increased TAG accumulation (0.5–1-fold) under nitrogen and sulphur starvation (Kajikawa et al. 2015).

Recently, knocking of Zn(II)2Cys6 – a TF of lipid regulation in *N. gaditana* – improved the total carbon portioning into lipids with an overall increased total lipid content to 40–55% under nutrient-replete conditions (Ajjawi et al. 2017). In another study, the proteomics of nitrogen-starved cells of *Chlorella* sp. showed ROC40 to be the most induced protein playing a key role in the circadian rhythm which is crucial for the survival of microalga under fluctuating conditions (Goncalves et al. 2016). However, this target has not been overexpressed till date and warrants further investigation.

4.4 RNAi Silencing Approaches

Double-stranded RNA (dsRNA) induce gene silencing in eukaryotes by degradation of homologous mRNA, a process termed as RNAi interference/silencing (Cerutti 2003). RNAi silencing can provide valuable insights into the gene expression which can then be developed as potential engineering targets for enhancing TAG accumulation in microalgae. For example, knockdown of phosphoenolpyruvate carboxykinase (PEPC) in *P. tricornutum* resulted in generation of two high lipid (~25%) accumulating lines (PEPCK19 and PEPCK21) as compared to control (21%) (Yang et al. 2016). PEPC is a key enzyme and is involved in the formation of oxaloacetate from phosphoenolpyruvate (PEP), providing influx to TCA cycle for proteins synthesis (Kao and Ng 2017). Thus, silencing PEPC will result in increase in carbon flux towards lipid synthesis. Further, PEPC complex has two classes, PEPC1 and PECP2, which have been identified in microalgae (Deng et al. 2014). The former is minor homotetramer (p109 subunit), while latter is an abundant heteromer (p109 and p131 subunits). Silencing of PEPC2 negatively correlated with lipid accumulation, while knockdown of PEPC1 significantly increased the lipid accumulation (20% higher TAG) as compared to wild type (Deng et al. 2014).

As noted in the TAG synthesis section, DGAT catalyses the terminal step for TAG formation, and thus, its silencing will lead to reduced TAG accumulation. In microalgae, two DGAT families have been identified DGAT1 and DGAT2, which share no sequence similarity. Thus, in order to identify which of the DGATs play a vital role in augmenting TAG accumulation, Dang et al. silenced five homologous genes in *C. reinhardtii* (Cr DGAT1, Cr DGAT2, Cr DGAT3, Cr DGAT4 and Cr DGAT5). Among these silenced DGAT genes, DGAT1 and DGAT5 were critical for TAG accumulation as their knockout resulted in 16–24% and 28–37% decrease in lipid content (Deng et al. 2012). Similarly, silencing of DGAT1A in *N. oceanica* resulted in 25% decrease in TAG content, while its overexpression enhanced TAG accumulation by 39% under nitrogen-deprived condition as compared to no transformed cell lines (Wei et al. 2017). Alternative to directly targeting the lipid augmenting genes, silencing the competing pathways genes could also increase the overall TAG accumulation. For example, Deng et al. silenced citrate synthase (CS) in *C. reinhardtii* and reported an increase in the total lipid content by 169% in transgenic lines as compared to wild type (Deng et al. 2013). CS is located in the mitochondria and catalyses the conversion of acetyl-CoA to citryl-CoA.

4.5 Advance Genome Editing Tools

All of the above-listed genetic engineering approaches regulate genes in cis and require replacement of native expression cassettes which alter the native regulation

leading to additional step of optimizing the codon or specific promoters required for a given set of physiological state (Baek et al. 2016; Gordon et al. 2016). Engineering trans acting tools eliminate the optimization of the native regulation along with successful transgenic cell lines. Two-component system CRISPR-Cas9 has emerged as a simple, efficient and accurate tool for generation of successful engineered strains (Jiang and Weeks 2017; Kao and Ng 2017). CRISPR-Cas9 has been derived from type II CRISPR system from *Streptococcus pyogenes*, relying on a single guide RNA (sgRNA) containing a sequence complementary to the target to lead Cas9, an endonuclease for initiating a double-strand break at the targeted gene on the host chromosome (Gordon et al. 2016; Kao and Ng 2017). A variant of CRISPR-Cas9 is CRISPR interference (CRISPRi) which is achieved by mutating the catalytic active site of Cas9 protein resulting in dead Cas9 (dCas9) (Posewitz 2017). The complex of sgRNA and dCas9 still binds to the target sequence and can be used for altering (repress/activate) the gene expression (Gordon et al. 2016).

To date a few genes have been targeted using CRISPR-Cas9 in different microalgal strains. The silencing of PEPC in cell wall-deficient strain of *C. reinhardtii* CC-400 cw 15 mt+ which resulted in increase in lipid content (28.5% of dry cell weight) being 74% higher than wild-type strain (Kao and Ng 2017). Further, attenuation of Zn(II)2Cys6 at 5′ UTR using CRISPRi resulted in twofold increase in the lipid productivity in *N. gaditana* (Ajjawi et al. 2017). Additionally, a few non-lipid augmenting targets have also been explored such as replacing the silacidin gene with resistance cassette (FCP: NAT) resulted in 85% NAT resistance colonies in *T. pseudonana* (Belshaw et al. 2017), knockout of CpFTSY gene in *C. reinhardtii* resulted in small and truncated chlorophyll antenna size resulted in overall increase in growth rate (Baek et al. 2016).

Another trans editing tool is TALEN, which comprise of TAL effector proteins that are secreted by *Xanthomonas* bacteria via type III secretion system (Christian et al. 2010). These TAL proteins are DNA-binding domains with multiple highly conserved 34 aa repeat modules with two divergent amino acids termed as repeat variable di-residues (RVD) (Miller et al. 2010; Serif et al. 2017). The RVD together are termed as targeting sequence and aid recognition of a specific sequence (Serif et al. 2017). The nuclease (Fok1) functions as dimer and requires two constructs of TAL proteins to generate double-strand break at the target site (Miller et al. 2010). The strand beak can then be repaired by the host machinery either by homologous recombination (HR) or non-homologous recombination (NHER). The former can be exploited for introduction of foreign sequence with a string homology to the DNA sequence adjacent to the target site, while the latter has high error rate and could be used to generate random insertions/deletions (Serif et al. 2017). The efficiency of TALE nucleases was demonstrated in *P. tricornutum*, by inactivation of UDP-glucose pyrophosphorylase which is involved in carbohydrate storage, and it thus increased the TAG content by 45% as compared to wild type (Daboussi et al. 2014).

5 Systemic Approaches for Integrating Algal Omics and Genetic Engineering for Custom Designing Microalgae for Biodiesel Production

Integration of the omics studies including transcriptomics, proteomics and metabolomics will aid in providing an accurate snapshot of the alterations in the metabolic pathways of potential microalgal strains cultivated under given set of conditions (Akula et al. 2009). This will in turn provide a hierarchical map of the gene, protein and metabolite regulation aiding in targeted and successful manipulation of microalgal strains for increasing the lipid productivity.

5.1 Omics Databases

System biology has the potential to integrate the results obtained from different omics studies with mathematical models and computational tools to develop detailed models illustrating the cellular metabolism of any microalga in question (Rodríguez-Moyá and Gonzalez 2010). To date, a few integrated platforms are available including ChlamyCys (for *C. reinhardtii*), Greenhouse (an online tool developed by Las Alamos National laboratory), Diatom EST database (for *T. pseudonana* and *P. tricornutum*), Alga-PrAs (algal protein annotation suite) and Cyan-Omics (omics database for cyanobacteria), respectively (https://greenhouse.lanl.gov/greenhouse) (Maheswari et al. 2009; Yang et al. 2015; Kurotani et al. 2017; Zhang et al. 2017).

5.2 Genome-Scale Models

Development of in silico mathematical models such as flux balance analysis (FBA), containing metabolic reactions and all the enzymes coding genes of the organism (Orth et al. 2010). A few of the FBA models constructed for different microalgal strains including C. reinhardtii, *Chlorella sorokiniana*, *Chlorella protothecoides*, and *Chlorella* sp. FC2 IITG, *P. tricornutum* and *Synechocystis*, respectively (Cogne et al. 2011; Boyle and Morgan 2009; Chang et al. 2011; Muthuraj et al. 2013a; Wu et al. 2015). Reconstruction of metabolic network of *C. reinhardtii* to illustrate primary metabolism leads to 458 metabolites and 484 metabolic reactions localized to three compartments: cytosol, mitochondria and chloroplast (Boyle and Morgan 2009). Further, the FBA model led to identification of one new gene (fructose 1,6-bisphosphatase) and 16 other genes which missing in the existing metabolic network. Another FBA model constructed for *C. reinhardtii* used a constraint-based approach to investigate the response of microalga under photoautotrophic conditions which highlighted the correlation of light-driven respiration and incident photon flux density (Cogne et al. 2011). Chang et al. constructed a FBA model for *C. reinhardtii*,

iRC1080 which comprised of 1080 genes, 2190 reactions and 1068 unique metabolites illustrating the importance of light-driven metabolism in green microalga (Chang et al. 2011).

Metabolic network construction of *C. sorokiniana* done in heterotrophic conditions comprising of 34 reactions and 37 metabolites including Embden Meyerhof-Parnas pathway (EMP), hexose monophosphates (HMP), TCA and fatty acid synthesis (Zhu and Huang 2017). Similarly, based on the genome of *C. protothecoides*, FBA model was reconstructed which was compartmented into four organelles including cytosol, chloroplast, mitochondria and peroxisome (Wu et al. 2015). The model consisted of 272 reactions, 270 enzymes and 461 encoding genes when the microalgal was grown in autotrophic and heterotrophic conditions. Muthuraj et al. constructed an FBA model of *Chlorella* sp. F22 IITG; under phototrophic and heterotrophic conditions, a shift in the intracellular flux was predicted during nutrient-sufficient to nutrient-deplete conditions (Muthuraj et al. 2013b). Under heterotrophic conditions, 50% of the total carbon flux was distributed towards glycolysis, while the rest was channelled into phosphate pentose pathway leading to carbohydrate synthesis. On the other hand, under autotrophic conditions, the carbon flux was directed towards Calvin cycle for fixation of CO_2, leading to the formation of dihydroxyacetone phosphate which then bifurcated acting as substrate for gluconeogenesis and glycolytic pathway (Muthuraj et al. 2013b). The FBA model for *P. tricornutum* was constructed utilizing the diatom genome, biochemical and online bioinformatics databases (Kim et al. 2016). The intracellular fluxes were calculated for autotrophic, mixotrophic and heterotrophic conditions leading to 587 metabolites and 849 reactions, compartmentalized into 4 main organelles including cytosol, mitochondria (matrix), chloroplast and peroxisome, respectively.

The genome-scale models developed so far include AlgaGEM (for *C. reinhardtii*), DRUM (for *T. lutea* and *C. sorokiniana*), iLB1027_lipid (for *P. tricornutum*), iCZ843 (for *C. vulgaris* UTEX 395), iN934 for *N. salina*, iRJL321 for *N. gaditana* and iSyp 821 for *Synechococcus* sp. PC 7002 (Gomes et al. 2011; Baroukh and Bernard 2016; Levering et al. 2016; Zuñiga et al. 2016). These genetic engineering techniques and system biology tools have not only aided in better understanding of the algal biology but also increased the lipid productivity of the microalgal strains, thereby providing a starting step towards sustainable biodiesel.

6 Conclusion and Future Avenues

Large-scale biodiesel production from microalgae relies on various factors including the achievement of high productivity per unit area, tolerance of the strain to outdoor fluctuating biotic and abiotic stress conditions, ease of harvesting along with sustainable lipid extraction techniques. Indeed, this requires better understanding of microalgal biology along with advancement of genome editing tools which will facilitate the development of high productive microalgal strains, capable of

autoflocculating, tolerating wide variety of stress conditions and ease of lipid extraction. The development and advancement of omic technologies, bioinformatics tools, genome editing approaches and system biology have improved our understanding and manipulation of microalgae biology. The omic studies conducted on different microalgal strains under various physiological conditions have provided insights into the TAG accumulation by identifying the key regulatory genes which could lead to hypothesis-driven strain engineering. On the other hand, the genome editing tools have not only verified the significant TAG augmenting genes but other significant genes pertaining to photosynthesis, CO_2 fixation and stress tolerance.

Ultimately, integration of algal omics, genome editing and system biology will play a fundamental role for achieving sustainable algal biodiesel in the near future. The future studies should focus on integration of the omics studies generated so far on different microalgal strains on a common platform for understanding the inter- and intraspecies variation which will lead to development of universal genome editing targets leading to industry desirable traits in one microalgal strain. However, it is crucial to demonstrate the performance of genetic engineered strains under outdoor conditions. Towards this, the US Environmental Protection Agency (EPA) recently, sanctioned outdoor cultivation of engineered *Acutodesmus dimorphus* which showed *Aequorea victoria* GFP expression (Szyjka et al. 2017). Cultivation of this microalgal strain outdoor for 50 days showed that it did not outcompete or adversely affected the native algal population, providing a staring step for engineered strains.

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Part II
Microalgae for Biofuel Production

Chapter 9
Manipulation of Microalgal Lipid Production: A Genetic Engineering Aspect

Su Chern Foo, Nicholas M. H. Khong, and Fatimah Md. Yusoff

Abstract Interests in microalgal lipids as green and renewable energy sources are piquing as cheap hydrocarbon fossil fuels reach their limit. Lipids from microalgae have important human uses, i.e., energy, food, and pharmaceuticals, depending on its quantity and quality. Genetic engineering is the introduction or suppression of a target gene for the selective expression of a bio-product, e.g., hydrocarbons for fuel or polyunsaturated fatty acids (PUFAs) for food, at a favorable quantity. Past studies like nitrogen starvation or salinity stress have shown to increase lipid contents of microalgae; however, studies on the molecular mechanisms underlying these stress-induced lipid productions remain limited. Next, complementing environmental stress manipulation with genetic engineering would potentially be a better and more effective approach to increase microalgae lipid production and accumulation. There are generally two approaches to enhance microalgae lipid production on a molecular level: firstly, overexpression and improvement of key enzymes involved in fatty acid and isoprenoid biosynthesis and, secondly, repression of lipid catabolic and competitive pathways such as beta-oxidation and starch synthesis. This review provides an update of microalgae lipid research findings to date and aims to address recent system biology discoveries and approaches on microalgae lipid production, the roadblocks encountered, and help needed to realize the ultimate goal, that is, microalgal lipids as sustainable resources for energy and high-value products.

Keywords Microalgae · Lipid content · System biology · Genes manipulation · Genetic engineering

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1 Introduction

Renewable energy is the world's fastest-growing energy sector set to replace fossil fuels, with projected consumption increasing by an average of 2.3% per year between 2015 and 2040 (EIA 2017). This is unsurprising as energy is the essential ingredient directly powering most, if not all aspects of economic-related developments. Thus, energy security is crucial in ensuring continuous progress. As cheap fossil fuels wane, a balancing act from unconventional and renewable energy sources like microalgae is needed to support the ever-increasing man's insatiable demand for energy. Three main evaluation criteria for potential renewable energy sources include (1) the availability and accessibility of resources; (2) the affordability of the resource acquisition and energy infrastructure; and (3) the environmental aspect or acceptability of the resources and its supply.

Bioenergy and biomass are terms used interchangeably describing energy source from a living or dead organism containing carbon. Biomass is used to produce biofuel types, i.e., biodiesel, biogas, or bio-alcohol. Specifically, biofuels are liquid oil extracted from crops like maize, soy, rapeseed, palm, or microalgae and subsequently transesterified to power our transportation systems. A past review by Pogson et al. (2013) compared long-term cost and environmental impact and concluded that although terrestrial energy crops are advantageous economically, the drawback was a threatening risk to food security where limited arable land to grow food crops will face tight competition with energy crops. In other words, a bioenergy source should be selected such that its biomass is capable of producing the highest net energy per unit area of space. In which case, microalgae biomass fits nicely to the energy security criteria due to the fact that they are ubiquitously found, have the ability to thrive in aquatic habitats thereby reducing land cost, and efficiently capture carbon through photosynthesis.

In addition, biofuels from microalgae are cleaner and greener than fossil fuels due to several reasons. Firstly, microalgae demonstrated high photosynthetic efficiency and ability to adapt to stressful conditions compared to terrestrial crops, stretching its potential for higher biomass productivity. For example, *Botryococcus braunii* has the ability to store lipids at more than 50% of their dry cell weight when subjected to stressed conditions, e.g., nitrogen deficiency (Chinnasamy et al. 2010). Secondly, in any strategic option to achieve sustainable energy advancement and fuel security within the means of research, innovation, and development, having a variety of biomass producers is ideal. There are several varieties of oleaginous microalgae species to bioprospect from, and they exist either as single cell (e.g., *Chlamydomonas reinhardtii*, *Dunaliella salina*) or colonies (e.g., *Thalassiosira pseudonana*, *Scenedesmus obliquus*). Thirdly, their small and primitive cell structure allows for rapid cell doubling and relatively easier oil extraction compared to higher plants. Besides being able to grow in aquatic habitats, there is a good chance for this phylum to be cultivated for its biomass in abandoned tin mining lakes, lagoons, or even unproductive water bodies like wastewater effluents. It is an added advantage when a sustainable energy source is produced at minimum cost to the environment,

while their photosynthetic activity helps to reduce CO_2 concentration in the atmosphere.

1.1 Selected Approaches to Increase Lipid Production

Microalgae are known to be important sources of triacylglycerols (TAGS) and high-value compounds such as long-chain polyunsaturated fatty acids (LC-PUFAs) and carotenoids (Begum et al. 2016; Foo et al. 2017; Medipally et al. 2015; Shang et al. 2018). Nutrient stress and physical culture variations (e.g., light intensity, CO_2 inputs) are some of the conventional strategies used to increase total lipids in microalgae, effective only to a certain degree (Table 9.1). A knowledge gap observed was that most studies performed did not delve further into elucidating the mechanisms for increased lipid production. Furthermore, applying external stress and culture manipulations (Singh et al. 2016; Zienkiewicz et al. 2016; Shang et al. 2016) tend to face problems during commercial-scaled productions (e.g., slow growth rate, culture crash, and inconsistency with laboratory-scale production). For instance, Breuer et al. (2013) described that the current lipid production in mass culture is about five times lower than that of the maximum theoretical production values due to uncontrollable factors such as pH, light intensity, temperature, and photoperiod that are subjected to environmental and climate changes when grown outdoors. Eventually, the key to manipulation of microalgae lipid production lies in a more thorough understanding of the behavior and biochemical metabolism of the organism. As such, integrative approaches like combining system biology in the comprehension of optimum lipid production in microalgae under specified levels of environmental stress would deem better and more reproducible results. It was reported that the integration of different approaches such as multi-omics technologies, gene manipulation, and synthetic biology design could potentially increase lipid production (Chen et al. 2017).

1.2 Molecular Approach to Increase Lipid Production

A quick search on the term "microalgae" using Scopus® search engine yielded 18,635 published documents, traceable since 1960. Among them, publications regarding "microalgae and lipid" ensued beginning from the year 1980, totaling 4631 publications till date (Fig. 9.1). More importantly, genetic studies related to microalgal lipids started in the 1990s, parallel to the genomic era and intensifying at the last quartile of 2000–2010. Molecular manipulations of microalgae especially involving expression of foreign genes within selected microalgae had been reported as early as 1992. However, most of these studies, involving gene splicing and biotransformation, produced transient to nonsignificant outcomes. Most molecular manipulations of microalgae for the enhancement their biofuel potential nowadays

Table 9.1 Selected approaches to increase microalgae lipid production

No.	Approaches	Findings	References	Suggestions
1	Environmental stresses, e.g., salinity, nutrient stress (N, P, Fe, Mg)	Enhanced total lipid production	Adenan et al. (2013), Chen et al. (2017), and Shang et al. (2016 #214);	To identify metabolic pathways
2	Cultivation conditions, e.g., media type, light, pH	Enhanced total lipid production	Medipally et al. (2015), Ji et al. (2013), and Cao et al. (2014)	To consider cost for economic sustainability
3	Combination of nutrient stress, chemical additives, and phytohormones	Production can be scaled up	Singh et al. (2016)	To consider economic and environmental costs
4	Membrane lipid augmentation	Regulation of membrane lipid composition and structure	Escribá (2017)	To identify molecular controlling factors in lipid production
5	Enhance lipid biosynthesis in microalgae cells	Kinetic modeling and metabolic flux analysis used to stimulate algal growth and lipid metabolism	Lenka et al. (2016)	To examine pathway, computational approaches, and molecular genetic manipulation
6	Isotopic labeling	Central carbon metabolic steps generate precursors for fatty acid biosynthesis and lipid assembly	Allen et al. (2015)	To quantify fluxes and metabolic operations in plant tissue
7	Metabolic acclimation mechanism	Increased uptake of industrial CO_2	Collet et al. (2014), Guo et al. (2017), and Aslam et al. (2018)	To examine compatibility of species to uptake industrial CO_2 waste for increased microalgae growth
8	Integration of environmental stresses and system biology	Enhanced production through genetic engineering for microalgae biorefinery	Chen et al. (2017), Winck et al. (2013), and Sankari et al. (2017)	To understand microalgae gene regulation under stressed conditions

fell into a few strategic categories including those that aimed to improve carbon capture and storage (carbon sequestration) or bypass redundant mechanisms in order to enhance energy efficiency and utilization; as well as those focusing on greener and low-carbon processes for the development and promotion of biomass growth.

The single most unique potential of microalgae is seen in the fact that they not only photosynthesize but have a relatively faster reproduction cycle than plants. In the meantime, they possess single-cell characteristics close to bacteria. Thus, strategies for the enhancement of lipid production in microalgae utilizing transgenic technology are emulated from those eminently used in bacteria and plant models:

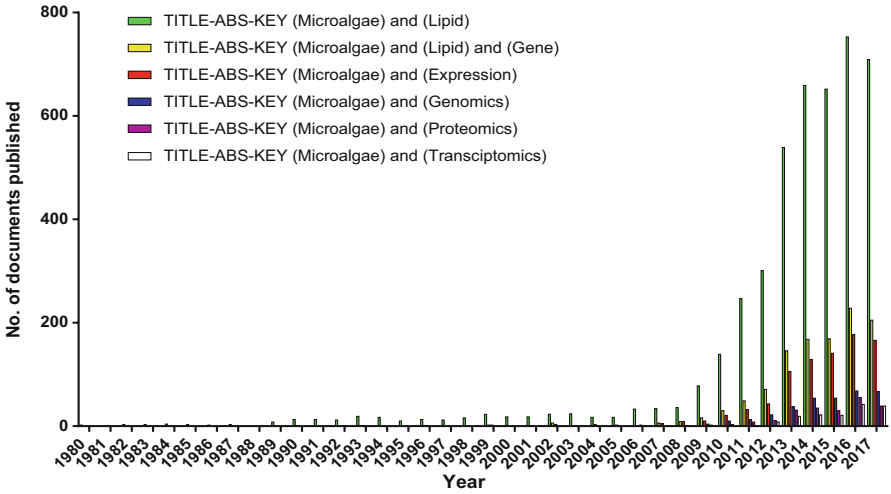

Fig. 9.1 The last 10 years show increased number of studies on system biology approaches to increase microalgae lipid production. (Source: Scopus, accessed on 5 March 2018)

three of which are especially well known, i.e., electroporation, particle bombardment, and *Agrobacterium* transformation. These methods have been proven successful to a certain extent in a few microalgae, e.g., *Chlamydomonas reinhardtii* (Doron et al. 2016; Jeon et al. 2013; Pratheesh et al. 2014). Then again, microalgae are neither bacteria nor plant. The most basic knowledge regarding susceptibility of microalgae to different antibiotics, which is imperative in biotransformation study (as common procedure to screen successful mutation), is only reported a decade after the new millenia. It became clearer that a more holistic understanding of microalgae biology and the regulation of metabolic pathways at the whole-cell level, rather than at the single pathway level, including environmental stress-induced metabolic responses, was needed. In this regard, "omic" technologies has come a long way with the establishment of universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) in selected microalgae models in a nontargeted and non-biased manner. These omic technologies can also be referred to as high-dimensional biology, and the integration of these techniques is referred to as system biology.

Genomics refers to the study of genes and their functions and related techniques (WHO 2018). Unlike genetics that only scrutinizes the functioning and composition of the single gene, genomics addresses all genes and their interrelationships to identify their combined influence on the growth and development of the organism. Comparative genomics analyses using bioinformatics tools have been performed to identify genes involved in lipid biosynthesis in various oleaginous plants especially maize *Arabidopsis*, *Brassica*, soybean, and castor. Similar approaches, thus, have been carried out since 2005, to alter fatty acid composition in microalgae through plant genetic engineering approaches. Nonetheless, recognition of genes responsible

for oil accumulation, specific to microalgae, is a prerequisite to targeting the organism for any metabolic engineering aiming to enhance biofuel yielding potentials. About 30 genomes of microalgae have been sequenced and published till date (https://genome.jgi.doe.gov/Algae/Algae.info.html). On the other hand, the transcriptome of an organism refers to the total RNA present in the cells and does not always correlate with the translatome or the proteome. The translatome is the total number of proteins within the cell under a given condition. The proteome is the total set of proteins produced by an organism, a cell, a tissue, or even by a genome. The reason for this is because many RNA molecules are not translated into protein, that is, they function in the cell as RNA only. Examples of this include rRNA and tRNA. Additionally, alternative splicing mechanisms, proteolysis, posttranslational modifications, etc. play a role in the disparity. Proteomics is a general term comprehending the analysis of the entire protein complement of a cell, tissue, or organism including the alterations or modifications produced in native protein of organisms under a specific, defined set of condition. The term proteomics first appeared in 1997 (Shah and Misra 2011) from the root word proteome which is the combination of protein and genome coined by Mark Wilkins in 1994. Meanwhile, study on microalgal proteomics was initially published in the early 2000s, describing protein fingerprinting of *Haematococcus pluvialis* in response to external stresses (Wang et al. 2003, 2004a, b) as well as variations of protein profile alterations of *Nannochloropsis oculata* following cadmium exposure (Kim et al. 2005). Microalgal proteomic analysis for lipid enhancement was only published around 2010 focusing discovery of new proteins involved in the lipid metabolism of selected microalgae (Nguyen et al. 2011; Terashima et al. 2010).

2 System Biology Approach to Increase Lipid Production

System biology is the study of several interacting complex biological networks as one integrated biological system. Empirically, system biology involves the following: firstly, the collection of large sets of omics data; secondly, mathematical modeling using bioinformatics tools; and finally, assessment of the model quality with experiment validation (Hood and Perlmutter 2004). Many scientific studies has been done to enhance microalgae lipid content (Dahmen-Ben Moussa et al. 2017; Hena et al. 2018; Peccia et al. 2013); however, it has come to realization that the full characterization and understanding of the complexity of the cell's biological system as a whole are limited. For example, most classical studies focused on a single isolated pathway thus neglecting uncontrollable variables occurring to other biological pathways. In addition, differences of lipid production pathways between microalgae classes as well as the effect of stress on lipid type (Hockin et al. 2012) are some of the research questions that need to be answered.

System biology addresses this gap by taking into account high-throughput data and passing it through intensive computation. Bioinformatics software organizes the data to ultimately obtain meaningful information. In this case, a scientist can make

use of this approach to not only identify the key pathways, i.e., fatty acid biosynthesis, isoprenoid biosynthesis, and other competitive metabolic pathways, but to understand how they interact with each other as an integrated biological system. The learnings can then be applied in achieving the final goal of enhancing microalgae lipid content.

In 2003, the human genome project was completed. It took scientists approximately 13 years to fully sequence the human genome and as a consequence, molecular studies conducted from the year 1990 to 2012 was termed the genomic era. At the same time, whole genome sequencing for a diatom, *Thalassiosira pseudonana* (Armbrust et al. 2004), and a rhodophyte, *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), was completed in 2004. Since then, an increased amount of molecular studies especially on *Thalassiosira pseudonana* was observed in the field of phycology research.

2.1 Microalgae Lipid Research in the Genomic Era (1990–2012)

A research study conducted in the late 1990s described the overexpression of acetyl coenzyme A carboxylase (ACCase) in the diatom, *Cyclotella cryptica* (Dunahay et al. 1996). As a result of introduction of this gene using microparticle bombardment, transformed lines produced two to three times more lipids than that of wild type. This was the first report of genetic engineering in chlorophyll *c* containing microalgae strain. In another study, insertion of a foreign gene, isoprene synthase (IspS) from plant vine, *Pueraria montana* into *Synechocystis* PCC 6803 accumulated as much as 50 μg isoprene per g dry weight/day (Lindberg et al. 2010). Isoprenoids are small volatile hydrocarbons useful as renewable biofuel (George et al. 2015). On the other hand, redirecting carbon flow away from starch synthesis and to neutral lipid synthesis increased lipid production. Li et al. (2010) showed that silencing of ADP-glucose phosphorylase increased total lipids by 32.6% in transgenes compared to wild-type *Chlamydomonas reinhardtii*. In summary, it is such studies that laid the foundation for microalgae lipid genetic engineering in the post genomic era (2012 to date).

2.2 Molecular Technique and Tools Development

The genomic era witnessed the completion of whole genome sequencing studies in selected microalgae species, i.e., *Thalassiosira pseudonana*, *Cyanidioschyzon merolae* 10D, and *Phaeodactylum tricornutum*. These research studies provided a wealth of genomic data to be taken to the next level, e.g., transformation and transcriptomic manipulation studies. It is thus assumed that the rest of the microalgae

to follow suit where accurate and reliable genomic data would be stored on a public platform. At the same time, more efficient molecular tools in nuclear transformation yielded a range of expressed sequence tag (EST) markers, DNA tagging fluorescence markers, as well as transcription and transformation techniques. Table 9.2 shows some of the major research efforts made in the genomic era (1990–2012) contributing toward today's advances in microalgae lipid engineering.

3 Genetic Engineering Metabolic Pathways for Increased Lipid Production

Post genomic era (since 2013 to date), a steady increase of scientific studies was found to focus on fundamental elucidation of different metabolic pathways and the involving enzymes and substrates in microalgae. This is a valid endeavor because in-depth information of different genes, pathways, and mechanisms involved in lipid synthesis is crucial to the improved comprehension of processes ultimately enabling development of interventions to an enhanced lipid production in microalgae. It is most critical to recognize the genes involved and their functional-physiology relationship in lipid biosynthesis of microalgae prior to any intention of manipulations for practical outcomes. Although some candidate genes involved in lipid biosynthetic pathway of selected microalgae have been suggested (Khozin-Goldberg and Cohen 2011; Misra et al. 2012), this area of study still presents plenty of opportunity for further discoveries.

Essentially, there are two major ways to enhance lipid production on a molecular level: firstly, improvement of fatty acid and isoprenoid production through upregulation of key biosynthetic enzymes, secondly, suppression of lipid catabolic activities as well as redirecting carbon flow from carbohydrate for lipid synthesis. Figure 9.2 presents an overview of genetically engineering microalgae for clean, green, and sustainable energy.

3.1 Upregulating Gene Expression in Fatty Acid and Isoprenoid Pathways

3.1.1 Fatty Acid Biosynthesis

Triacylglycerols (TAG) is the resulting ester of three fatty acid molecules and one glycerol which takes place in the endoplasmic reticulum of the cell. There are three major steps in fatty acid biosynthesis. The first step involves carboxylation of acyl-CoA to form malonyl CoA, catalyzed by the acyl-CoA carboxylase enzyme (ACCase). Next, elongation step takes place with loading of malonyl CoA onto acyl carrier protein (ACP); catalyzed by fatty acid synthase (FAS) enzymes, i.e., 3-ketoacyle-ACP synthase (KAS), 3-ketoacyl-ACP reductase (KAR),

Table 9.2 Molecular technique and tools employed in the genomic era (1990–2012)

Molecular technique	Molecular tool	Gene of study	Microalgae strain	Phylum	Use	References	Potential application to microalgae lipid genetic engineering
Genome sequencing	Whole genome shotgun approach	Whole genome	*Thalassiosira pseudonana*	Heterokontophyta	Sequence and annotation of whole genome enabled establishment of diatom evolutionary history (secondary endosymbiosis), nitrogen metabolism pathway, etc. In particular, two pathways of beta-oxidation of fatty acid was elucidated	Armbrust et al. (2004)	Building upon this discovery, future studies on nitrogen starvation and its effect on gene expression can be compared. Also, silencing of genes involved in beta-oxidation with RNAi is a good opportunity to explore into
Genome sequencing	Whole genome random sequencing method	Whole genome	*Cyanidioschyzon merolae* 10D	Rhodophyta	Sequence and annotation of genome provide a model system with a simple gene composition for studying origin, evolution, and fundamental mechanisms of eukaryotic cells	Matsuzaki et al. (2004)	A good platform for recombinant proteins and valuable compounds

(continued)

Table 9.2 (continued)

Molecular technique	Molecular tool	Gene of study	Microalgae strain	Phylum	Use	References	Potential application to microalgae lipid genetic engineering
Genome sequencing	Diatom genome was annotated using JGI annotation principles	Whole genome	*Phaeodactylum tricornutum*	Heterokontophyta	Sequence and annotation allowed for comparison with *Thalassiosira pseudonana* genome in terms of evolutionary history	Bowler et al. (2008)	This work provided a complete DNA database of another oleaginous species for genetic engineering
Gene characterization	Bioinformatics programs like sequence analysis using MACAW and ALIGN program	Acetyl-coenzyme A carboxylase (ACCase)	*Cyclotella cryptica*	Heterokontophyta	Genomic library construction contributed to full characterization of ACCase gene	Roessler and Ohlrogge (1993)	Genetic information will enable examination of mRNA expression of ACCase gene, a rate-limiting enzyme in lipid biosynthesis
Transcriptome analysis	Expressed sequence tag (EST)	Short gene sequences expressed from mRNA	*Chlamydomonas reinhardtii*	Chlorophyta	EST generated from this study are useful source for gene analysis related to carbon stress acclimatization	Asamizu et al. (2000)	Similar approach can be done related to nitrogen stress to induce lipid accumulation
Transcriptome analysis	Expressed sequence tag (EST)	Short gene sequences	*Fragilaria cylindrus* Grunow	Heterokontophyta	The EST information generated	Mock et al. (2006)	Understanding the gene expression can allow for

		expressed from mRNA			provided insights to cold adaptation		manipulation of lipid type
Transcriptome analysis	Expressed sequence tag (EST)	Short gene sequences expressed from mRNA	*Ankistrodesmus convolutus* Corda	Chlorophyta	Useful for characterizing genes and sequences of a new species	Tanh et al. (2011)	Studies on stress responsive genes is encouraged to understand isoprenoid synthesis under nutrient-limiting conditions
Transformation	Microparticle bombardment and plasmid vector pNICgfp	Fucoxanthin chlorophyll a-/c-binding protein (fcp) and nitrate reductase (NR) from *Cylindrotheca fusiformis*	*Phaeodactylum tricornutum* Bohlin	Heterokontophyta	An efficient transformation method for gene knockout using homologous recombination or RNAi was introduced	Miyagawa et al. (2009)	A tool for high-throughput analysis of gene function
Transformation	RNAi	A gene X inverted repeat (IR) is nested within MAA7 gene on the transformation plasmid	*Chlamydomonas reinhardtii*	Chlorophyta	An efficient link between level of resistance to 5-fluoroindole (5-FI) to the silencing of MAA7 gene, which in turn silences target gene	Rohr et al. (2004)	Posttranscriptional gene silencing achieved by generating transformants that expressed an inverted repeat (IR) gene construct thus, reducing the abundance of a gene product. In the case of lipid engineering, this transformation model could potentially be applied

(continued)

Table 9.2 (continued)

Molecular technique	Molecular tool	Gene of study	Microalgae strain	Phylum	Use	References	Potential application to microalgae lipid genetic engineering
DNA tagging	Fluorescence tag	Green fluorescence protein (GFP)	*Chlamydomonas reinhardtii*	Chlorophyta	cgfp is a useful tool to visualize DNA and protein expression	Fuhrmann et al. (1999)	Possibility to apply to related green algal species
DNA tagging	Fluorescence tag	Luciferase gene (lue)	*Synechocystis* 6803	Cyanobacteria	An optimized quantification of luciferase from sediment was described	Moller et al. (1995)	Possibility of quantification of lipids via flow cytometry

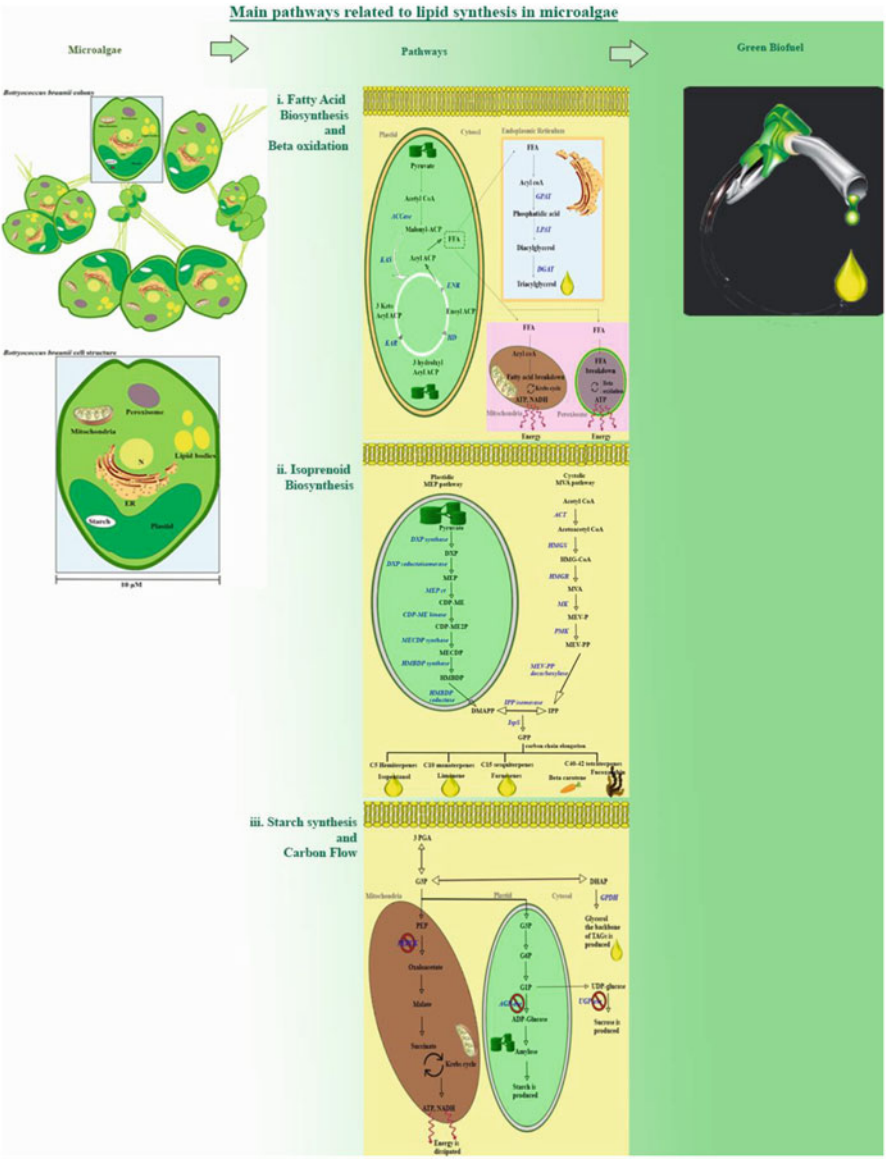

Fig. 9.2 Overview of genetic engineering lipid-related pathways in microalgae

3-hydroxyacyl-ACP dehydratase (HD) and enoyl-ACP reductase (ENR). Finally, termination step takes place when fatty acid reaches its designated carbon chain length. The enzyme thioesterase (TE) hydrolyzes ACP, thus releasing the newly synthesized fatty acid either to the chloroplast (C16 and C18; Sn-2) or endoplasmic reticulum (C16 and C18; Sn-1). Figure 9.3 elucidates the fatty acid biosynthesis

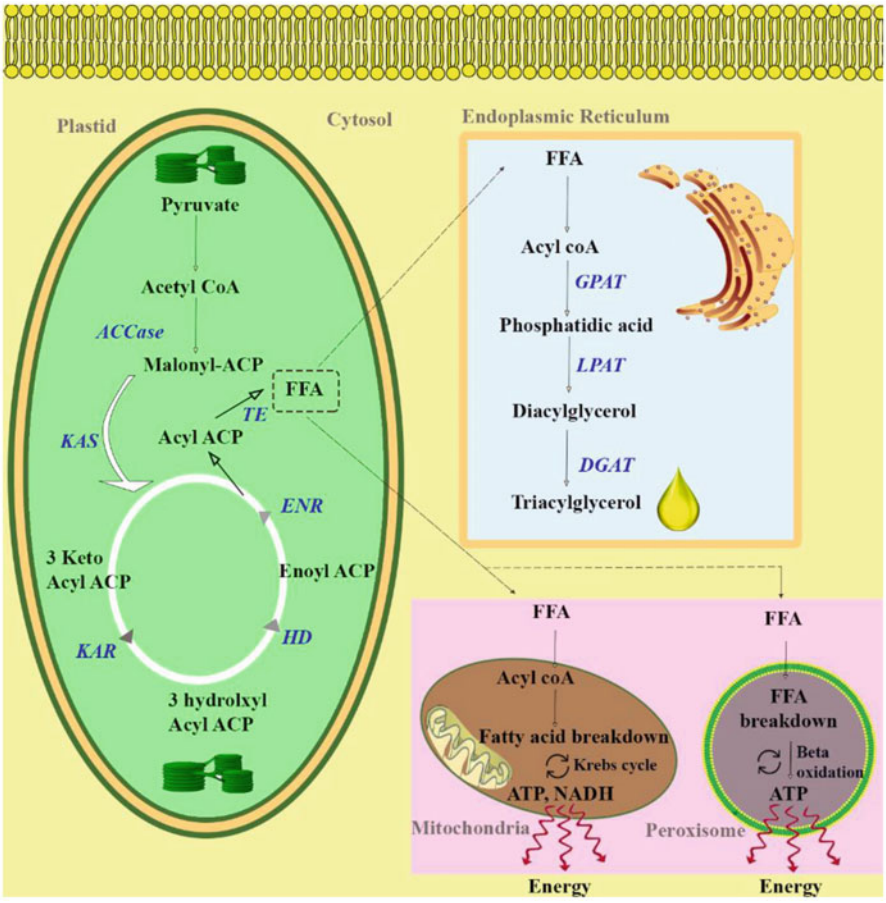

Fig. 9.3 Fatty acid biosynthesis and beta-oxidation pathway metabolites in black font and enzymes in blue italicized font. Free fatty acids are elongated in the endoplasmic reticulum to form triacylglycerol (TAG), whereas beta-oxidation occurs to generate ATP. *ACCase* acetyl-coA carboxylase, *ACP* acyl carrier protein, *COA* coenzyme A, *DGAT* diacylglycerol acyltransferase, *ENR* enoyl-ACP reductase, *TE* Thioesterase, *GPAT* glycerol-3-phosphate acyltransferase, *HD* 3-hydroxyacyl-ACP dehydratase, *KAR* 3-ketoacyl-ACP reductase, *KAS* 3-ketoacyl-ACP synthase, *LPAT* lyso-phosphatidylcholine acyltransferase, *NADH* nicotinamide adenine dinucleotide, *FFA* free fatty acid. (Figure adapted and modified from Radakovits et al. 2013)

occurring in the chloroplast and subsequent elongation and unsaturation process in the endoplasmic reticulum.

Table 9.2 further elaborates on the different enzymes, e.g., ACCase, DGAT1/2, and TE subjected to genetic engineering in the fatty acid biosynthesis pathway. It was observed that these gene targets were chosen mainly due to their rate-limiting nature under the assumption that by increasing availability of rate-limiting enzyme, more lipids will accumulate.

3.1.2 Isoprenoid Biosynthesis

Isoprenoids, also known as terpenes, are suitable as carbon neutral biofuels owing to their methyl branching and cyclic structure which lower freezing point and increase energy density, respectively (George et al. 2015). Isoprenoid-based biofuels have similar properties (e.g., high cetane numbers of at least 40) to conventional commercial fuels. An added advantage of microalgae-based isoprenoids is water immiscibility, i.e., spontaneously separating from biomass into culture media. This is preferred as no additional cost and effort are needed during extraction process (Lindberg et al. 2010).

Isoprenoid-based biofuels are classified into groups based on their number of carbons, i.e., C5 hemiterpenoids (e.g., isopentanol, isoprene), C10 monoterpenoids (e.g., limonene, pinene), and C15 sesquiterpenoids (e.g., farnesenes, bisabolenes) (Phulara et al. 2016). Subsequent elongation of terpenoids to C40–43 terpenoids produces commonly known bioactives like beta-carotene and fucoxanthin. Fucoxanthin is a potent bioactive able to confer anticancer (Jin et al. 2018), antiviral (Unnithan et al. 2014), and significant antioxidant (Foo et al. 2017) properties.

Two major pathways take place for isoprenoid biosynthesis, i.e., cystolic mevalonate (MVA) pathway and plastidic methylerythritol 4-phosphate (MEP) pathway (Paniagua-Michel et al. 2012). To date, there are still limited studies on pathway elucidation of isoprenoids in microalgae. Nevertheless, among them showed a pattern in the preferred isoprenoid pathway of synthesis in different phylogeny groups. The greens (*Scenedesmus* sp., *Dunaliella salina*) followed the MEP pathway for isoprenoids and sterols (Schwender et al. 2001), while the brown diatoms (*Nitzschia* sp., *Phaeodactylum* sp.) employed the use of both MEP pathway for carotenoids and MVA pathway for sterols (Lichtenthaler 2004). Following, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) with five-carbon chain length are the two building blocks for isoprenoid biosynthesis. Figure 9.4 shows the production of IPP and DMAPP.

3.2 Downregulating Lipid Catabolic Enzymes and Redirecting the Carbon Flow

3.2.1 Beta-Oxidation Pathway

Principally, beta-oxidation is a catabolic process of storage lipids with the aim of harvesting energy. This is done by breaking down fatty acids to two-carbon fragments, i.e., acyl-CoA which enters the Krebs cycle to produce ATP (Campbell and Reece 2002). This process occurs in both mitochondria and peroxisomes (Fig. 9.3). Catabolic enzymes like lipase, phospholipase, and acyltransferase are important in regulating beta-oxidation thus becoming targets in genetic engineering for the selective lipid accumulation in microalgae. Earlier, Trentacoste et al. (2013) demonstrated that by knocking down genes expressing lipase, phospholipase, and

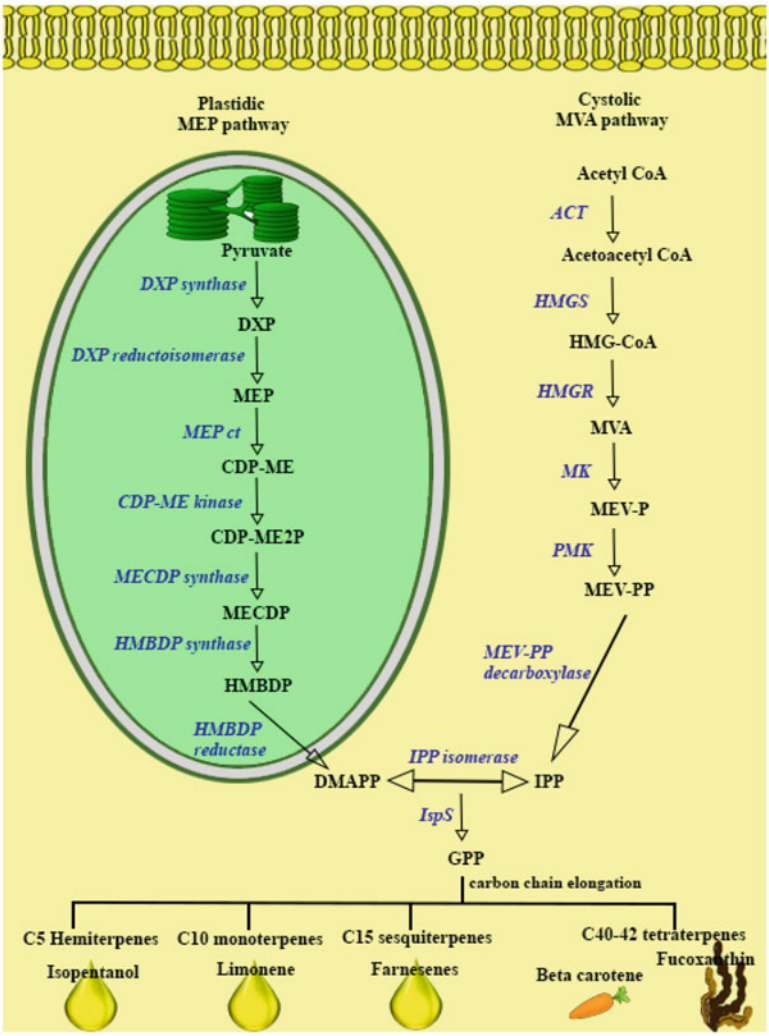

Fig. 9.4 Two major pathways of isoprenoid biosynthesis, MEP and MVA where metabolites are in black font and enzymes in blue italicized font. *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-D-erythritol 4-phosphate, *MEP ct* 2-C-methyl-D-erythritol 4-phosphate cytidylytransferase, *CDP-ME* 4-(cytidine 5B-diphospho)-2-C-methyl-D-erythritol, *CDP-ME2P* 2-phospho-4-(cytidine 5B-diphospho)-2-C-methyl-D-erythritol, *MECDP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBDP* (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate, *ACT* Acetoacetyl CoA thiolase HMGS, 3-hydroxy-3-methylglutaryl synthase, *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *HMGR* 3-hydroxy-3-methylglutaryl reductase, *MVA* mevalonate, *MK* mevalonate kinase, *MEV-P* phosphomevalonate, *PMK* phosphomevalonate kinase, *MEV-PP* diphosphomevalonate, *IPPi* isopentenyl diphosphate isomerase, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *IspS* isoprene synthase. (Figure adapted and modified from Kuzuyama and Seto 2012)

acyltransferase in *Thalassiosira pseudonana*, an increase of 3.2–4.1-folds in total lipids, as compared to the wild strain, is observed. Similarly, Zhang and Liu (2016) also reported an increase of 23% total lipids in transformed *Isochrysis galbana*, compared to wild type, by salicylhydroxamic acid inhibition of the mitochondrial alternative oxidase pathway. Salicylhydroxamic acid, the mitochondrial alternative oxidase inhibitor of the electron transport pathway, freed up substrates and energy, i.e., NADH, thereby increasing lipid yield.

3.2.2 Starch Synthesis and Carbon Flux

Carbon flux or carbon partitioning is defined as the competition between synthesis of starch and TAG for common metabolites, i.e., 3-phosphoglycerate (3PGA) and glyceraldehyde 3-phosphate (GA3P) (de Jaeger et al. 2014). As both starch and TAG biosynthesis competes for these substrates, the assumption was that blocking starch formation will divert carbon flux toward TAG accumulation. Consequently, studies to genetically rechannel carbon flow in the plastid of green microalgae, *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*, were reported (de Jaeger et al. 2014; Li et al. 2010).

It is important to identify the cog that turns the wheel, and in this case, blocking the crucial enzymes of starch synthesis will allow TAG accumulation (Table 9.3). For example, phosphoenolpyruvate kinase (PEPCK) functions to flux out oxaloacetate which subsequently redirects carbon flow to fatty acid biosynthesis. Figure 9.5 elucidates suppression of key enzymes in carbon flux.

4 Roadblocks in Genetically Engineering Microalgae for Lipids

Research findings in the last 10 years in microalgae genetic engineering have unveiled to scientists and industries a glimpse of the vast potential it holds. Among the applications of the bio-products from these tiny cells are not limited to biofuel but also pharmaceuticals, value-added food products, cosmeceuticals, animal feed, carbon sinks and even bioaccumulators in wastewater treatments. Nevertheless, it is important to note that not every genetic manipulation results in stable transformed cells with the desired phenotypic expression. Often, there is a butterfly effect from genetically manipulating genes, i.e., a slower growth rate in transformed cells. For example, silencing AGPase in starch synthesis to redirect carbon flux resulted in 32.6% increase of total lipids in transformed *Chlamydomonas* cells; however, the trade-off was a slower growth rate due to altered energy partitioning in PSII that was dissipated as heat rather than photochemical conversion to promote growth (Li et al. 2010). Despite so, some studies reported no negative effects on the growth of their transformed microalgae observed 5 days after gene knockdown of

Table 9.3 Pathway and genes involved in the growth and regulation of microalgae lipid biosynthesis and its competitive metabolic activities (beta-oxidation and starch synthesis)

Mode of genetic engineering	Specific pathway	Genes	Microalgae strains	Form of delivery	External factor	Increase in total lipids	Lipid class	References
Fatty acid biosynthesis	Thioesterase (TE) overexpression	Genes insertion of TE and ACP genes from *Cuphea lanceolata*	*Chlamydomonas reinhardtii* TKAC 1017	Electroporation	None	1.25-fold vs. wild type	C14:0 increased by 1.25–1.58-fold	Inaba et al. (2017)
Fatty acid biosynthesis	Thioesterase (TE) overexpression	Gene insertion of lauric acid-biased TE (C12TE) from *U. californica* and KAS (KASIV) from *C. hookeriana*	*Dunaliella tertiolecta*	Glass bead bombardment	None	fourfold vs. wild type	C12:0 increased by sevenfold and C14:0 increased by fourfold vs. wild strain	Lin and Lee (2017)
Fatty acid biosynthesis	Thioesterase (TE) overexpression	Gene insertion of fatty acyl-ACP thioesterase from *Dunaliella tertiolecta*	*Chlamydomonas reinhardtii* CC 424	Glass bead bombardment	None	56% improvement in total lipids	63% and 94% more neutral lipids ideal for biofuels	Tan and Lee (2017)
Fatty acid biosynthesis	Diacylglycerol acyltransferase 2 (DGAT 2) overexpression	Gene insertion using vector pAlgaeDGAT-eGFP	*Chlamydomonas reinhardtii* wild type CC-125	Electroporation	None	12% increase in ALA in transformed line	PUFA increased, while SFA decreased	Ahmad et al. (2015)
Fatty acid biosynthesis	Diacylglycerol acyltransferase 2 (DGAT 2) overexpression	Gene insertion using vectors encoding Thaps3_25595 Tag RFP or eGFP contransformed with pMHL_9 encoding selective marker nourseothricin acetyltransferase, NAT	*Thalassiosira pseudonana*	Microparticle bombardment with Bio-Rad biolistic particle delivery system	Silicon starvation	1.52–1.95-fold increase vs. wild type	Substantial increase in C20:5 (EPA) and C22:6 (DHA)	Manandhar-Shreshtha and Hildebrand (2015)

Fatty acid biosynthesis	Diacylglycerol acyltransferase 1 (DGAT1) overexpression	Gene insertion of plasmid pfcpA: EGFP-HOGP and pDGAT1: EGFP	*Phaeodactylum tricornutum* CCAP 646	Microparticle bombardment with Bio-Rad biolistic particle delivery system	Nitrogen starvation	25–30% increase vs. wild type	Increase in C16:0 (>30%), C16:1 (>40%)	Shemesh et al. (2016)
Fatty acid biosynthesis	Acetyl CoA carboxylase (ACCase)	Overexpression of ACCase	*Cyclotella cryptica*	Microparticle projectile bombardment	None	2–3-fold increase of ACCase expression vs. wild type	NA	Dunahay et al. (1996)
Isoprenoid biosynthesis	Mevalonic acid pathway	Overexpression of isoprene synthase genes (IspS)	*Synechocystis* PCC 6803	Eaton-Rye (2011) method	None	Isoprene yield improvement of 2.5-fold for 4 genes as compared to one isoprene synthase gene only	N/A	Bentley et al. (2014)
Isoprenoid biosynthesis	Switching on MVA pathway	Expression of isoprene synthase (IspS gene)	*Synechocystis* PCC 6803	Foreign and characterized gene insertion (IspS) from a plant called Kudzu vine (*Pueraria montana*)	None	Accumulation of around 50 ug isoprene per g dw/day	N/A	Lindberg et al. (2010)
Isoprenoid biosynthesis	Ketosynthase (FabH) overexpression	Gene replacement of FabH (ATCC 27264D-5) into *E. Coli* and transferred into *Synechococcus*	*Synechococcus* PCC 7002	Restriction enzymes and DNA ligase	None	N/A	N/A	Kuo and Khosla (2014)
Beta-oxidation	Mitochondrial alternative oxidase suppression	N/A	*Isochrysis galbana* IOAC 724S	Chemical inhibition by salicylhydroxamic acid	Nitrogen starvation	23% increase vs. control	N/A	Zhang and Liu (2016)

(continued)

Table 9.3 (continued)

Mode of genetic engineering	Specific pathway	Genes	Microalgae strains	Form of delivery	External factor	Increase in total lipids	Lipid class	References
Beta-oxidation	Lipid catabolic genes inhibition	Gene knockdown of lipase, phospholipase, acyltransferase	*Thalassiosira pseudonana*	RNAi and antisense	40 h silicon starvation	3.2 to 4.1-fold increase in lipids vs. wild type	N/A	Trentacoste et al. (2013)
Starch synthesis and carbon flux	Redirecting carbon flux	Silencing of chrysolaminarin synthase Thaps3_12695	*Thalassiosira pseudonana* CCMP 1335	Antisense knockdown	Silicon starvation	Transiently increased TAG level	N/A	Hildebrand et al. (2017)
Starch synthesis and carbon flux	Redirecting carbon flux	Silencing of UDP-glucose phosphorylase using RNAi	*Phaeodactylum tricornutum* Bohlin CCMP 2561	RNAi	None	1.25 times more vs. WT	N/A	Zhu et al. (2016)
Starch synthesis and carbon flux	Redirecting carbon flux	Silencing of UDP-glucose pyrophosphorylase using TALEN	*Phaeodactylum tricornutum* Bohlin CCMP 2561	TALEN (transcription activator-like effector nucleases)	None	45-fold TAG accumulation compared to controls	N/A	Daboussi et al. (2014)
Starch synthesis and carbon flux	Redirecting carbon flux	Silencing of phosphoenolpyruvate carboxykinase (PEPCK)	*Phaeodactylum tricornutum* Bohlin CCMP 2561	RNAi	None	22–40% higher vs. WT	No significant difference between transformed vs. wild type	Yang et al. (2016)
Starch synthesis and carbon flux	Redirecting carbon flux	Random mutagenesis	*Scenedesmus obliquus* UTEX 393	Monochromatic UV light	None	49.4% increase vs. wild type	N/A	de Jaeger et al. (2014)
Starch synthesis and carbon flux	Redirecting carbon flux	Silencing of ADP-glucose phosphorylase	*Chlamydomonas reinhardtii*	Mixed transformation procedures employed	None	32.6% increase than wild type	N/A	Li et al. (2010)

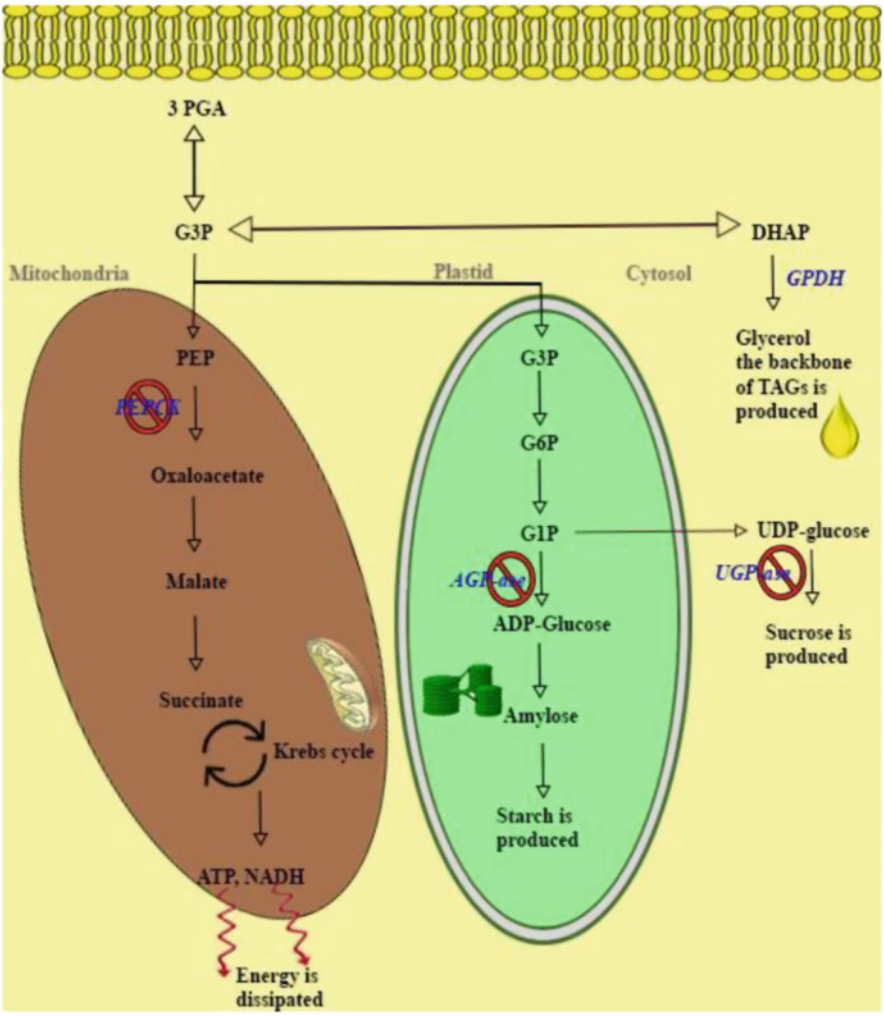

Fig. 9.5 Shows rechanneling carbon flow from starch synthesis to TAG accumulation where metabolites are in black font and enzymes in blue italicized font. *3PGA* 3-phosphoglycerate, *G3P* glyceraldehyde 3-phosphate, *DHAP* dihydroxyacetone phosphate, *GPDH* glycerol-3-phosphate dehydrogenase, *PEP* phosphoenolpyruvic acid, *ATP* adenosine triphosphate, *G6P* glucose-6-phosphate, *G1P* glucose-1-phosphate, *UDP-glucose*, uridine diphosphate glucose, *PEPCK* phosphoenolpyruvate kinase, *AGPase* ADP-glucose phosphorylase, *UGPase* UDP-glucose phosphorylase. (Figure adapted and modified from Ho et al. 2017)

lipase, phospholipase, and acyltransferase (Trentacoste et al. 2013). It is important to note that lipids are secondary energy source after carbohydrates which are used only when essential elements of growth like light, water, or nutrients become limiting. Hence, long-term blocking of starch pathways will lead to impairment in cell growth and maintenance (Hong and Lee 2015). Eventually, a successful engineered

microalgae is one that achieves the delicate balance between sufficient extent of gene silencing in multiple pathways and desirable cell growth and physiology and that is long lasting.

Microalgae have unique cell wall characteristics reflecting their taxonomic lineages, e.g., chlorophytes have a cellulosic cell wall, while diatoms own siliceous cell wall. This cell wall poses a barrier to introduce foreign DNA entry, but microprojectile bombardment or transcription activator-like effector nucleases (TALEN) are some of the targeted gene editing tool able to overcome this hurdle. Nevertheless, this has hindered development of standardized protocols for transformation. A proposed solution was to genetically engineer cells without cell walls to enable protoplast transformation (Yang et al. 2015). An industrial advantage of microalgae lacking cell wall is cost of extraction process can be alleviated, but it is unknown in terms of cell survivability. Another roadblock faced in current genetic engineering endeavor is secondary mutations in transformed cells. Firstly, microalgae has a natural and efficient defense mechanism rendering introduced gene ineffective as observed in *Chlamydomonas* (Jeong et al. 2002). To counter, Kim et al. (2015) proposed temporary knockdown instead of permanent mutation to improve transformation efficiency. Secondly, even if successful during transformation initial stage, it is not guaranteed that the desired trait will retain after long-term maintenance and mass culture. In light to this, Ahmad et al. (2015) recommended genetically transformed *Chlamydomonas reinhardtii* to be maintained in liquid nitrogen to reserve its acquired lipid accumulative ability. As demonstrated by their study, transformed cell was still able to exhibit the same effect after a year as compared to maintaining transformed lines on Tris-acetate-phosphate (TAP) agar medium.

On the contrary, transformed cells that are too strong and resistant to the environment may pose as a threat as invasive species through species domination, surpassing the growth of native phytoplankton species and ultimately altering food web dynamics and ecosystem biogeochemical cycles. Therefore, rigorous ecological risk assessments encompassing ecological, economic, and health impacts as well as strict government regulations need to be put in place prior to mass culture of the transgenic strain. In 2013, Henley et al. reported an overall "low but not zero" risk by transgenic cells advising to proceed with caution. That is to continue research in genetically modified microalgae production but stressing on the importance of rigorous monitoring via mesocosm experiments to minimize risk while promoting public and regulatory acceptance. At the same time, this is also a golden opportunity to genetically engineer microalgae with an enhanced antioxidant system to slow down lipid oxidation and degradation, i.e., via the fatty acid biosynthesis and isoprenoid biosynthesis pathway. This will ensure final lipids harvested at its highest quality with longer shelf life, a desirable attribute of fatty acid feedstock in industries. Isoprenoids or carotenoids as well as phenolic acids present in the cell are natural defense against oxidants and have shown good antioxidant activities (Foo et al. 2017). Thus, microalgae are not just cell factories of biofuel but have value-adding qualities as carotenoid feedstock in food, flavor, pharmaceuticals, nutraceuticals, cosmeceuticals, and the synthetic chemistry industry.

Another concern shrouding microalgae genetic engineering is that buildup of substrates or fatty acid precursors in the cell can slow down growth. For example, artificially inserting thioesterase gene into *Dunaliella tertiolecta* indeed increased medium chain fatty acid (MCFA) substrates by fourfolds vs. wild type (Lin and Lee 2017) in the fatty acid biosynthesis pathway. Nonetheless, a side effect of MCFA buildup caused changes to the cell membrane properties and affected growth, whereas in the isoprenoid biosynthesis pathway, IPP accumulation resulted in growth reduction (George et al. 2015) too. More studies are needed to address this concern as this trade-off resulting in slower growth rate may not be sustainable in the long term because it would be economically feasible to mass cultivate microalgae in continuous culture compared to using batch cultures that costs more time, effort, and money.

Genetically modified organisms (GMO) are defined as an organism in which its genetic material has been altered via in vitro techniques with the aim to introduce a new trait, which does not occur naturally in the organism. One of the biggest challenges arising from genetic engineering microalgae is the acceptance by government, industries, and, finally, the consumer toward GMOs. Firstly, government policies and vision can dictate if a GMO variety is accessible or restricted to the public. For example, in Malaysia, parallel to the National Biotechnology Policy (NBP) to develop biotechnology as a platform for an innovation-led economy, the Malaysian government allows the use of GMOs provided that GMOs are transferred, handled, and used according to measures consistent with international obligations and domestic legal frameworks (Andrew et al. 2018). The Biosafety Act was approved in 2007 as an enabling legislation with the interest in protecting the country's mega diversity. This is done by regulating GMOs via a regulatory body, i.e., the National Biosafety Board (NBB) with active technical and scientific assessment with the Genetic Modification Advisory Committee (GMAC). Secondly, a study finding with business companies and industry reported farmers accept the reality of GMOs and understand the importance of this eventuality while companies' support of business for transgenic organisms in the Europe is growing (Sarno and Malgeri Manzo 2016). Certain parties may be concerned that presence and growing GMOs in the country can have a negative impact on the perception image of the country; however, Knight et al. (2005) reported in their study that this was not the case.

In the third group, consumer concerns of GMO as food resources to be potentially carcinogenic and possibly toxic to health is a valid point which calls for more clinical research to prove otherwise. In order to increase consumer acceptance toward GMOs, governments, manufacturers, and researchers need to address the harmful aspects of GMO food, endorsing that it is less risky with scientifically proven data highlighting both health benefits (D'Souza and Quazi 2005) and risk to consumers. This is to enable consumers to ultimately make informed decisions prior to purchasing GMO crops. Although health concerns does not have a direct effect if genetically altered microalgae are used for biofuel, i.e., energy applications, there is still a risk of escaping GMO to the environment leading to potential gene transfer to other livestock.

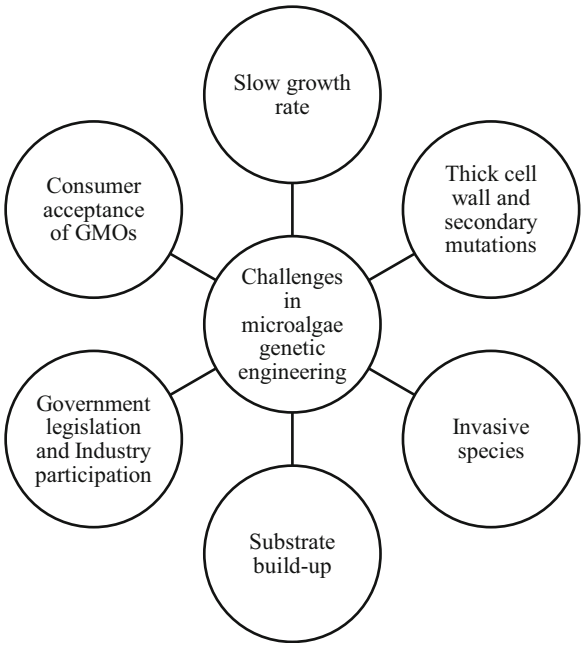

Fig. 9.6 Summarizes challenges in genetically engineering microalgae for increased lipids

In summary, it is acknowledged that there are challenges faced by GMO that needs to be addressed on an environmental, socioeconomic, and health point of view. Nevertheless, the advantages that it brings clearly outweigh the disadvantages in that it is the key to solving our energy crisis. Proper information policies and legislation as well as adequate safety measures will ensure protection of human health and the environment. Also, continuous information dissemination educating the public will influence public perception toward GMO and subsequently its acceptance. With the pressure to protect human and ecosystem health from potential negative impacts of GMOs and, at the same time, the inevitability of declining fossil fuel supply, the reality is microalgae is our next best chance as a sustainable energy source. Despite its challenges (Fig. 9.6), microalgae genetic engineering is still something worthy of exploring and investing.

5 Future Work for Microalgae Genetic Engineering

This section recommends potential research work in the attempt to bring genetically engineering microalgae for lipids to move forward (Fig. 9.7). Microalgae lipid biosynthesis increases during nitrogen starvation (Shemesh et al. 2016); thus, it would be ideal to employ the use of wastewater deficient in nitrogen as a culture medium. In particular, there are three types of wastewater having characteristic nitrogen to phosphorus (N/P) ratios, i.e., aquaculture (1.137:1), industrial (0.83:1), and municipal (0.568:1) wastewaters (Chinnasamy

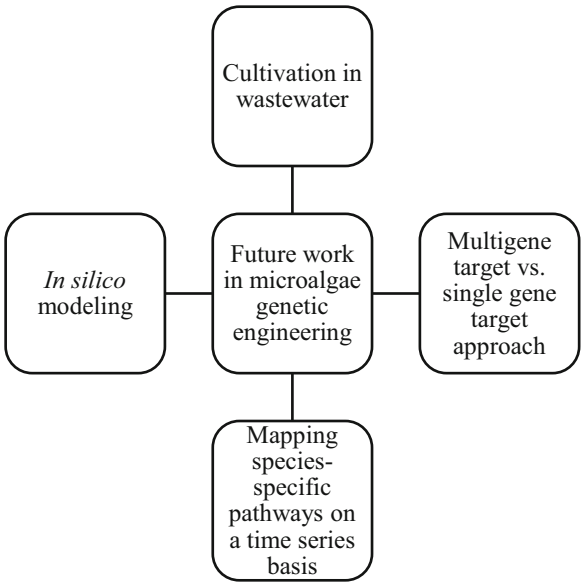

Fig. 9.7 Recommends future work for microalgae genetic engineering

et al. 2010; Hena et al. 2015, 2018). Among them, municipal wastewaters promoted as much as 21.54–41.98% total lipids in dry weight consisting of C16:0, C18:0, C18:2, and C18:3 lipid classes while fulfilling International Biodiesel Standard for Vehicles (EN14214) (Hena et al. 2015). In addition to nutrient recycling, microalgae cultivation in wastewater medium can considerably reduce production cost for biofuels as well as heavy metal removal (Salama et al. 2017), ultimately returning clean freshwater to the ecosystem in the meantime manufacturing valuable biochemical products in their cells.

Secondly, single gene targeted approach was often employed because it could clearly compare changes in transgenes vs. wild strain. Two separate studies on silicon starved *Thalassiosira pseudonana* and yielded dissimilar outcomes in lipid content. Hildebrand et al. (2017) silenced a single gene, chrysolaminarin synthase Thaps3_12695 to redirect carbon flux, only to momentarily increase TAG level. On the other hand, multiple gene knockdowns (i.e., lipase, phospholipase, and acyltransferase) increased total lipids by 3.2- to 4.1-fold (Trentacoste et al. 2013). This highlights that modification of more than one gene results in a higher lipid content. Also, a suggestion during multigene targeted approach is to employ statistical tools like multivariate analysis to show the degree of change exerted by different genes as compared to a simplified statistical student T-test. Of course, it is likewise important to compare if targeting several pathways at one time would be more effective than targeting one pathway.

Besides that, more research should endeavor to continuously gather high-throughput omics data because these data sets are the core of in silico modeling, which aids in understanding cell response at the system level (system biology). In silico work allows for the analysis and prediction of cellular behavior under genetic and environmental changes (Hong and Lee 2015). In particular, it is encouraged that

more in-depth work is needed to reveal species-specific metabolic molecular networks as each strain is unique. Currently, full genomic sequencing and metabolic reconstruction in microalgae strains (Chen et al. 2017) are among the efforts taking lead in revealing metabolic networks. Furthermore, it is suggested that an integrative approach with in silico modeling be done before actual experiment to take into account the complex nature of biochemical networks occurring in the cell. This is important to aid in the prediction of the effects of gene insertion or deletion of a pathway. Also, it would save a great deal of time and cost if experiments are used to validate hypothesis as opposed to random trial-and-error-based experiments. Once a simulated experiment with the support of valid data regulation and mechanism, biochemistry as well as comprehensive genomic information is laid out through the model, a reliable outcome can be predicted. Additionally, it would be useful to model species-specific lipid production on a time series basis. The information would allow for prediction of real-time biochemical occurrences and product yield (De Bhowmick et al. 2015) of the cell. This can then alert harvesting of biomass prior to the onset of lipid catabolic activities and growth declination thus saving resources (i.e., electricity, space, and media) while minimizing contamination.

In summary, there are still opportunities to improve lipid content of oleaginous microalgae as a sustainable source of energy and high-value bio-products which can be achieved through genetic engineering approaches.

6 Conclusion

Understanding the genes and pathways revolving around lipid biosynthesis is the port key toward genetic engineering microalgae for sustainable sources of biofuels. This is evident as increasing studies have demonstrated that facilitating overexpression of fatty acid biosynthesis enzymes and suppressing lipid oxidation and its competitive pathways are effective strategies (Bellou et al. 2014) to increase lipid production. This success largely owes to the development of genomic tools which has brought microalgae genetic engineering leaps ahead of its years. For example, genomic sequencing tools like next-generation sequencing (NGS) can now fully sequence whole microalgae genomes. This genetic information has provided the necessary line of sight for scientists to perform in silico analysis like flux balance analysis, allowing prediction of microalgae's metabolic responses to disturbances (e.g., N stress) as well as product yields. Besides, targeted gene insertion (TGI) like TALEN or clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) are some of the techniques that can bring microalgae production to the next level via targeted gene editing and insertions. Overall, genomic advancements in techniques and tools are especially useful to allow further understanding of different pathways leading to lipid synthesis, enabling the achievement of gene regulation to increase lipid production.

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Chapter 10
Harvesting of Microalgae for Biomass Production

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Abstract Microalgae have a great commercial potential as a source of several compounds of interest to the food, pharmaceutical, and chemical industries. The market was estimated to be around US$1.25 billion per year with more than 20 different commercial products and the genera commercially produced are mainly *Chlorella, Arthrospira* (*Spirulina*)*, Dunaliella,* and *Haematococcus*, with a production of 5.000 tons of dry matter in 2004, increasing to 9.000 tons in 2010. However, despite extensive research carried out to date, harvesting is still one of the most costly processes in microalgae production. Consequently, the microalgae that are currently produced commercially are mainly indented for high value products (>$10,000 t^{-1}). The aim of this chapter is to give an overview of the major constraints and technologies available for harvesting microalgae.

1 Introduction

Microalgae have a great commercial potential as a source of several compounds of interest to the food, pharmaceutical, and chemical industries (Borowitzka 2013). For instance, the microalgal biomass can be used in feed for livestock (Madeira et al. 2017) and in human nutrition (Wells et al. 2016), as it contains high levels of essential nutrients such as minerals, proteins, carbohydrates, lipids, and vitamins (Becker 2007). Moreover, microalgae produce several compounds with bioactive

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properties (Michalak and Chojnacka 2015) that promotes health (Jahan et al. 2017), prevent diseases (de Jesus Raposo et al. 2013), or combat pathogenic microorganisms (Shannon and Abu-Ghannam 2016). Numerous chemicals (Hess et al. 2017) and potential alternative biofuels such as methane, diesel, and hydrogen can also be derived from microalgae biomass (Preez 2016). The microalgae market was estimated to be around US$1.25 billion per year with more than 20 different commercial products, and the genera commercially produced are *Chlorella*, *Arthrospira* (*Spirulina*), *Dunaliella*, and *Haematococcus*, with a production of 5.000 tons of dry matter in 2004, increasing to 9.000 tons in 2010 (Pulz and Gross 2004; Brennan and Owende 2010).

However, despite extensive research carried out to date, harvesting is still one of the most costly processes in microalgae production (Vandamme et al. 2013). Consequently, the microalgae that are currently produced commercially are mainly indented for high-value products (>$10,000 t^{-1}). Contrarily, for low-value commodities (<$1000 t^{-1}), specifically biofuels, the production costs must be reduced by well over an order of magnitude to be economically viable (Benemann 2013). The aim of this chapter is to give an overview of the major constraints and technologies available for harvesting microalgae.

2 Why Is It Difficult to Harvest Microalgae?

2.1 Intrinsic Characteristics of Microalgae

The majority of microalgae of commercial interest are planktonic, meaning that they have evolved to remain suspended in the water column. First, they are microscopic in size, with species generally ranging from picoplankton (0.2–2 μm) to microplankton (20–200 μm), though some species may reach up to mesoplankton (2 mm) (Finkel et al. 2010). For example, *Nannochloropsis* is recognized as a potential biofuel feedstock; however, due to its small size (2–5 μm), most harvesting techniques are ineffective or too expensive for commercial purposes (Chua and Schenk 2017). On the other hand, the filamentous cyanobacteria *Arthrospira* can be easily and inexpensively harvested using rather coarse screens (Vonshak and Richmond 1988). The microscopic size of microalgae has direct impact on its settling velocity (Stokes' Law), since smaller cells settle slower than larger cells (Finkel et al. 2010). Besides size, the shape (i.e., surface-area-to-volume ratio) of the microalgae also influences the settling velocity. For example, diatoms have a cell wall composed of weighty silica (2.1 g mL^{-1}), which makes them theoretically denser than water (~1.0 g mL^{-1}), yet, several diatoms have long projections (i.e., spines) or form chains, which increase the surface area, counteracting their settling (Miklasz and

Denny 2010). Another adaptation that prevents settling is density as, in overall, microalgae have densities similar to that of water due to production of light-weight compounds such as lipids (0.86 mg L^{-1}), presence of gaseous vacuoles (0.12 mg L^{-1}), or exchanges of high for low molecular weight ions in its cellular constitution (Miklasz and Denny 2010). Some species such as *Chlamydomonas*, a potential candidate for biofuels and bio-products production (Scranton et al. 2015), uses flagella for motility. Microalgae naturally release organic matter into water as a by-product of photosynthesis, secondary metabolism, or simply by cell lysis, which negatively impacts harvesting and causes fouling in membrane filtration (Pivokonsky et al. 2016).

Microalgae are more productive than conventional agricultural crops. While conventional crops such as wheat, corn, or rice produce only about 10 ton of useful biomass ha^{-1} $year^{-1}$, microalgae can produce more than 30 tons dry biomass ha^{-1} $year^{-1}$, even in extensive open raceway ponds. Higher productivities can be achieved in closed photobioreactors. In conventional agriculture, the harvested biomass is equal to the standing crop biomass at the end of the growing season. The standing crop biomass is harvested in a single operation at the end of the growing season and harvesting one hectare of a crop takes little more than 10 min using highly efficient combines. Although microalgae can achieve very high growth rates, growth of microalgae is rapidly limited by light limitation caused by self-shading of the cells in the culture. In a typical 20-cm-deep raceway pond, growth stops when microalgal biomass is about 0.5 g L^{-1}, which corresponds to about 1 ton ha^{-1}. To harvest 30 tons microalgae biomass ha^{-1} $year^{-1}$, the entire volume of the raceway pond has to be processed 30 times, which corresponds to processing 60.000 m^3 of culture broth. From this comparison, it is evident that harvesting is much larger challenge in microalgae production than in production of conventional agricultural crops. Moreover, microalgae have high growth rates, requiring frequent harvesting cycles.

Additionally, microalgae have a negative surface charge, which mainly originates from the presence of carboxylic (-COOH) and amine ($-NH_2$) groups on the cell surface. Above pH 4–5, the carboxylic groups dissociate and become negatively charged, whereas the amine groups get neutral, resulting in a net negative surface charge (−10 to −35 mV). Due to electrical repulsion, the microalgae cells do not aggregate easily, remaining suspended in the water column (Box 10.1) (Henderson et al. 2008a; Vandamme et al. 2013). Thus, planktonic microalgae very rarely sediment naturally, usually requiring a very long settling time or special environmental conditions as we will discuss in the following sections.

Box 10.1 Coagulation Theory

Zeta potential

The behavior of microalgae in water is influenced by their surface charge, which is usually negative. Those negative ions will attract positive ions (counterions) from the surrounding solution while repelling negative ions (co-ions). Close to the microalgae surface, the counterions will form a dense layer called *stern layer*. This creates a dynamic equilibrium where the counterions are attracted to the negative surface of the microalgae but are repelled by the positive stern layer, whereas the co-ions are repelled from the negative surface and attracted to the positive layer. This *diffuse layer* extends into the solution until the concentrations of counterions and co-ions are in equilibrium, i.e., a zero electrical potential is established. Together, the stern and the diffuse layers form an *electrical double layer* around the microalgae cells (Fig. B1).

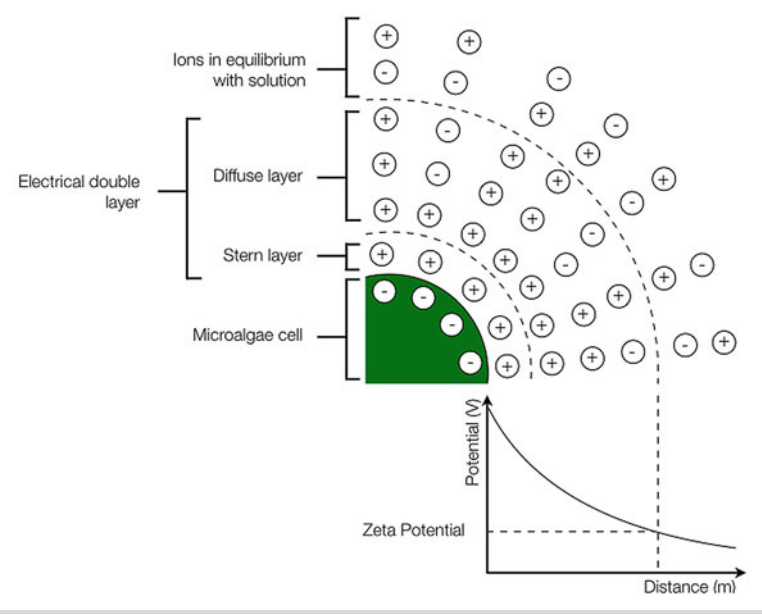

Fig. B1 Structure of the electrical double layer of charged ions in solution surrounding a negatively charged microalgal cell and the potential difference between the particle and the bulk fluid as a function of the distance from the particle surface

(continued)

Box 10.1 (continued)
The microalgae cells are repelled by each other due to its surface charge. So, by measuring the surface potential, we can evaluate the strength of the repulsive force. Unfortunately, the surface potential is not easily measured. On the other hand, the *zeta potential* at the outer border of the diffuse layer (i.e., the slipping plane) can be measured in a fairly simple manner, thus being a good approximation for surface potential.

Energy barrier x Energy trap

The DLVO theory, named after Derjaguin, Landau, Verwery, and Overbeek, is the classic mechanistic theory of how particles (i.e., microalgae cells) interact, by balancing electrostatic repulsion and van der Waals attraction forces (Fig. B2). Electrostatic repulsion becomes significant when two microalgae cells approach each other and their double layers begin to overlap. The energy required to overcome this repulsion increases dramatically as the microalgae come close together. On the other hand, van der Waals attraction increases when two microalgae cells approach each other. The energy balance between each force can shift from attraction to repulsion and back to attraction with increasing distance between cells. In order to agglomerate, two cells on a collision course must have sufficient kinetic energy to overcome the energy barrier. Once this barrier is cleared, the energy trap region is reached, and attraction forces aggregate the cells. To improve coagulation, the energy barrier can be lowered either by compressing the double layer or by reducing the surface potential. The double layer can be compressed by increasing the concentration or valence of ions in solution, while the surface potential, estimated by measuring the zeta potential, can be reduced by the addition of coagulants.

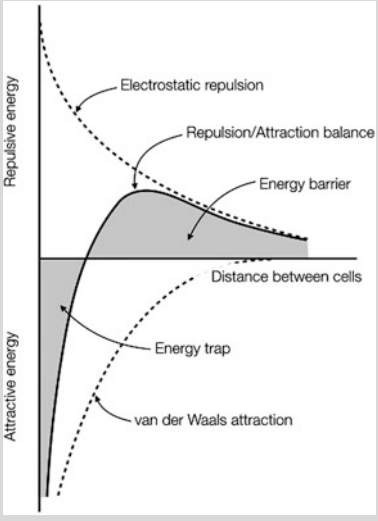

Fig. B2 Potential energy diagram for the interaction of two microalgae cells. Electrostatic repulsion is shown as a positive curve, whereas the van der Waals attraction is shown as a negative curve. The balance curve is formed by subtracting the attraction curve from the repulsion curve

2.2 Production System Limitations

A factor that makes microalgae harvesting a laborious and expensive process is the cell density that cultures generally reach and the amount of biomass required for any commercial application. On the most productive systems, such as photobioreactors, microalgae reach densities ranging from 2 to 6 g L^{-1} (Davis et al. 2011), although densities up to 10 g L^{-1} can be attained (Stephens et al. 2010). However, when we convert these high densities into dry mass content, we find that the biomass represents less than 1%, while water corresponds to 99%. So, to harvest 1 ton of biomass, it will be necessary to process around 100 m^3 of culture medium. Still, photobioreactors are expensive in both capital and operational costs and, consequently, are not widely employed for commercial microalgae production, except for high-value products (Acién-Fernández et al. 2013). Instead, raceway-type open ponds are widely employed commercially due to its lower capital and operational costs (Benemann 2013). Unfortunately, open ponds are less productive than photobioreactors, thus being necessary to manipulate larger volumes of culture to obtain the same amount of biomass (Vandamme et al. 2013). This factor, associated with the harvesting technology employed, will strongly influence the biomass production costs. For instance, centrifugation is a highly efficient and fast harvesting technology; nonetheless, it will turn impractical and energy demanding (up to 1 MJ Kg^{-1} of dry biomass) to process large volumes of low-producing open ponds, making necessary the adoption of a pre-concentration step, which also adds to the cost (Fasaei et al. 2018) being profitable only to high-value products (Acién-Fernández et al. 2013). The species produced also influence the choice of harvest technology. *Dunaliella salina*, for instance, which is commercially cultured for β-carotene and astaxanthin production, have no protective cell wall (Monte et al. 2018), and centrifugation generally tends to damage the cells due to shearing stress, leading to loss of contents. Moreover, filtration is not recommended for this species as the cells may deform and pass through the filters (Borowitzka 2010). So, there is no technology applicable to all species, being a species-specific decision. Besides, the intended product also dictates the harvest technology. For instance, flocculation is considered a low-cost harvesting method (Vandamme et al. 2013); however, depending on the chemicals employed, it may interfere with the downstream processing (Borges et al. 2011) or its applications for human or animal nutrition (Van Haver and Nayar 2017). The operational mode also dictates the technology due to harvesting frequency (Moheimani et al. 2016). In batch operation, the entire culture will have to be harvested when the desired density is reached, however at a lower frequency. If a semicontinuous mode is employed, where 20–40% of the culture is harvested daily, a smaller volume will have to be processed yet more often. At the other hand, in a continuous mode, a small fraction of the culture is continuously harvested.

In brief, the most daunting task is not harvesting the microalgae itself but making it an economically viable and environmentally sustainable process, due to the current high net energy ratio (i.e., the energy required to produce the dry biomass divided by

its energy content) and carbon balance (Slade and Bauen 2013). Consequently, microalgae are not yet cost-effective for bulk production of commodities, as market prices are low and revenues do not counterweight production costs, nor yet sustainable to energy production (Slade and Bauen 2013; Ruiz et al. 2016), being currently more attractive to high-value products (Bilad et al. 2014; Chew et al. 2017).

3 Approaches to Harvest Microalgae

3.1 Unit Operations

Basically, microalgae harvesting is a separation process involving a heterogeneous mixture composed of a discontinuous solid (the microalgae) and a continuous liquid phase (the culture medium). Depending on the way that the solids are collected, the separation processes can be classified in two groups, as gravity or as filtration-based unit operations (Fig. 10.1) (Svarovsky 2001). In gravity-based unit operations, the liquid phase is constrained in a stationary or rotating vessel, and the solids move freely within the liquid. The separation is due to mass forces acting on the solids and to a difference in density between the solids and the liquid. In filtration-based unit operations, on the other hand, the solids are constrained by a membrane, and the liquid is allowed to flow freely through the membrane. Therefore, the separation is due to differences in solids and membrane pore sizes rather than by differences in density.

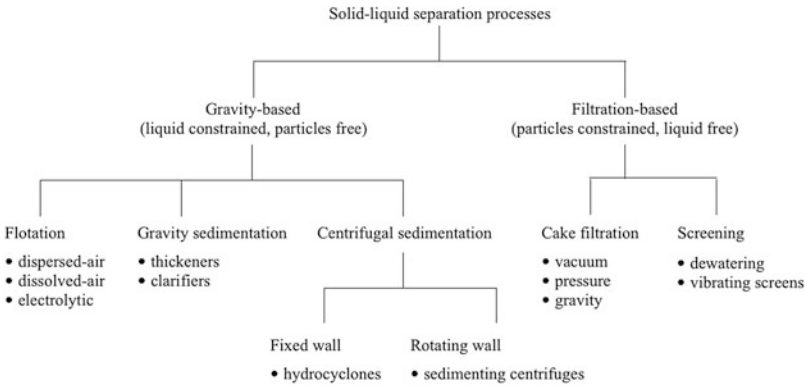

Fig. 10.1 Classification of solid-liquid separation processes

3.2 Requirements to Harvest Microalgae

Irrespective of which unit operation is employed, the process must be designed to handle large volumes of culture and be efficient at recovering and concentrating the biomass (Pahl et al. 2013). The recovery efficiency (RE) of a separation process can be defined as the ratio of the biomass harvested to the total biomass in the culture before harvesting, expressed in percentage (Eq. 10.1). The concentration factor (CF) is defined as the ratio of the concentration before and after harvesting, usually expressed in times (Eq. 10.2). In brief, the recovery efficiency tells if the separation process was effective in getting all the biomass from the culture, whereas the concentration factor tells how many times the original concentration was increased and, hence, the volume was reduced. Additionally, the separation process must have a low energy demand and be constructed with resistant materials, especially if employed to harvest marine species. Furthermore, the separation process should not contaminate the biomass or its residuals nor alter the biomass chemical composition or interfere with downstream processes (i.e., product extraction, stabilization, or purification). Although it is not a prerequisite related to harvesting itself, but to sustainability, it is best practice that the separation process has low net energy ratio and carbon balance, as well as it does not contaminate the culture medium or alter its chemical composition drastically, allowing its reusability and reducing cultivation costs due to water use and nutrient input.

$$\text{RE}\ (\%) = \frac{\text{biomass harvested}}{\text{total biomass in culture}} \tag{10.1}$$

$$\text{CF}\ (\times) = \frac{\text{concentration after harvest}}{\text{initial concentration}} \tag{10.2}$$

3.3 Single- or Multiple-Step Harvesting

Though the unit operations can be employed individually (i.e., single step) to harvest microalgae, they are frequently combined in sequential steps (Fasaei et al. 2018) (Fig. 10.2). In this case, each step is designed to increase the microalgae concentration by employing the most suitable and economical method, increasing the concentration and reducing the volume to be processed by the subsequent step (Pahl et al. 2013). Recently, Fasaei et al. (2018) performed an economic evaluation of 28 different combinations of unit operations for large-scale microalgae harvesting, concluding that single step (e.g., centrifugation) can be satisfactory if the separation process reaches high biomass concentrations. However, due to the large volumes that need to be processed, centrifugation requires around 14 MJ kg^{-1} of dry biomass, representing about 55% of the energy content of the biomass (Norsker et al. 2011). On the other hand, a multiple-step approach (e.g., membrane

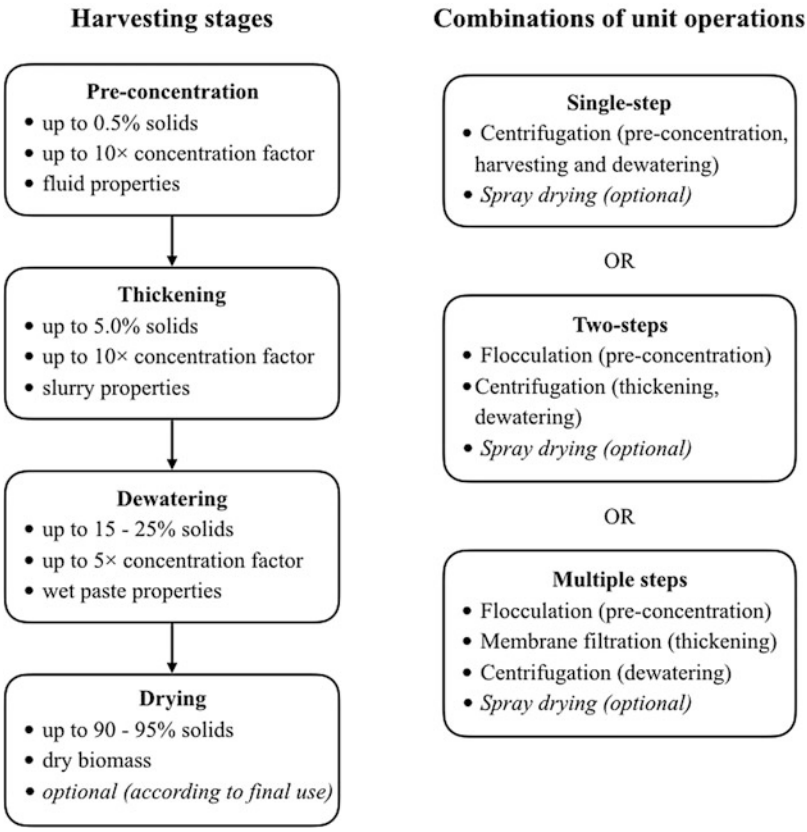

Fig. 10.2 Harvesting stages and possible combinations of unit operations for microalgae harvesting

filtration followed by centrifugation) is more attractive from an economic point of view (Fasaei et al. 2018).

In general, the sequence of unit operations can be classified into four stages, i.e., pre-concentration, thickening, dewatering, and drying (Fig. 10.2). At pre-concentration, the biomass is collected from the culture pond and increased from *ca.* 0.05% up to 0.5% solids, resulting in a 10× concentration factor. At this stage, the concentrate still retains its fluid-like consistency. The following is thickening, which increases the harvested biomass up to 5% solids, an additional concentration factor of 10×, producing a slurry-like concentrate. The third is dewatering, where the solid concentrations are increased up to 15–25%, resulting in a wet paste. Depending on the intended use of the biomass, a drying stage can be employed to allow further downstream processing (i.e., dry extraction) or increase the biomass stability and minimize spoilage. Drying removes unbound water generating a 90–95%

dry biomass. Although being an important step, drying is energy demanding as more than 85% of a wet paste is composed of water, adding to production costs.

3.4 Microalgae Treatment Prior to Harvesting

Due to intrinsic characteristics of microalgae (e.g., cell size, density, and charge), no single separation process is totally effective or economical regardless of the type of product (Moheimani et al. 2016). However, the efficiency can be significantly improved, and energy demand can be reduced, if microalgae are treated prior to harvesting. The factor that most hampers harvesting is the negative surface charge of microalgae cells, which generates an electrostatic repulsion that prevents individual cells from aggregating (Box 10.1). This electrostatic repulsion can be overcome by destabilizing the microalgae cells through coagulation, i.e., by turning them less negative by addition of a positive chemical additive (Henderson et al. 2008b). With the electrostatic repulsion between microalgae cells weakened, then van der Waals attraction forces can act and form large, with smaller surface-area-to-volume ratio, and heavier microalgae aggregates (i.e., flocculation) (Fig. 10.3). Coagulation and flocculation are low-cost processes commonly employed in water and wastewater treatment for the removal of suspended solids in large volumes of liquid, making it a perfect pre-concentration step to either gravity of filtration-based processes for microalgae harvesting (Vandamme et al. 2013; Fasaei et al. 2018).

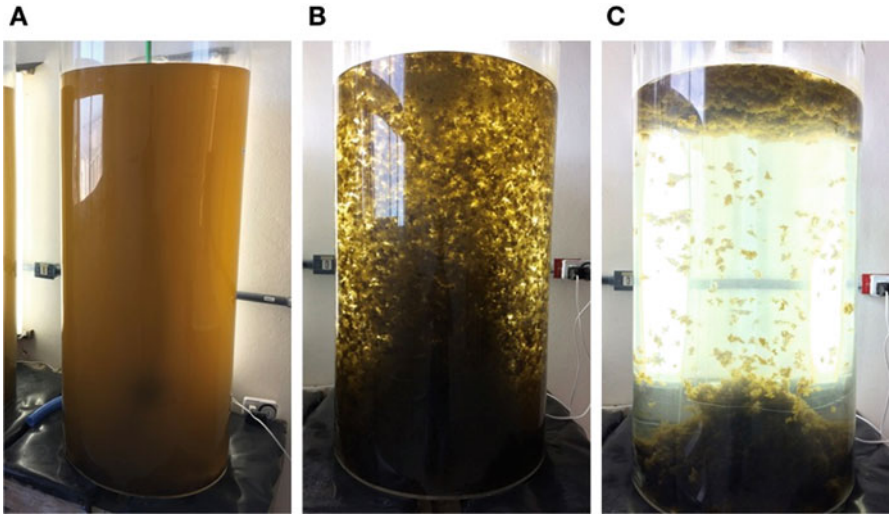

Fig. 10.3 Picture of a *Conticribra weissflogii* culture in a 330 L photobioreactor prior (**a**) and after addition of flocculant, showing floc formation (**b**) and separation from the medium (**c**). Note the occurrence of both sedimentation and flotation of the biomass

4 Coagulation and Flocculation

Coagulation and flocculation can be achieved by employing positively charged chemical additives. Although, in theory, coagulation and flocculation are distinct processes, in practice it is difficult to establish this difference as the additives can perform in both ways (Bratby 1980; Gregory 2013). Basically, coagulation is a process in which the particles are destabilized by the action of low molecular weight polyelectrolytes, either by the compression of the double layer or by reducing the surface charge, allowing aggregates to form due to a lower electrical repulsion. On the other hand, flocculation is a physical process where usually high molecular weight flocculants bridge particles, aggregating them into a larger, random, three-dimensional structure, which is loose and porous (Gregory 2013). Flocculation can be described in two stages, adsorption of the polyelectrolytes and formation of flocs. There are several mechanisms for the formation of flocs, such as charge neutralization, electrostatic patch, and bridging and sweeping flocculation (Gregory 2013). In charge neutralization, the zeta potential of the particle is reduced by adsorbed cationic polyelectrolytes, allowing the particles to come together by van der Waals attraction (Fig. 10.4a). In the electrostatic patch mechanism, the polyelectrolyte

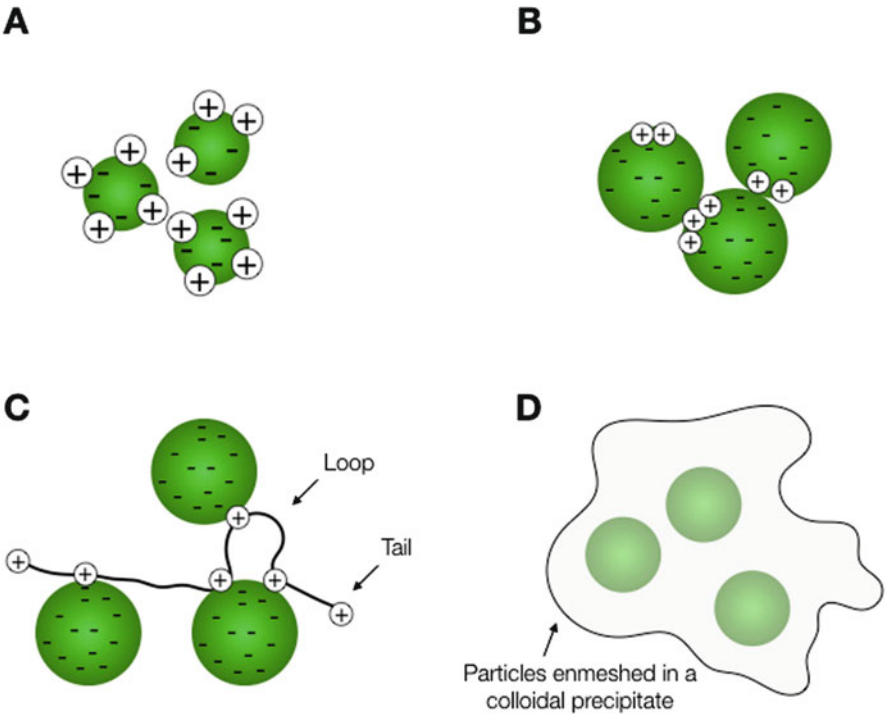

Fig. 10.4 Overview of different flocculation mechanisms, charge neutralization (**a**), electrostatic patch mechanism (**b**), bridging mechanism (**c**), and sweeping flocculation (**d**)

locally reverses the charge of the particle surface, resulting in patches of opposite charge, and the particles connect with each other through those patches (Fig. 10.4b). In bridging, as the polyelectrolyte absorbs onto the particle, tails and loops are formed, extending further into the solution and attaching simultaneously to several particles (Fig. 10.4c). Sweeping is the process in which particles are entrapped in a (mostly mineral) colloidal precipitate (Fig. 10.4d). Several additives can be employed for flocculation, ranging from inorganic hydrolyzing metal salts to organic polymeric flocculants (synthetic or natural). However, flocculation can also be induced spontaneously by other methods without the addition of manufactured chemicals, as in autoflocculation (section 4.4) and bioflocculation (section 4.5).

4.1 Hydrolyzing Metal Salts

Hydrolyzing metal salts are the most widely used coagulants for water treatment and mining, being mostly based on aluminum ($AlCl_3$ and $Al_2(SO_4)_3$) or ferric salts ($FeCl_3$ and $Fe_2(SO_4)_3.3H_2O$). When dissolved in water at low concentrations, the iron and aluminum ions hydrolyze forming positively charged hydroxides resulting in coagulation by charge neutralization (Fig. 10.4a). However their effectiveness is greatly dependent on medium pH, with the optimal range between 5.0 and 5.5 when using aluminum and 3.7–4.2 when using iron (Gregory 2013). When dosed at higher concentrations, well above solubility, the hydroxides form amorphous precipitates that enmeshes the suspended particles (Fig. 10.4d). Sweep flocculation usually leads to faster aggregation than charge neutralization, producing stronger and larger flocs, yet rather weak compared to polymeric flocculants (Gregory 2013). The use of metal salts for microalgae harvesting presents several disadvantages, such as the high doses required, ranging from 120 to 1000 mg/L, and the biomass being contaminated with metals, thus limiting its use (Şirin et al. 2012; Granados et al. 2012). Moreover, the residual metal salts dissolved in the culture medium can interfere with its reuse (Renault et al. 2009; Granados et al. 2012).

The more effective coagulants alternative to hydrolyzing metal salts are those based on pre-hydrolyzed forms of aluminum-like polyaluminum chloride (PACl), which present highly charged cationic species (Şirin et al. 2012). PACl give more rapid flocculation, stronger flocs, and lower volumes of residual solids (sludge) than traditional hydrolyzing metal salts, working in a wider pH range (5.0–9.0) (Duan and Gregory 2003; Şirin et al. 2012). However, the mechanisms of action are not still well understood (Duan and Gregory 2003).

4.2 Polymeric Flocculants

Polymeric flocculants are long-chain molecules consisting of at least one type of repeating unit (i.e., a monomer), being characterized according to its nature

(synthetic or natural), electrical charge (cationic, anionic, or nonionic), molecular weight, or charge density (Bratby 1980; Bolto and Gregory 2007; Roselet et al. 2015). If the polymer is charged, then it is denominated as a polyelectrolyte. Polymeric flocculants can act by bridging, charge neutralization, and electrostatic patch, with efficiency depending on the structural arrangement adopted when dissolved. The most usual arrangement is as a random coil, which is less effective, being influenced by the polymer molecular weight, by charge density, and by the medium ionic strength, whereas a fully stretched arrangement is highly unlikely, though more efficient (Sukenik et al. 1988; Gregory 2013; Roselet et al. 2015). Ideally, a flocculant should be cheap and nontoxic though most synthetic polymers present some level of toxicity, especially to aquatic organisms. Cationic polymers are more toxic than anionic or nonionic ones, whereas the monomers (particularly acrylamide) are more toxic than the polymers (Hamilton et al. 1994; Beim and Beim 1994; Bolto and Gregory 2007; Costa et al. 2014; Roselet et al. 2017; Pereira et al. 2018). Alternatively, natural polymers (e.g., chitosan, guar gum, cassia gum, starch, and tannin) are being employed to harvest microalgae as an environment-friendly approach (Renault et al. 2009; Vandamme et al. 2010; Banerjee et al. 2014; Roselet et al. 2015; Peng et al. 2017). Although efficient at harvesting freshwater microalgae, most natural polymers present a poor performance for marine microalgae (Sukenik et al. 1988; Vandamme et al. 2010). This may be related to its molecular structure as most present are linear polymers, being easily compressed at higher ionic strengths (Banerjee et al. 2013, 2014; Zargar et al. 2015). An exception seems to be tannin-based polymers, which are branched and present a more rigid backbone, apparently being not affected by salinity (Roselet et al. 2015).

4.3 Optimal dosing of chemicals in flocculation

Generally, the efficiency of a particular flocculant is dependent on its dose (Fig. 10.5), being determined in a case-by-case basis employing a simple jar test methodology. At low flocculant dose, the flocculation efficiency is low as the particles are still negative, due to insufficient particle surface coverage (Zone 1). As the dose is increased, efficiency also increases until a maximum is achieved (Zone 2), due to optimum surface coverage and particle charge neutralization. If the dose is further increased, then the particle surface will be totally covered by the flocculant, resulting in poor efficiency due to restabilization by charge reversal (Zone 3). For hydrolyzing metal salts, a fourth zone is observed where very high doses result in efficiency recovery due to sweep flocculation. Although efficient, sweep flocculation results in high flocculant demand, increasing the production costs. Apart from polymer dose and medium ionic strength, the organic matter naturally produced by microalgae also is an important interferent in the flocculation process (Henderson et al. 2010; Hulatt and Thomas 2010; Vandamme et al. 2012b, 2016; Garzon-Sanabria et al. 2013; Roselet et al. 2017). In brief, due to its anionic nature, the organic matter competes with microalgae for the added flocculant, increasing its demand in order to be

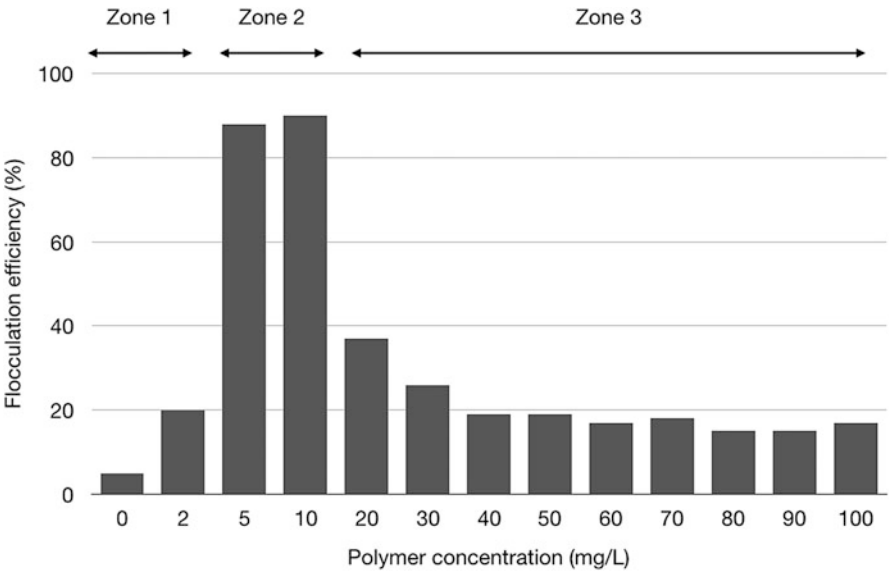

Fig. 10.5 Example of flocculation efficiency as a function of polymer concentration. Zone 1: Concentration lower than optimum results in ineffective flocculation. Zone 2: Optimal concentration results in maximum efficiency. Zone 3: Concentration higher than optimum results in ineffective flocculation due to microalgae restabilization

effective and, consequently, increasing the production costs. Interestingly, a recent study demonstrated the capacity of organic matter excreted by *Microcystis aeruginosa* strain CS-564/01 in enhancing flocculation in dispersed air flotation (Hanumanth Rao et al. 2018b). Moreover, the organic matter produced by *M. aeruginosa* CS-564/01 enhanced flotation when added to cultures of *Chlorella vulgaris* CS-42/7, *Mychonastes homosphaera* CS- 556/01, and *Microcystis aeruginosa* CS-555/1 (Hanumanth Rao et al. 2018b), indicating a possible bioflocculation effect.

4.4 Autoflocculation

Autoflocculation, alkaline flocculation or pH-induced flocculation, occurs spontaneously in microalgal cultures with pH above 9.0, due to photosynthetic CO_2 depletion or addition of bases (Sukenik and Shelef 1984; Vandamme et al. 2012a; Tran et al. 2017). The fact that flocculation occurs at a high pH is surprising, since the surface charge of microalgae cells is expected to become more negative at a high pH, thus inhibiting aggregation (Lavoie and de la Noüe 1987). However, autoflocculation is triggered by ions naturally available in the water (Smith and Davis 2012). For example, in waters rich in phosphate (>0.35 mM), positively charged calcium phosphate precipitates are formed at high pH, absorbing and

reacting with the negatively charged microalgae cell, resulting in flocculation by charge neutralization or simply by sweep flocculation (Knuckey et al. 2006; Christenson and Sims 2011; Beuckels et al. 2013; Besson and Guiraud 2013). However, at low phosphate concentrations, autoflocculation can also be induced by calcium carbonate or magnesium hydroxide precipitation (Vandamme et al. 2012a). This is important for marine species, as the concentrations of magnesium and calcium are much higher in seawater (Wu et al. 2012; Vandamme et al. 2015). Autoflocculation is not toxic as metal salts (Muylaert et al. 2017) and don't interfere with downstream processing (Vandamme et al. 2018). Moreover, these precipitates can be removed from the biomass after harvesting by mild acidification (Beuckels et al. 2013; Vandamme et al. 2015). However, in commercial production the pH is usually maintained in a range suitable to microalgae growth, by addition of CO_2. So, if pH is artificially raised, by addition of bases, then the production costs will increase. The best solution would be to shut the CO_2 addition and leave the pH increase naturally due to photosynthetic CO_2 depletion (Sales and Abreu 2015).

4.5 Bioflocculation

Bioflocculation has emerged as a promising eco-friendly strategy to harvest both freshwater and marine microalgae, owing to its biodegradable and nontoxic nature (Ummalyma et al. 2017; Shahadat et al. 2017). Basically, it is a flocculation process promoted by extracellular polymeric substances (EPS) produced by bacteria, microalgae, filamentous fungi, or yeast or by interaction of these organisms with the cultured microalgae (Van Den Hende et al. 2011; Zheng et al. 2012; Alam et al. 2016; Tran et al. 2017). The EPS is mainly composed of polysaccharides and proteins, though the first have better flocculating properties due to a higher molecular weight and the presence of many functional groups. A bioflocculant widely employed is poly (γ-glutamic acid), produced by the bacteria *Bacillus subtilis* (Zheng et al. 2012). However, the mechanism underlying bioflocculation is often not clearly understood (Alam et al. 2016). Bioflocculation can be performed by coculturing the target microalgae with the bioflocculating organism or adding it just prior to harvesting; however, both result in microbiological contamination that may interfere with the final application. Another disadvantage is that culturing a heterotrophic bioflocculating organism requires supplementary substrates as well as extra energy sources to support its growth (Alam et al. 2016).

5 Gravity-Based Processes

The rationale of gravity-based processes can be easily understood by the settling velocity of particles in a liquid, as described by Stokes' law (Svarovsky 2001). According to Stokes' law (Eq. 10.3), the settling velocity (v, m s^{-1}) of a particle (i.e., the microalga cell) is directly influenced by gravity (g, m s^{-2}), its radius (R, m), and

the mass density difference between the fluid (ρ_f, kg m^{-3}) and particle (ρ_p, kg m^{-3}). Regarding the mass density difference, the particle will move downward if $\rho_p > \rho_f$ or upward if $\rho_p < \rho_f$. On the other hand, the viscosity of the water (μ, kg m^{-1} s^{-1}) inversely affects settling velocity.

$$v = \frac{2}{9} \frac{(\rho_p - \rho_f)}{\mu} gR^2 \tag{10.3}$$

In summary, if the density difference between the particle and the water is significant, the particle will sediment (or float) naturally; otherwise, we can manipulate the radius and density of the particle (e.g., by coagulation/flocculation) or even the gravitational force (e.g., centrifugation) in order to increase the settling velocity. But if the density (e.g., salinity) and viscosity (e.g., temperature) of water increase, the more difficult it will be to microalgae to settle.

5.1 Gravity Sedimentation

In this process, the microalgae are separated from the culture medium by the simple action of gravity, based on cell density or size (Eq. 10.3). Thus, the larger or heavier the microalgae, the fastest they will sediment. Harvesting by sedimentation is attractive as the energy demand is very low and requires a relatively low-cost infrastructure. However, as discussed in Sect. 2.1, microalgae have several adaptations to remain suspended in water, which hampers their natural sedimentation. In this way, harvesting by sedimentation alone requires long detention periods as settling velocity is low (about 1 cm h^{-1}) and only works for large nonmotile or heavier species, such as *Pediastrum*, *Scenedesmus*, *Arthrospira*, or immotile cysts of *Haematococcus* (Wang et al. 2013; Depraetere et al. 2015; Moheimani et al. 2016). Due to the long detention period, the biomass can eventually be lost by respiration or undergo bacterial decomposition, which adversely alters its physical and chemical characteristics. Moreover, sedimentation generates a rather dilute concentrate (2–3% solids), being mostly employed as a pre-concentration step prior to other thickening and dewatering technologies. As the primary purpose in microalgae harvesting is to produce a highly concentrated slurry, then the process is called thickening (Svarovsky 2001). Therefore, the term clarifier is employed which purpose is to clarify the liquid, being mostly employed for water treatment. Moreover, the feed concentration to a thickener is usually higher than that to a clarifier.

Basically, settling tanks can be of two types, conventional or lamella thickeners. Conventional clarifiers are cylindrical in shape with a funnel-shaped bottom (Fig. 10.6). The microalgae culture enters from the bottom to the top of the tank, through a central inlet. As the turbulence is minimal, the microalgae settle and accumulate at the bottom, while the clarified medium flows through outlets at the edge of the tank. A rotating arm recovers the biomass, leaving through the bottom of the tank.

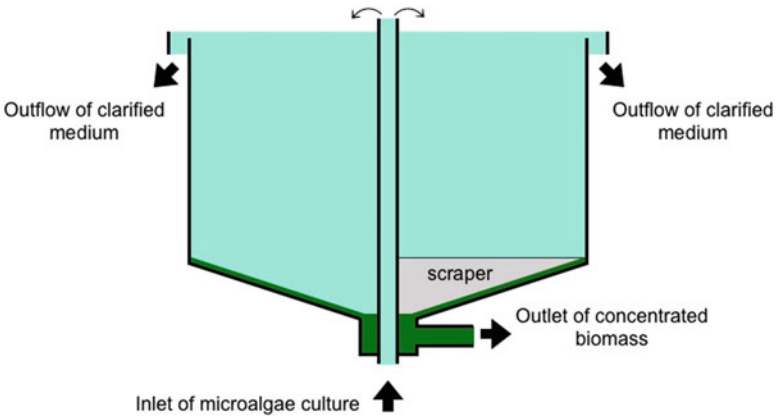

Fig. 10.6 Schematic drawing of a conventional circular thickener

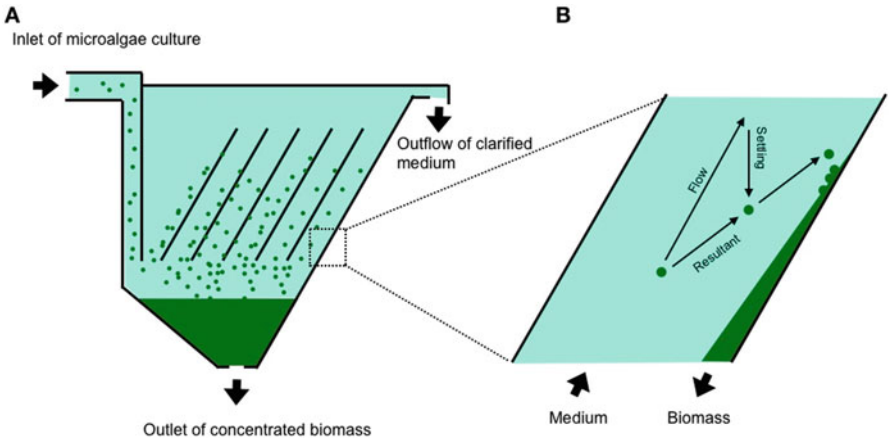

Fig. 10.7 Schematic drawing of a lamellar thickener (**a**) and detail of the settling process (**b**)

The lamella thickener is a tank with inclined parallel plates (lamellae at 55° from the horizontal) installed in its interior, whose main function is to increase the settling surface (Fig. 10.7). They are more efficient than conventional thickeners, providing a large effective settling area for a small footprint (Janelt et al. 1997). As the culture moves through the thickener, the biomass accumulates on the inclined lamellae. As gravity overcome friction, due to the inclination angle, the biomass slides out of the tank (Janelt et al. 1997; Smith and Davis 2013). Janelt et al. (1997) employed a lamella thickener to harvest *Chlorella vulgaris*, without addition of flocculating agent, obtaining a recovery efficiency around 30–35% and a concentration factor of 1.5–1.6. Despite being more efficient than conventional thickeners, those results

are still low for a commercial production. Smith and Davis (2013) optimized an inclined thickener, also to harvest *C. vulgaris*, significantly increasing both recovery efficiency (70%) and concentration factor (80-fold). Thus, it is possible to increase the process efficiency. Nevertheless, sedimentation is more suitable for organisms larger than *C. vulgaris* (5 μm), with larger settling velocities, such as the cyanobacterium *Arthrospira* (Janelt et al. 1997).

Sedimentation can be considerably improved, both in time and in efficiency, if a flocculating agent is employed (Janelt et al. 1997; Smith and Davis 2013). This agent will aggregate the microalgae cells, thus forming larger and heavier flocs than the isolated cells, which will settle faster.

5.2 Centrifugal Sedimentation

Unlike sedimentation, almost all microalgae species can be harvested using centrifugation, irrespective of cell size or density. A centrifuge is essentially a sedimentation tank with a high rotational speed that raises the gravitational force (up to 14,000× g) and, consequently, increases the settling velocity of microalgae (Fig. 10.8). The use of centrifuges offers many advantages when compared to other commonly used processes. For example, the biomass is free of chemicals (e.g., flocculants), recovery efficiency (100%) and final concentration (22% solids) are high, and detention period is short, thus maintaining the biomass quality and avoiding its deterioration. High quality and absence of chemicals are fundamental requirements if the biomass is intended to be used in the food, pharmaceutical, and

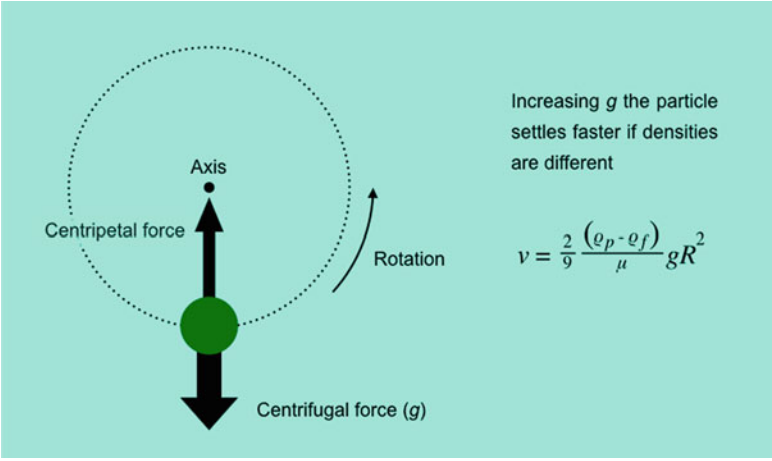

Fig. 10.8 Settling velocity equation (v) under conditions that favor the sedimentation of microalgae by the increase of the centrifugal force. g gravity, R cell radius, ρ_f density of water, ρ_p density of the microalga, μ viscosity of water

cosmetic industries. Still, the high gravitational force may damage species with fragile cells (e.g., *Dunaliella salina*) due to shearing stress, causing the loss of metabolites of interest during the process (Xu et al. 2015). In addition, the energy demand of centrifuges is very high (up to 8 kWh m^{-3}), and, thus, they are mainly employed for high-value products. For biofuel production, centrifugation would result in harvesting costs of \$4.52 L^{-1} of oil (Dassey and Theegala 2013). Yet, harvesting costs would be reduced to \$0.864 L^{-1} of oil if a low-efficiency (28%) and a high-flow (18 L min^{-1}) approach were adopted, instead of a high-efficiency and low-flow approach, resulting in 82% energy reduction (Dassey and Theegala 2013).

Regarding production scale, the use of larger centrifuges becomes problematic because, due to the mechanical limitations, the gravitational force they can reach is smaller, thus reducing their efficiency. Also, capital costs increase with scale, not mentioning maintenance costs.

Centrifuges are more advantageous for harvesting low volumes of concentrated cultures. So, centrifuges can be employed as a single step for harvesting photobioreactors (~5 g L^{-1}) or combined with a primary concentration step such as sedimentation, flotation, flocculation, or filtration, to reduce the volume to be centrifuged. Flocculation, in particular, not only reduces the volume but also the energy demand as the flocs are larger and heavier, thus requiring lower gravitational force. However, flocculation limits the uses of this biomass if it is required to be free of chemicals.

The conical plate (or disc stacked) centrifuge is the most employed model to harvest microalgae (Milledge and Heaven 2011). This centrifuge has a series of parallel (0.4–3 mm) conical discs, which increases the settling surface and reduces the distance of the particle settling, using the same principle of a lamellae clarifier (Fig. 10.9). The energy consumption for separation varies from 0.7 to 1.4 kWh m^{-3}, depending on the microalgae harvested. Besides the conical plate, perforated basket, imperforated basket, decanters, and hydrocyclones, centrifuges are also employed on industrial scales (Pahl et al. 2013).

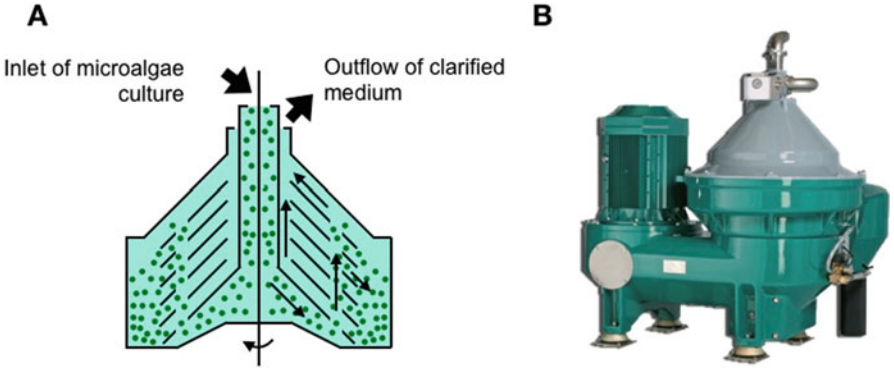

Fig. 10.9 Cross section showing the operation of a comical plate centrifuge (**a**). Example of continuous self-cleaning industrial centrifuge (**b**)

5.3 Flotation

Contrarily to sedimentation and centrifugation, flotation consists of making the microalgae less dense than the water (i.e., $\rho_p < \rho_f$), so they simply float to the surface, being collected by skimming (Laamanen et al. 2016). This is achieved by the addition of air bubbles that adhere to the microalgae cells, decreasing their density and increasing buoyancy (Fig. 10.10) (Singh et al. 2011). The adhesion of air bubbles to microalgae depends on many factors, including the size of the microalgae, the likelihood of collision, and the adhesion between them. Small-sized microalgae are easier to bring to the surface compared to larger-sized microalgae; however, decreasing size also decreases the likelihood of collision with bubbles. Moreover, since microalgae and bubbles have negative surface charges, causing electrostatic repulsion, it is necessary to employ additives such as surfactants (Garg et al. 2012; Zhou et al. 2017), polymers (Hanumanth Rao et al. 2018a), or even the organic matter produced by microalgae (Hanumanth Rao et al. 2018b) to modify the surface properties. Despite this, flotation is faster and more efficient than sedimentation (Besson and Guiraud 2013; Laamanen et al. 2016). While sedimentation produces a concentrate of about 3% solids, flotation achieves up to 6%, producing a thicker concentrate (Laamanen et al. 2016). Furthermore, as biomass builds up on the surface, it is easier to remove from the tank by skimming or scraping the surface.

Flotation can be classified according to how bubbles are produced, into dissolved-air (DAF), dispersed-air (DiAF), or electrocoagulation flotation (ECF) (Svarovsky 2001; Laamanen et al. 2016). In DAF the atmospheric air is pressurized (25–90 PSI),

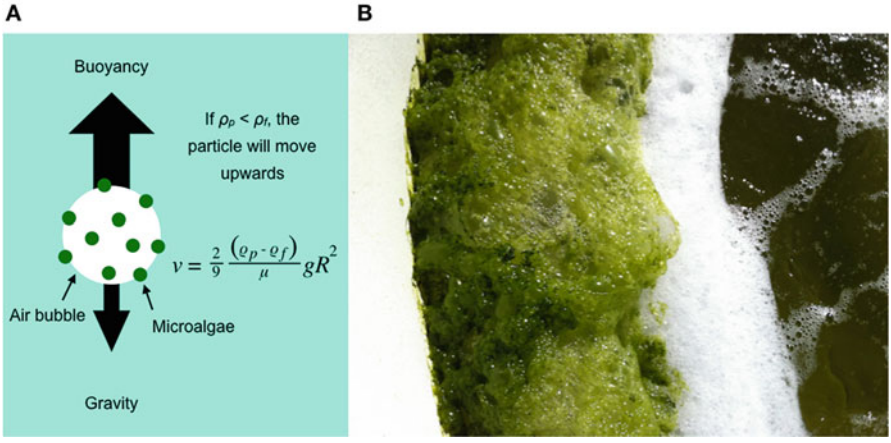

Fig. 10.10 Settling velocity equation (v) in conditions that favor the flotation of microalgae by adsorbing air bubbles on its surface and decreasing its density (**a**). Detail of foam with biomass produced by flotation, adhered to tank wall (**b**). g gravity, R cell radius, ρ_f density of water, ρ_p density of the microalga, μ viscosity of water

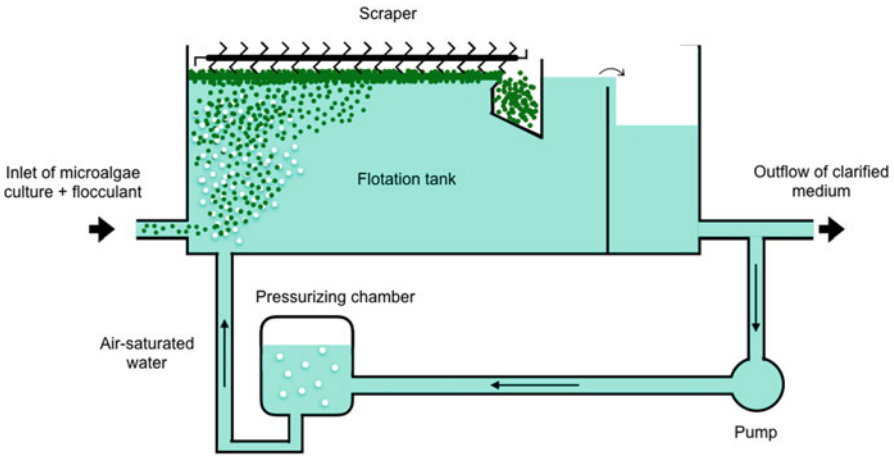

Fig. 10.11 Schematic drawing of a dissolved-air flotation (DAF) tank

increasing its saturation in the water, according to Henry's Law of solubility of gases. When this saturated water (5–15%) is injected into the flotation tank, which is under atmospheric pressure, the decompression causes the air to return to its gaseous form, forming microbubbles (10–100 μm) (Laamanen et al. 2016). As they rise to the surface, the microbubbles collide and adhere to the microalgae cells. The biomass then aggregates and accumulates on the surface, forming a foam which is then removed by a scraper or skimmed. Thereafter, the clarified medium exits the flotation tank as effluent, while a portion is pumped into the pressurizing chamber to be reintroduced into the flotation tank as saturated water (Fig. 10.11). This process works well in freshwater and is able to handle the large volumes required in a commercial scale plant (10,000 m^3 day^{-1}). However, DAF has a high electrical consumption (7.6 kW h m^{-3}) due to the high pressures required to saturate the water (Wiley et al. 2009).

In DiAF the bubbles are larger (700–1500 μm) because the air is directly inserted into the flotation tank through a porous media by a low-pressure compressor (Fig. 10.12) (Laamanen et al. 2016). Thus, the size of the bubbles varies according to the type of injector. By using a low-pressure compressor (15 PSI), the electrical consumption of this type of system is lower (3 kW h m^{-3}) than by DAF. However, due to the larger size of the bubbles, their efficiency is lower, being common is the use of flocculating agents to increase the efficiency of the process (over 90%). This is a significant disadvantage as the contaminated biomass may not be used as an animal feed or a food supplement (Draaisma et al. 2013). However, the effectiveness of DiAF in saline waters is increased as the surface tension is reduced, resulting in smaller bubbles than in freshwater (Laamanen et al. 2016). Because it is a simpler and cheaper system, it can be easily assembled with aeration hose (3 mm bubbles), a low-pressure air compressor, and flocculant (Fig. 10.13).

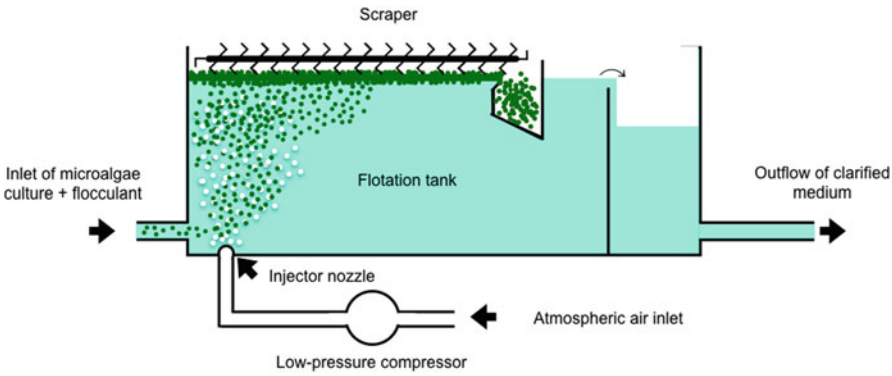

Fig. 10.12 Schematic drawing of a dispersed-air flotation (DiAF) tank

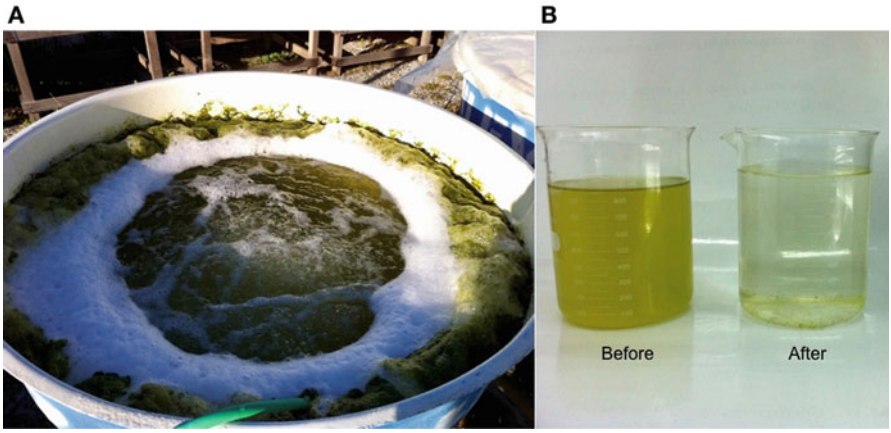

Fig. 10.13 Example of a dispersed-air flotation (DiAF) system built with simple materials. Notice the formed foam with *Nannochloropsis oculata* biomass (**a**) and water transparency after harvesting (**b**)

Electrolytic flotation (EF) is a process where the passage of an electric current through an electrode (sacrificial or non-sacrificial) causes the decomposition of water, generating hydrogen and oxygen microbubbles (Fig. 10.14) (Vandamme et al. 2011; Lee et al. 2013). As in the previous processes, the microbubbles adhere to the microalgae and transport them to the surface. However, because the microbubbles generated are extremely small (15–45 μm), EF is much more efficient than DAF or DiAF. To perform the electrolysis of the water, two electrodes are employed, being a cathode and an anode. The cathode is usually made of an inert material, while the anode can be made of aluminum or iron (sacrificial electrodes), or made of carbon (non-sacrificial electrodes) (Misra et al. 2015). When an electric potential is applied through the electrodes, a flow of electric current is established,

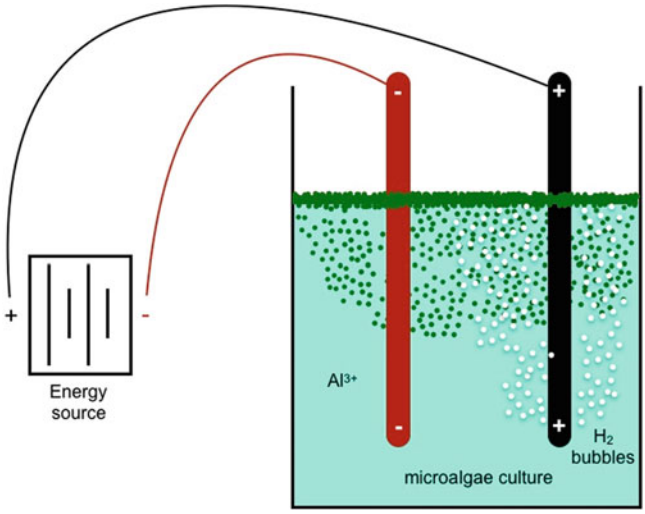

Fig. 10.14 Schematic drawing of the electrolytic flotation (EF) process, with a sacrificial aluminum anode. The formation of bubbles occurs at the cathode, while at the anode, there is the release of aluminum ions that act as a flocculating agent

transferring an electron from the anode to the cathode. The electrolysis reaction occurs at the cathode, where two electrons ($2e^-$) break two molecules of water ($2H_2O$) into hydrogen gas (H_2) and two hydroxide ions ($2OH^-$). In addition to the size of the bubble, the efficiency of the system is higher because, as the sacrificial anode is oxidized, it releases metal ions (Al^{3+} or Fe^{3+}) which react with the hydroxide ion forming a flocculating agent ($Al(OH)_3$ or $Fe(OH)_3$) that aggregates the microalgae (Duan and Gregory 1996; Misra et al. 2015; Laamanen et al. 2016). However, this process causes the electrode depletion, requiring periodic replacement and adding to the production costs. The efficiency of EF is considered high (96%), especially for marine microalgae, because the electrical conductivity is higher in salt water (Gao et al. 2010; Vandamme et al. 2011). However, iron electrodes perform poorer than aluminum electrodes, probably due to the lower current efficiency (Gao et al. 2010; Vandamme et al. 2011; Baierle et al. 2015). The energy expenditure is also low, consuming only 0.3 kWh m^{-3} for an operational period of 75 min (Poelman et al. 1997). However, as the metallic hydroxides from the sacrificial electrodes remain adhered to the biomass, EF is not recommended if the biomass is intended to be used for human or animal nutrition (Uduman et al. 2011; Draaisma et al. 2013). For instance, Vandamme et al. (2011) reported 1.5% of aluminum in the biomass. To avoid the formation of metallic hydroxides, the application of non-sacrificial carbon electrodes is recommended (Misra et al. 2015). In this case, no flocculating agent is formed, so aggregation is achieved by adjusting the applied current and pH and the addition of an electrolyte (sodium chloride). Although being a flotation process, the biomass usually sinks after some time due to its weight.

6 Filtration-Based Processes

Basically, harvesting of microalgae by filtration-based process is a solid-liquid separation where a permeable membrane acts as a physical barrier, allowing the liquid phase to pass (permeate) while the solid phase is retained (retentate) (Svarovsky 2001; Bilad et al. 2014). Therefore, contrarily from gravity-based processes, the separation is due to differences in size between solids and the membrane pores. In order to obtain a flow through the membrane, it is necessary to apply a pressure difference across the membrane (i.e., a transmembrane pressure), which can be positive or negative. Essentially, microalgae harvesting is achieved using surface filters for cake filtration in which the solids are deposited on the upstream side of a thin membrane. The main disadvantage is that a cake is allowed to form on the filter surface (dead-end filtration), gradually decreasing the permeance at a constant transmembrane pressure (Fig. 10.15a). Consequently, to maintain the permeance constant, it is necessary to increase the transmembrane pressure over time, which increases the energy consumption, being recommended for filtering low-concentration cultures. However, the permeance can be maintained constant, and energy consumption low, if cake formation is prevented. This is usually achieved by employing cross-flow filtration (or tangential flow filtration) in which the slurry is moving tangentially to the membrane so that the cake is continuously sheared off (Fig. 10.15b). Cross-flow filtration is more efficient and recommended for large-scale harvesting than dead-end filtration (Petruševski et al. 1995; Bilad et al. 2014).

Filtration can either be continuous or discontinuous, being classified based on the membrane pore size as macrofiltration (100–10 μm), microfiltration (10 μm–900 Å), ultrafiltration (100–40 Å), nanofiltration (80–8 Å), and reverse osmosis (50–5 Å)

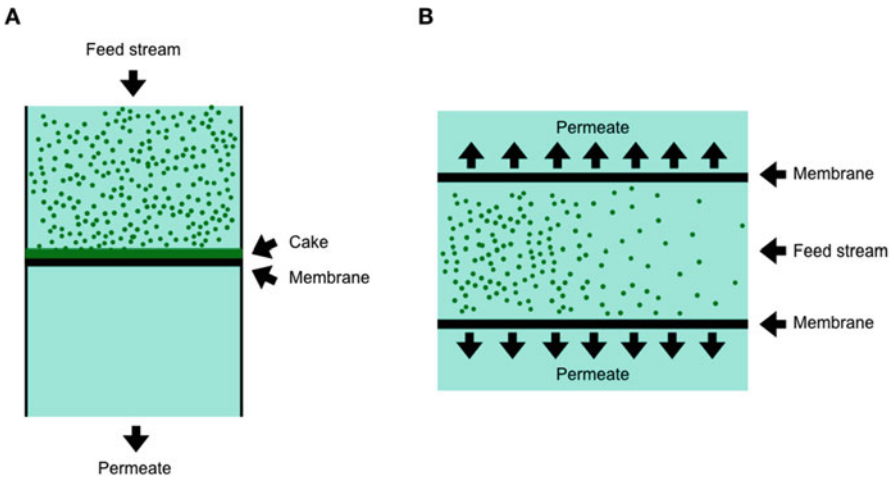

Fig. 10.15 Schematic of dead-end (**a**) and cross-flow filtration (**b**)

(Drexler and Yeh 2014). Microfiltration and ultrafiltration are commonly employed for discontinuous microalgae harvesting, although for full-scale systems, continuous operation is recommended (Bilad et al. 2014). The choice of membrane should be defined in a case-by-case basis as membrane characteristics (e.g., material, pore size, hydrophobicity), hydrodynamics conditions (e.g., transmembrane pressure), and suspension characteristics (e.g., algae species, cell format, concentration, organic matter release) are relevant to membrane performance (Mo et al. 2015). Membrane materials used in microalgae harvesting are mostly organic, being made of polyvinylidene fluoride (PVDF), cellulose acetate (CA), polytetrafluoroethylene (PTFE), polypropylene (PP), poly(ether sulfones) (PES), polyvinyl chloride (PVC), polyacrylonitrile (PAN), or polyethylene terephthalate (PET) (Bilad et al. 2014). Negatively charges membranes are preferable as they are believed to reduce the interaction between microalgal cells and the membrane surface, thus reducing cake formation (Marbelia et al. 2016).

Although the efficiency increases as the pore is reduced, the higher will be the energy requirement for pumping, as high transmembrane pressure and membrane shear rate are necessary to maintain high permeances and decrease fouling, respectively (Mo et al. 2015). Moreover, maintenance costs are also high due to membrane replacement due to pore clogging and fouling, as microalgal culture are composed of potential foulants (e.g., biomass, colloidal, and dissolved fractions) (Rawat et al. 2013; Bilad et al. 2014). Therefore, due to low membrane life-span, filtration is not recommended as a single step for large-scale processes for producing biomass (Molina Grima et al. 2003). Instead, filtration can be successfully employed as a primary concentration process (up to 2–7%), removing the majority of water and leaving a minimal load for a secondary concentration step (up to 20–25%) using centrifugation or other technologies (Bilad et al. 2014). In general, the energy consumption of membrane filtration is 0.2–2 kWh m^{-3} (Mo et al. 2015).

6.1 Screening

Screening with large mesh (>25 μm) is an effective gravity separation method employed to harvest large filamentous algae (>100 μm) such as *Arthrospira* and *Aphanizomenon* (Vonshak and Richmond 1988; Carmichael et al. 2000). Screening devices are basically of two types, inclining and vibrating. Efficiencies of biomass removal are high (up to 95%), processing up to 20 m^3 h^{-1} and producing a concentrated slurry of 8–10% solids (Vonshak and Richmond 1988). Vibrating screens are as efficient as inclining screens, filtering the same volume per unit time but requiring one-third of the area, as they can be arranged in stacks. However, a problem observed for vibrating screens is that, after repeated harvesting, the filaments in culture become shorter decreasing harvesting efficiency (Vonshak and Richmond 1988; Depraetere et al. 2015).

6.2 Membrane Filtration

Microfiltration and ultrafiltration are the most commonly studied membranes for microalgae filtration. Dead-end filtration is considered not economically viable for most microalgae harvesting purposes due to membrane fouling and pore clogging, being mainly applied for low concentrated solutions (Bilad et al. 2014; Drexler and Yeh 2014). Instead, cross-flow filtration is preferred as it is less susceptible to fouling, producing a concentrated sludge of 8–15% solids (Mo et al. 2015). Permeate fluxes in cross-flow filtration generally ranges from 15 to 120 L m^{-2} h^{-1}, and energy requirement have been estimated to be 3–10 kWh m^{-3} (Rossignol et al. 1999). For minute species, flocculation can be employed to aggregate the cells, increasing harvesting efficiency.

Basically, the equipment employed for microalgae harvesting can be divided into pressure (like plate-and-frame press), vacuum (like rotary drum), and gravity filters (like belt press) (Svarovsky 2001). In the plate-and-frame press (Fig. 10.16a), the microalgae culture is pumped into a sequence of interspaced hollow plates covered with a filtering membrane. The plates are pressed together with hydraulic or screw-driven arms (around 6 bar), forcing the liquid through the membrane, whereas the biomass is retained within the plate, forming a cake (dead-end filtration). The process is then stopped, the plates are separated, and the biomass is discharged (batch-wise). Despite being an efficient process to recover microalgae biomass, plate-and-frame filters are not widely employed as it is inadequate to recover small microalgae species, and filters have to be cleaned or replaced regularly (Mo et al. 2015). In the rotary drum vacuum filters (Fig. 10.16b), the driving force for filtration results from the application of suction on the filtrate side of the medium (Svarovsky 2001; Shao et al. 2015). The rotary drum is partially submerged in the microalgae culture, and the medium is drawn into the drum through a membrane. The microalgae cells accumulate on the external

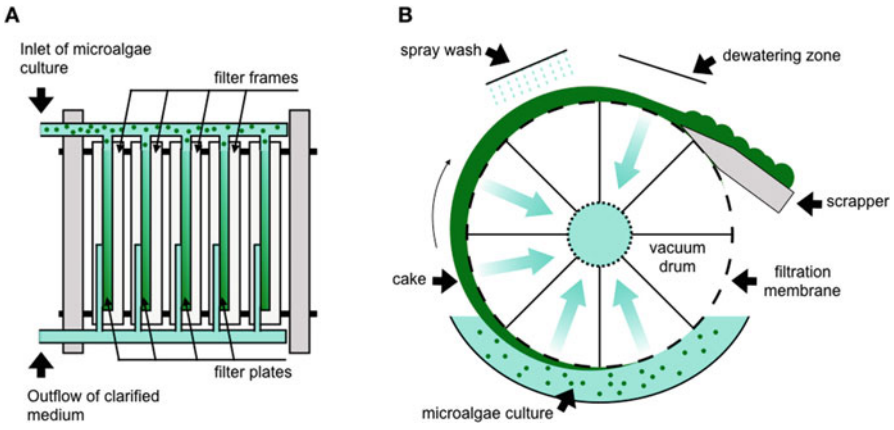

Fig. 10.16 Schematic of a filter and plate (**a**) and rotary drum filters (**b**)

surface of the membrane as the drum slowly rotates (1–10 min per revolution), continuously forming a cake layer that is peeled of the filter surface and collected. In the belt press, the principle of separation is gravity drainage, where the microalgae feed is filtered by a fabric mesh that moves over rollers (Sandip et al. 2015). Belt press has a lower energy consumption and operational costs, besides being operated continuously and upscaled. However, belt press requires a previously concentrated microalgae suspension (10–40 g L^{-1}), being employed in combination with other unit operations like flocculation (Sandip et al. 2015).

7 Conclusion

Microalgae have a great potential as a source of several compounds of interest to the food, pharmaceutical, and chemical industries. The annual production is 9.000 tons of dry matter, with a market value above US$1.25 billion. Yet, developing a low-cost and energy-efficient large-scale harvesting method remains as one of the major challenges to be overcome. Microalgae developed several adaptations to remain in suspension. Moreover, the culture systems employed are not so efficient as even the most producing systems cannot attain high biomass densities. Consequently, large volumes of dilute culture (0.05%) need to be processed and concentrated to obtain a paste with a dry matter content of 15–25%. If microalgae are intended for high-value products, the harvesting costs are compensated. However, for low-value products, the current costs are prohibiting. No harvesting method is universal as several factors should be considered, such as species, culture system, and the final product envisioned. However, different harvesting methods can be sequentially employed in order to reduce the production costs. One example could be flocculation followed by membrane filtration to pre-concentrate the biomass, combined with centrifugation to obtain the microalgae paste. Nevertheless, care should be taken that the harvesting method does not result in biomass contamination or interfere with its downstream processing. Moreover, to be environmental and economically sound, the harvesting method should allow the recycling of the spent culture medium. Therefore, a significant demand exists for research in microalgae harvesting.

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Chapter 11
Methods for Extraction of Valuable Products from Microalgae Biomass

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Abstract Microalgae are regarded as a promising and feasible source of diverse products for application in the nutraceutical, pharmaceutical, cosmeceutical, and chemical industries. Such products include biofuels, lipids, polyunsaturated fatty acids, pigments, enzymes, polysaccharides, and proteins. The recovery of products from algal biomass is a matter of constant development and progress. In the present chapter, the standard techniques for the extraction of biofuels and high-value metabolites from microalgae in the laboratory are reviewed. Traditional methods such as solvent extraction, as well as novel techniques, like supercritical fluid extraction, microwave-assisted extraction, and ultrasound-assisted extraction, are presented in this work, including the suitability of these methods for specific types of metabolites. Pretreatment techniques for the enhancement of product recovery are discussed. This chapter is intended as a reference of the existing methods for the researcher looking forward to study the production of metabolites by microalgae, in order to aid the selection of a suitable technique for specific metabolites.

Keywords Microalgae · Lipid extraction · Pretreatment · Traditional methods · Advanced method

1 Introduction

Microalgae are photosynthetic microorganisms that represent an attractive alternative source of lipids for biodiesel production. Because of their unicellular structure, they have the potential for high oil production without challenging the use of agricultural resources. Microalgae are able to grow in saltwater, brine, nutrients from residual wastewaters, and waste CO_2 (Griffiths et al. 2012; Wijffels et al. 2013;

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Hernández et al. 2014). Microalgae include prokaryotic cyanobacteria and eukaryotic photosynthetic organisms (Brasil et al. 2017) that utilize solar energy, nutrients, and carbon dioxide (CO_2) to produce a wide variety of complex molecules. The primary metabolism of these microorganisms synthesizes proteins, carbohydrates, and lipids; and secondary metabolites, such as carotenoids, alkaloids, terpenoids, and polyketides, are species dependent (Cuellar-Bermudez et al. 2015; Mazard et al. 2016).

Some of these metabolites are of particular interest to the industry because of their high-value metabolites. Examples of these are antioxidants, pigments, pharmaceuticals, polyunsaturated fatty acids (PUFAs), and biomass for animal feed, fertilizers, or energy (Mata et al. 2010).

The study of microalgae has gained interest due to their capacity to produce valuable molecules under different environmental conditions, a feature that makes them an attractive candidate for the production of energy, like biodiesel and biogas (Nie et al. 2018).

Another area worth exploring is the use of microalgae as a platform for wastewater treatment. Phycoremediation is a feasible solution to mitigate the environmental impact of industries with high waste production. For example, there are reports of microalgae biomass production from sewage, swine wastewater, or even poultry waste. *Spirulina platensis*, *Spirulina maxima*, and *Chlorella* sp. have been used for this purpose (Wu and Wilson 1981).

A critical step for the recovery of valuable products from microalgae is the extraction method. To achieve the extraction of the desired products, microalgae cells have to go through cell disruption for the release of the molecules of interest and then to apply one of several methodologies, such as solvent extraction, mechanical methods, freeze-thawing, enzymatic lysis, microwave-assisted extraction, and ultrasound extraction, among others. For cell rupture at large-scale settings, mechanical methods are preferred, since they allow complete disintegration of the cells, with high product yield (Moraes et al. 2010).

The factors to be taken into account for microalgae product extraction are cell disruption method, type of solvent, solvent-to-biomass ratio, and extraction time. These factors should be studied altogether, so the extraction process can be optimized, by minimizing costs and maximizing yield (Moraes et al. 2010). A set of factors have a simultaneous effect on a process. Design of experiments is the most effective tool to identify and optimize the most significant factors with the least experimental runs (Sharif et al. 2014). These designs are divided into two categories: screening (full factorial, fractional factorial, and Plackett-Burman designs) and optimization (central composite or Box-Behnken design) (da Silva et al. 2016).

This chapter reviews the existing methods for the extraction of biodiesel and other metabolites from microalgal biomass, at a laboratory and large scales, in order to aid researchers with the selection of a suitable technique for specific metabolites in their studies.

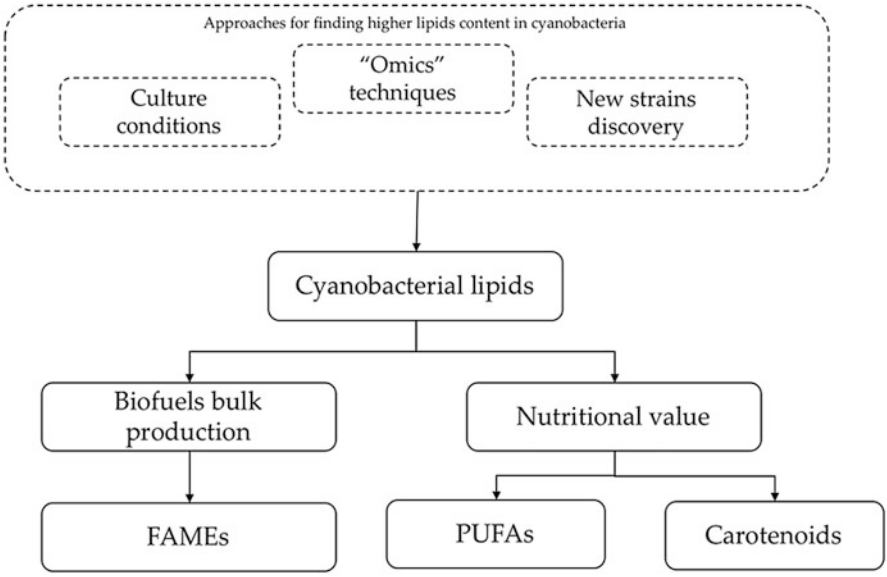

Fig. 11.1 Schematic classification of cyanobacterial application for lipids and approaches for finding/producing higher lipid content strains

2 Microalgae Products: Biofuels and Metabolites of Nutritional Value

The role of lipids in the microalgae cell is critical for cellular function. In cyanobacteria and microalgae, lipids can be classified according to their potential applications as fatty acids for biofuels (suitable for transesterification) and lipids with nutritional value, like polyunsaturated fatty acids and carotenoids (Fig. 11.1).

Microalgae produce lipids through the conversion of carbon dioxide emissions into glucose, which in turn is converted to fatty acids for membrane synthesis. Under stress conditions, fatty acids are converted into lipids for biodiesel production. Total oil productivity depends on the oil content of the biomass and growth rate (Aarthy et al. 2018).

2.1 Biodiesel

Biodiesel is a fuel produced by transesterification of triacylglycerides (TAGs) usually obtained from plant oils with high amounts of fatty acid methyl esters (FAMEs) and also is produced by some cyanobacteria from CO_2 and sunlight. Particularly for biodiesel production, three important factors have been identified when using photosynthetic microorganisms for bulk production: lipid productivity, fatty acid profile, and harvesting potential. Cyanobacteria present different advantages over microalgae since they provide high

biomass concentration and grow under relatively harsh environments; also, they can be easily harvested by flotation or filtration (Griffiths et al. 2012). The utilization of organisms such as cyanobacteria and microalgae for biofuels has many advantages over fossil fuels and plants: higher growth rate compared to plants, availability of genetic tools and sequenced genome, and their ability to grow in areas where crop fuels cannot (Machado and Atsumi 2012).

Since a few decades ago, intensive research has been carried out on biofuel production with *Synechocystis* and *Synechococcus* and in a minor extent with *Anabaena* and *Cyanothece* (a nitrogen-fixing strain). "Omics" techniques have been extensively used in the introduction of pathways for ethanol, butanol, fatty acids, and other organic acids, all focused on biofuel bulk production (Wijffels et al. 2013). Recently, genetic engineering techniques have been applied to *Synechocystis* sp. to increase the intracellular concentration of fatty acids, reaching up to 197 mg/L (Machado and Atsumi 2012).

The discovery of new strains with higher fatty acid production is another approach. Cyanobacterial isolates of the genera *Synechococcus*, *Trichormus*, *Microcystis*, *Leptolyngbya*, and *Chlorogloea* have been investigated regarding quantity and quality of lipid feedstock. Their biomass productivity ranged from 3.7 to 52.7 mg/L-day, and the lipid productivity varied between 0.8 and 14.2 mg/L-day. The most promising species were the ones of genera *Synechococcus* and *Microcystis*; they also showed similar fatty acid profiles to those of seeds already used for biofuel production (Da Rós et al. 2013). Strains of *Pseudanabaena* sp., *Synechococcus* sp., and *Nodosilinea* sp. were isolated and exhibited lipid contents from 7.4% to 15.66% (dry cell weight), being *Synechococcus* sp. the strain with maximal biomass and lipid productivity. These strains were then subjected to mixotrophic cultivation with ostrich oil as a carbon source, resulting in a high content of palmitoleic, palmitic, linoleic, linolenic, and oleic acids (Modiri et al. 2015).

An important and simplest approach, other than genetic engineering, to increase the number of fatty acids in cyanobacteria is the modification of culture conditions such as nutrients and light intensity. *Synechocystis* sp. cultures were optimized for nutrient supply and light intensity, showing lipid productivities of 722 mg/L after 12-day cultivation (Monshupanee and Incharoensakdi 2014).

It has been reported that for biomass and lipid productivity, mixed cultures possess higher potential than single cultures. Cyanobacteria *Synechocystis salina* was co-cultured with three different species of microalgae to assess their potential to remove nitrogen and phosphorus from wastewater and lipid productivity. Lipid productivities determined for single cultures ranged from 4.39 to 7.13 mg/L-day, whereas for dual-species cultures, these values ranged from 7.76 to 11.10 mg/L-day (Gonçalves et al. 2016). *Leptolyngbya* sp. was co-cultured with *Chlorella vulgaris* in heterotrophic and mixotrophic conditions. Mixotrophic cultures with sodium acetate produced the highest biomass and neutral lipid productivity (156 g/m^3-day and 24.07 g/m^3-day, respectively) (Silaban et al. 2014).

Enrichment of the culture media with sodium carbonate (Na_2CO_3) and high light intensities also resulted in an increase in unsaturated fatty acids (particularly linoleic acid) in *Synechococcus* sp. (Silva et al. 2014). Recently, lipid droplet (inclusions of neutral lipids) location and identification have been studied because of their content

of TAGs for biodiesel. *Nostoc punctiforme* lipid droplet locations were identified to assess new possible ways to increase their presence (Peramuna and Summers 2014).

2.2 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids are 18–22 hydrocarbon chains and are classified as omega-3 and omega-6 fatty acids. PUFAs are reported to have a positive impact on health such as reduction of the risk for cardiovascular disease, neurological disorder, inflammation, and cancer. By overexpression of endogenous and exogenous genes encoding for PUFA desaturases in the blue-green algae *Synechocystis* sp., the accumulation of α-linolenic acid (ALA, 18:3n-3) and stearidonic acid (SDA, 18:4n-3) was increased (Chen et al. 2014). *Synechocystis aquatilis* is one strain of cyanobacteria that has high CO_2 uptake and has been tested for lipid production in batch and continuous culture. The extracted fatty acids were mostly composed of saturated acid (palmitic acid) as well as polyunsaturated fatty acids (linoleic and a-linolenic acid) (Kaiwan-arporn et al. 2012). Genetic engineering has also been used in the cyanobacteria *Synechococcus* sp. to include desaturase enzymes, resulting in 5.4 times greater accumulation of ALA compared to the wild-type strain and production of both GLA (gamma-linoleic acid) and SDA (Dong et al. 2016b).

2.3 Carotenoids

Carotenoids are a group of isoprenoid compounds comprising a wide range of structures; they possess high antioxidant and pigmentation properties. In *Arthrospira platensis* the carotenoids β-carotene, β-cryptoxanthin, zeaxanthin, and astaxanthin have been identified (Esquivel-Hernández et al. 2017). β-Carotene is a worldwide used carotenoid with industrial applications in cosmetic and food industry (Malik et al. 2012; Havaux 2014). Cyanobacteria have been studied in regards of carotenoid production; recombinant DNA technology has been applied to strains of *Synechocystis* to increase the number of carotenoids in the cell, resulting in different metabolic pathway studies (Al-Haj et al. 2016).

3 Methods for the Extraction of Microalgae Products

Algae biomass can be submitted to different processes in order to extract different compounds. There are several conversion processes; some of the most important are the mechanical-based treatments (Cherubini et al. 2009). After microalgae cultures reach the stationary phase of growth, the biomass is concentrated, and the products of interest can be extracted by using dry or wet biomass. For the first case, a

dewatering process can be performed by centrifugation and is followed by a cell disruption process with the objective to break the cellular walls and favor the release of microalgae components that are not secreted outside the cell. The methodologies used involve disruption, breakage, or disintegration (Dong et al. 2016a). On the other hand, to get dry biomass after the process of dewatering, it undergoes a thermal drying process that usually produces a paste-like biomass, with a dry weight above 85% (Xu et al. 2011).

The pretreatment of microalgae biomass is a very important step for the recovery of lipids and other valuable products, as a tool to improve extraction yields (Karemore and Sen 2016). Microalgae cell walls consist of a fibrillar matrix and crystalline polymers, and the proportion varies between species, so it is necessary to develop a specific pretreatment method (Aarthy et al. 2018).

3.1 Solvent Extraction

Most of the solvent-based extraction techniques used for extraction of lipids from microalgae are based on the traditional methods used for plant oil extraction, like organic solvent extraction, Folch method, Soxhlet, and others. Organic solvents are absorbed within the cell wall, where they cause swelling and rupture of the microalgae cell, making the cell contents available to be separated on the following step (Molina Grima et al. 2013). The main parameters to consider in the choosing of a solvent for the extraction of lipids from microalgae are polarity or extractability, lipid solubility, water miscibility (ability for two-phase systems), and low toxicity (Bensalem et al. 2018).

3.1.1 Folch Method

Lipid extraction by the Folch method relies on the use of a 2:1 chloroform-methanol mixture for extraction of intracellular lipids and is the basis of many solvent extraction methods used nowadays. First, a cell homogenate is equilibrated with 25% volume of saline solution and stirred. This mixture is left to stand until biphasic separation, so the lipids settle on the upper layer (Ranjith Kumar et al. 2015). After, the organic phase is recovered and is evaporated under nitrogen flux. The lipids are weighed, and the lipid content is calculated as the ratio of the weight of lipids and the weight of total microalgae biomass (Fig. 11.2).

Since this method was originally designed for animal cells and tissues, a previous step of disruption of microalgae cell walls has to be included (Molina Grima et al. 2013).

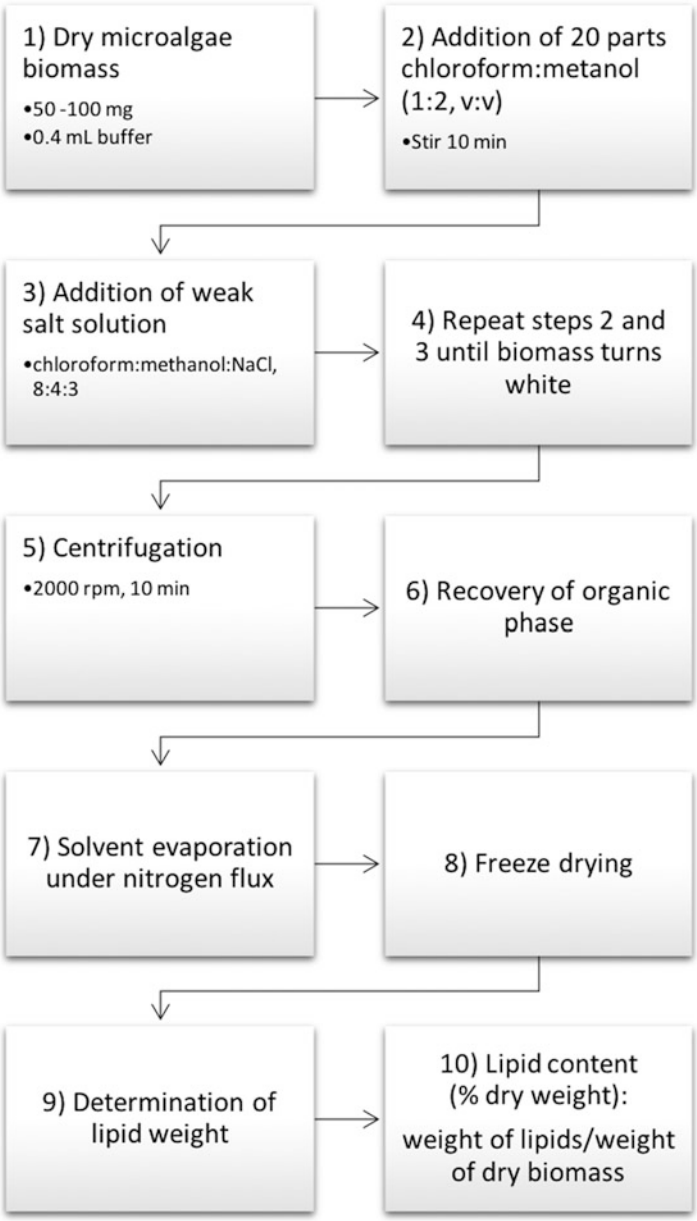

Fig. 11.2 Scheme of a modified Folch method for the extraction of lipids from microalgae cells

3.1.2 Soxhlet Extraction

Soxhlet extraction (SE) is a process in which partially soluble components of a solid sample are transferred to a liquid phase (solvent), by means of a Soxhlet extractor. This technique employs nonpolar solvents, like hexane, to obtain neutral lipids. The extraction consists of the placement of the solid sample in a filter paper thimble into the main chamber of the Soxhlet apparatus. Then the solvent is heated to reflux and travels into the main chamber, so the less soluble compounds are recovered by the solvent (Royal Society of Chemistry 2018). As the extraction solvent polarity increases, a higher extraction yield of microalgae can be achieved, due to recovery of complex lipids and pigments (Baumgardt et al. 2016). This is an important consideration since total lipid extracts with polar solvents are complex and other metabolites different than lipids are present. Soxhlet extraction parameters include a choice of solvent, sample particle size, and extraction time (Sharif et al. 2014). A modified methodology for microalgal lipid extraction is presented in Fig. 11.3 (Molina Grima et al. 2013).

Soxhlet extraction is usually carried out in laboratory scale, requires high solvent consumption and long extraction time. After, the total fatty acid methyl esters (FAMEs) are measured to evaluate the efficiency of the extraction procedure (Baumgardt et al. 2016).

3.1.3 Other Solvent-Based Methods: Bligh and Dyer Method

The Bligh and Dyer method consists in the simultaneous lipid extraction and partitioning, with protein precipitation in the interface between two liquid phases, similar to the Folch method. However, the solvent mixture composition and ratios are different (Fig. 11.4).

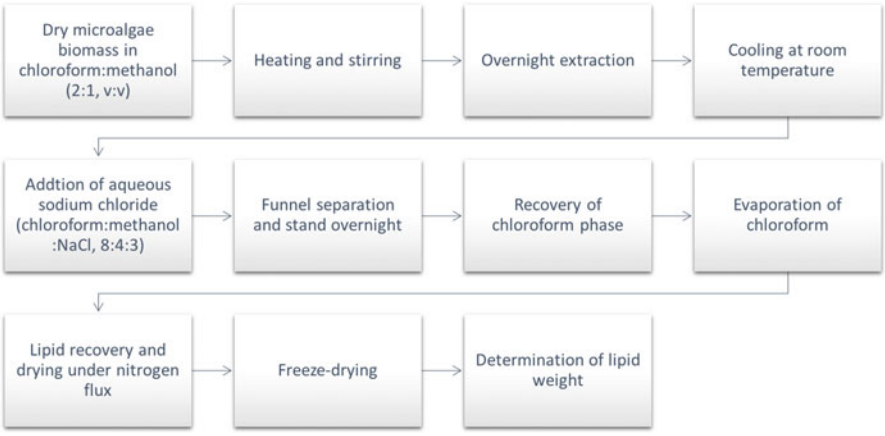

Fig. 11.3 Diagram of the Soxhlet extraction method for the extraction of lipids from microalgae

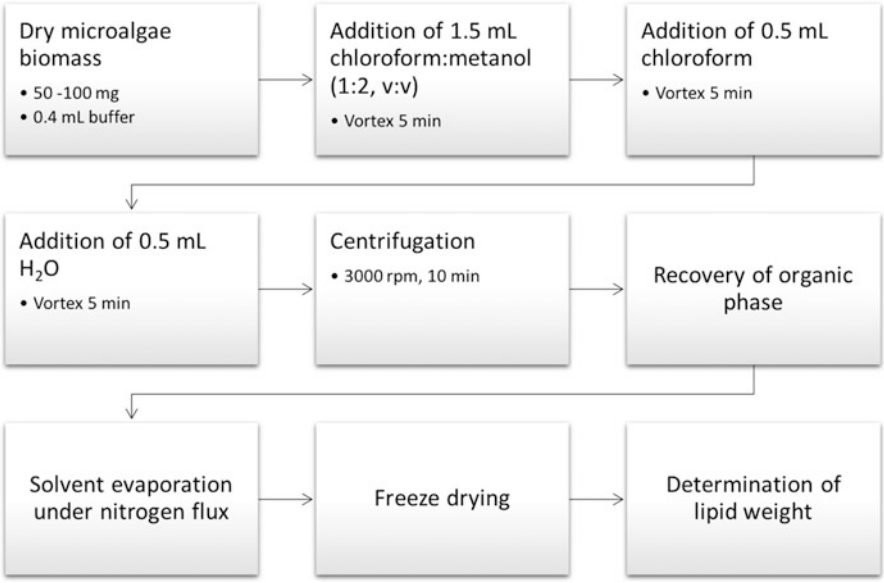

Fig. 11.4 Diagram of the Bligh-Dyer method for the extraction of lipids from microalgae

First, the lipids from a cell homogenate are extracted with 1:2 chloroform/methanol, and the chloroform phase (lipid-rich) is recovered. Microalgae lipids are extracted and measured by gravimetry. This procedure is employed in the pilot- and large-scale operations (Ranjith Kumar et al. 2015).

An improvement of this method is the addition of 1 M NaCl, instead of water, in order to avoid binding of acidic lipids to denatured lipids. Shorter separation times have been achieved by the addition of 0.2 M phosphoric acid and HCl. Increased recovery of acidic phospholipids has been achieved by the addition of 0.5% acetic acid (v/v) (Ranjith Kumar et al. 2015).

Organic solvent extraction remains as one of the main strategies for the recovery of valuable products from microalgae. Solvents should be chosen based on the polarity of the target compounds. For instance, TAGs are nonpolar molecules and the main lipid target for biodiesel production; hence a nonpolar solvent is a suitable choice for extraction.

3.2 Mechanical Methods

Cell disruption methods include solid shear, cavitation and collapse, pulsed electric fields, chemical hydrolysis, enzymatic digestion, subcritical water extraction, and high-pressure homogenization and bead milling.

3.2.1 Milling

Bead milling consists in disrupting cell walls of microalgae by grinding and agitation of the cells on a solid surface of glass beads (Ghasemi Naghdi et al. 2016). The size of the beads for an effective disruption is in the range of 0.3–0.5 mm. The beads can be made up of zirconia-silica or zirconium oxide. The process efficiency is determined by the biomass concentration, flow rate, agitator movement type and speed, and temperature.

The process of milling can be done in shaking vessels or through agitated beads. In the shaking vessel method, the culture vessel is under shake by using a vibrating platform, allowing the beads to move the microalgae cells and forcing them to collide with each other. The highest recovery of lipids through this method was performed by Ryckebosch and collaborators, recovering 40% of lipids from a culture of *Phaeodactylum tricronutum* (Ryckebosch et al. 2012). On the other hand, Zheng and collaborators extracted 11% of lipids from a culture of *Chlorella vulgaris*, using a bead milling vessel (Zheng et al. 2012). In the case of agitated beads, a method in which the beads and the culture are agitated by a rotatory agitator inside the culture vessel at the same time provides heat that helps in the disruption process, as reported by Lee and collaborators. The authors used this methodology and obtained an oil yield inside the range of 7.9–8.1 g/L, using cultures of *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. (Lee et al. 2010).

3.2.2 Pressing

The use of presses is one of the classical methods used to perform the extraction of value-added products from many sources. This method is based on the mechanical crushing of materials with a very low content of moisture. First of all, dried biomass is submitted to high mechanical pressure for crushing and breaking the cells and then squeezing the oil out of the biomass. The extraction efficiency can be improved by variations in the pressure force used, the algal strain used, and configuration of the press and pistons used. In the gel-press alternative, algae are first washed before carbohydrate extraction using diluted alkali. Centrifugation separates residues, followed by filtration through porous silica and concentration by evaporation. The material recovered is extruded through spinnerets into a cold solution of potassium chloride, and the gelled threads are then dewatered by pressure (Amin 2009).

Shear-based devices such as French press and Hughes press use high pressures to force a biomass solution through a small aperture. Usually, the oil recovery is in the range of 70–75%. Sometimes for enhanced oil recovery, mechanical crushing is used in addition with chemical methods. The principal drawbacks of this method are the requirement of high-cost maintenance and less efficiency compared to other methods (Ranjith Kumar et al. 2015).

Different products produced by microalgae, including lipids, proteins, and pigments, have been extracted by means of mechanical extraction. Table 11.1 shows the relation of mechanical extraction used and the yield obtained in different studies.

Table 11.1 The yield of value-added products extracted by the mechanical extraction

Method	Microorganism	Product	Yield (%)	References
Milling	*Chlorella vulgaris*	Lipids	11	Zheng et al. (2012)
	Chlorella protothecoides	Lipids	18.8	Shen et al. (2010)
	Botryococcus sp.	Lipids	28	Prabakaran and Ravindran (2011)
	Phaeodactylum tricronutum	Lipids	40	Ryckebosch et al. (2012)
Pressing	*Nannochloropsis* sp.	Proteins	91	Grimi et al. (2014)
	Spirulina platensis	Phycocyanin	82.48	Moraes et al. (2010)
	Haematococcus pluvialis	Astaxanthin	3.4	Olaizola (2000)
	Chlorococcum infusarium	Lipids	96.2	Karemore and Sen (2016)

3.3 Freeze-Thaw Method

The freeze-thaw method favors lipid extraction from microalgae biomass, since it decreases to a minimum the loss of volatile lipids due to evaporation. This method consists of the crystallization of the intracellular water by freezing the wet biomass at a temperature near −80 °C. After, the samples are thawed, so the frozen cells are lysed by the expansion of the ice crystals. This method is usually employed in combination with another method, such as ultrasonication, microwave-assisted extraction, or bead milling, with the purpose to increase yield efficiency (Aarthy et al. 2018). However, freeze-thawing cycles must be carefully managed. A study of the metabolic profile of marine microalgae after freeze-thawing under standard freeze-storage temperatures (−20 °C and −78 °C), for 1 and 2 cycles of 7 days each, reports that unfrozen samples showed a decrease of 10% in reproducibility after 1 cycle and decreased 7% further after the second cycle (Chr. Eilertsen et al. 2014).

3.4 Enzymatic Methods

In enzymatic extraction processes, a combination of enzymes is employed to break down the algal cell wall, expel lipid bodies outside the cell, and separate the lipid fraction from the lipid/protein matrix (Sierra et al. 2017). Enzymatic lysis is an alternative to mechanical cell disruption. The lytic enzymes have to be specific for the microalgae species, the most common being cellulase and lipase, due to the presence of polysaccharides, like cellulose and hemicellulose, in algal cell walls and lipids, contained in a sac surrounded by phospholipids (Aarthy et al. 2018).

Aqueous enzymatic assisted extraction (AEAE) is a cell disruption technique for the extraction of lipids from microalgae. Remarkable features include high selectivity, mild reaction conditions (neutral pH, incubation from 25 to 37 °C), and absence of intensive drying steps (Sierra et al. 2017). A report from Chen and collaborators presents an improved method for enzymatic lysis coupled with thermal treatment for

Table 11.2 The yield of value-added products extracted by enzymatic extraction

Microalgae	Enzyme	Product	Yield (%)	References
C. vulgaris	Cellulase	Lipids	25	Zheng et al. (2011)
	Lysozyme		23	
	Snailase		7	
N. oceanica	Cellulase	Lipids	28.8	Chen et al. (2016)
		Protein	29.9	
Mixed culture of microalgae (Northwest Iran)	β-Glucosidase/cellulase + α-amylase + amyloglucosidase	Reducing sugars	93.64	Shokrkar et al. (2017)
	α-Amylase + amyloglucosidase		61.19	

extraction of lipids from *N. oceanica*, finding the optimal extraction parameters at 37 °C, pH 5.0, 1.3% of cellulase, liquid/solid ratio 15 mL/g, and 5 h. These conditions yielded up to 28.8% of lipids (Chen et al. 2016).

The main steps for enzymatic extraction of lipids from microalgae include biomass harvesting, conditioning and addition of enzymes, stirred incubation for disruption of algal cell walls, addition of solvent (if needed), centrifugation, and lipid fraction recovery (Sierra et al. 2017). Furthermore, enzymatic digestion can be used as means for saccharification of the carbohydrate biomass, for bioethanol production, after the removal of lipids (Heo et al. 2017).

The main microalgae products of interest obtained by enzymatic methods are lipids, carbohydrates, and proteins and are presented in Table 11.2.

3.5 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) employs the solvating properties of a supercritical fluid, by applying pressure and temperature above the critical point of a compound or mixture. The tunable parameters to consider for SFE include solvent, temperature, pressure, extraction time, solvent flow rate, sample size, use of the modifier, and particle size (Sharif et al. 2014).

Supercritical fluid extraction with carbon dioxide (SFE-CO_2) has been employed as an alternative green extraction technique, to spare the use of toxic solvents (Hernández et al. 2014). The advantages of SFE-CO_2 consist of the low critical point of CO_2 at near room temperature at relatively low pressure (30.9 °C and 73.9 bar) and it being generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and environmentally friendly (Reverchon and De Marco 2006; Esquivel-Hernández et al. 2016). Moreover, CO_2 becomes gaseous after depressurization, thus separated from the sample without residual traces of solvent, can be collected for recycling for subsequent extractions, which in itself brings economic and environmental benefits. In Fig. 11.5, a diagram of a semi-batch SFE system is shown (Laitinen 1999).

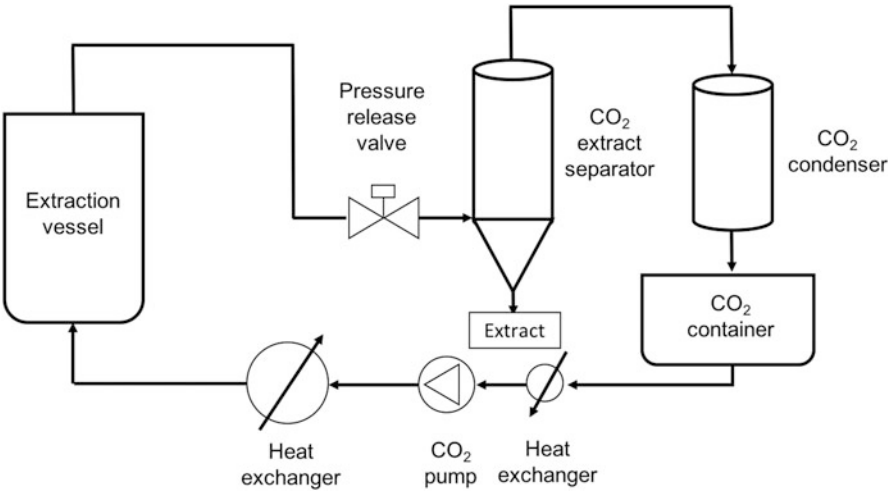

Fig. 11.5 Schematic diagram of a semi-batch supercritical fluid extraction apparatus. Reproduced from Laitinen 1999.

Particularly useful for biodiesel extraction, supercritical CO_2 is highly selective for nonpolar lipids, such as triglycerides, and does not solubilize phospholipids (Hernández et al. 2014). Other solvents used in SFE include hydrocarbons (hexane, pentane, butane), nitrous oxide, sulfur hexafluoride, and fluorinated hydrocarbons (Reverchon and De Marco 2006).

3.6 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) relies on the contact of a dielectric polar substance (water, for instance) and a fast oscillating electric field, produced by microwaves, that generates heat due to the friction caused by inter- and intramolecular movements. The heat induces the formation of water vapor in the cell, which eventually causes rupture and further leakage and release of intracellular components, led by an electroporation effect (Ghasemi Naghdi et al. 2016). Thus, MAE is regarded as a rapid, simple, safe, effective, and economical method for the extraction of lipids that does not require the previous dewatering of samples (Ranjith Kumar et al. 2015). Furthermore, microalgae pretreated by microwaves present multiple micro-fissures within the cell wall, which yields higher bio-oil recoveries (Šoštarič et al. 2012).

Besides extraction, microwaves can be employed for transesterification of oils into biodiesel and represent an attractive option since it requires short reaction time (15–20 min), low operational cost, and efficient extraction of algal oils. One

important drawback of this method is the high maintenance cost in commercial scale settings (Ranjith Kumar et al. 2015).

For MAE the main parameters to be taken into account include extraction time, temperature, dielectric properties of the process mixture, solid/liquid ratio, and type and concentration of solvent (Ghasemi Naghdi et al. 2016).

3.7 Ultrasound-Assisted Extraction

Ultrasonic-assisted extractions (UAE) can recover oils from microalgae cells through cavitation (Harun et al. 2010). During the low-pressure cycle, high-intensity small vacuum bubbles are created in the liquid. When the bubbles attain a certain size, they collapse violently during a high-pressure cycle. During the implosion very high pressures and high-speed liquid jets are produced locally, and the resulting shear forces break the cell structure mechanically, favoring the extraction of algal lipids (Wei et al. 2008). Ultrasonic waves produce high-pressure cycles that allow the diffusion of solvents, like hexane, into the algal cell wall. The transfer of lipids from the cell into the solvent is aided by ultrasound through the mechanical breakage of the cell wall due to the cavitation shear force (Cravotto et al. 2008).

Lipid recovery can be enhanced by increasing the exposure time and by using mixtures of polar and nonpolar solvents. Also, UAE favors the release of cell contents into the solvent, through mass transfer and penetration of solvent within the cell. UAE can be performed at low temperatures, an ideal feature when dealing with the extraction of thermally sensitive molecules (Ghasemi Naghdi et al. 2016).

3.8 Other Methods

Microalgae are composed of single cells surrounded by an individual cell wall, which includes “unusual” lipid classes and fatty acids that differ from those in higher animals and plants (Guschina and Harwood 2006). In some cases, for extraction of lipids from microalgae, regular extraction methods may not be applicable (Ryckebosch et al. 2012). Extracting and purifying oil from algae is considered challenging due to its energetic and economically intensive nature (Fajardo et al. 2007; Mercer and Armenta 2011).

3.8.1 Pressurized Liquid Extraction

The wet lipid extraction process uses wet algae biomass by using solvent proportionately (Sathish and Sims 2012). This method resembles the solvent extraction process but varies with the nature of biomass (wet). The advantage of the process includes the elimination of a drying step; the interference of moisture content with

the extraction solvents and lack of wide applicability to all kinds of solvents are the major limitations of this extraction procedure.

Hydrothermal liquefaction is a process in which biomass is converted in hot compressed water to a liquid biocrude (Brown et al. 2010; Biller et al. 2012). Processing temperatures range from 200 to 350 °C with pressures of around 15–20 MPa, depending on the temperature, because the water has to remain in the subcritical region to avoid the latent heat of vaporization (Biller et al. 2012). At these conditions, complex molecules are broken down and repolymerized to oily compounds (Peterson et al. 2008). This procedure is ideal for the conversion of high-moisture-content biomass such as microalgae because the drying step of the feedstock is not necessary.

3.8.2 Osmotic Pressure

Osmotic shock or osmotic stress is a sudden change in the solute concentration around a cell, causing a rapid change in the movement of water across its cell membrane (Fajardo et al. 2007). This shock causes a release in the cellular contents of microalgae. The method is more applicable for the strains cultivated in marine environments (e.g., *Nannochloropsis* sp.). Osmotic shock is also induced to release cellular components for biochemical analysis (Larach 2010). This method is also applied for *Halorubrum* sp. isolated from saltern ponds. The results showed increased lipid productivities and variations in lipid compositions (Lopalco et al. 2004).

3.8.3 Pulsed Electric Field Technologies

Pulsed electric field (PEF) processing is a method for processing cells by means of brief pulses of a strong electric field (Guderjan et al. 2007). Algal biomass is placed between two electrodes and the pulsed electric field is applied. The electric field enlarges the pores of the cell membranes and expels its contents (Guderjan et al. 2005).

4 Concluding Remarks

Extraction of lipids is a key aspect involved in the biomass-to-biodiesel production; the selected method directly influences the potential lipid productivity of the process. So far, several methods have been employed for extracting the cellular contents (lipids) of microalgae. Each method has its own advantages and disadvantages for practical applicability. Among the processes described, solvent extraction is suitable for extracting lipids from mass cultures, but requires large volumes of solvent. The Soxhlet extraction method is applicable only when a single solvent is used and is not suitable for binary solvent applications. On the other hand, recovery and reusability of the solvent are possible with this method. The ultrasonic-assisted extraction can

perform well when coupled with the enzymatic treatment, but both methods lack cost-effectiveness and feasibility for large-scale applications. Supercritical carbon dioxide extraction (SFE-CO_2), pulsed electric fields, osmotic shock, hydrothermal liquefaction, and wet lipid extraction require more optimization efforts for large-scale applications. A suitable method that is operable with both binary and single solvents, applicable at large scales and yielding higher lipid productivities, is yet to be optimized to achieve enhanced microalgae lipid yields for biofuels and metabolite extraction.

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Chapter 12
Different Cell Disruption and Lipid Extraction Methods from Microalgae for Biodiesel Production

Vinod Kumar, Neha Arora, Manisha Nanda, and Vikas Pruthi

Abstract The global energy demand is increasing at an exponential rate, and available petroleum sources are rapidly decreasing. In this context, microalgae regained attention for biodiesel production due to its high growth rate and high lipid content. One of the major obstacles for large-scale production of biodiesel from microalgae is extracting intracellular lipids which are present inside the cell wall and membrane. Therefore, there is a substantial necessity to develop a cost-effective, safe, environment-friendly, and efficient extraction method of microalgae lipids. In downstream processing, algal cell disruption and lipid extraction techniques are important for biodiesel production due to high energy consumption and high costs involved. Several techniques for lipids extraction from microalgae have been reported by various researchers. This chapter provides an overview on latest advancements that have been made on the different cell disruption methods including mechanical, chemical, and biological cell disruption methods and different lipid extraction methods including conventional extraction lipid methods, green solvent-based extraction methods, and solvent-free extraction methods.

Keywords Microalgae · Lipids · Biodiesel · Cell disruption · Extraction

1 Introduction

Escalated energy demands due to industrialization and population growth have led to depletion of fossil fuel reserves. Currently, the consumption of existing petroleum sources is reported to be 10^5 times higher than its rate of formation with estimated

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increase in energy demands by 50% in the coming years (Shuba and Kifle 2018). According to the International Energy Agency (IEA), a 2.1% escalation in global energy demand while 1.4% enhanced CO_2 emission was reported (https://www.iea.org/geco/). Indeed, such a dependability on fossil fuels is associated with serious consequences including uncertainty of continuous supply, environment degradation, resource constraint, and escalating oil prices (Adenle et al. 2013). In order to circumvent the above problems, exploring biofuels is the way forward for a sustainable and safe future. The burning of biofuels results in reduced emission of noxious gases and is biodegradable and less toxic (Popp et al. 2014). Bioethanol, biogas, and biodiesel are the biofuels which are produced by using different processes like transesterification, fermentation, and gasification (Kwon et al. 2012). Among these, biodiesel either directly or with little modifications can be utilized in diesel engines thereby providing a sustainable and eco-friendly transportation fuel.

Biodiesel can be produced from edible feedstock such as seeds of sunflower, rapeseed, palm, and soybean which are rich in oil (Cheng and Timilsina 2011). However, these first-generation biofuels are not suitable for accomplishing the requirement as it leads to "food versus fuel" controversy (Ullah et al. 2015; Singh and Olsen 2011; Nogami et al. 2014; Ross et al. 2010; Bharathiraja et al. 2015; Sambusiti et al. 2015). On the other hand, the second-generation fuel based on the utilization of non-edible crops such as jatropha and castor requires fertile land for cultivation, which competes with arable land and nutrients available for agricultural crops (Vassilev and Vassileva 2016; Mueller et al. 2011; Achten et al. 2010; Adenle et al. 2013). Biodiesel production from microalgae, termed as third generation, has attracted global attention in recent years for their simple structure, sustainable features, CO_2 sequestration efficiency, and immense capability as energy crops (Adenle et al. 2013). Microalgae are considered to be a technically viable alternative energy resource over conventional biofuels. Microalgae are simple photosynthetic eukaryotic microorganisms that can accumulate lipids (20–70% dry cell weight) with less generation time in comparison to terrestrial plants by utilizing light, simple nutrients, and CO_2 (Brennan and Owende 2010). Additionally, microalgae cultivation doesn't compete with arable land as they can flourish extensively in different wastewaters as well as saline waters (Widjaja et al. 2009).

Although microalgae have prodigious capacity to accommodate future energy demands, certain techno-economic constraints associated with its cultivation, harvesting, lipid extraction, and downstream processing restrict its utilization at large scale (Hannon et al. 2010). Among these, lipid extraction from microalgae is the crucial part which particularly involves expenditure of more energy and hinders economic execution of the process (Jeevan et al. 2017). Currently, various numerous mechanical and nonmechanical processes have been reported for cell-wall disruption including bead milling, high-pressure homogenization, hydrodynamic cavitation, ultrasonic/microwave/pulsed electronic field treatment, steam explosion, as well as solvent, ionic liquid, osmotic shock, surfactant, hydrolytic enzyme, and algicidal treatment, respectively (Lee et al. 2017). Indeed, the selection of suitable method for efficient extraction of lipid largely depends on the biology and cell-wall characteristics of microalgae.

The present chapter provides an overview of the algal biomass and cell-wall characteristics to shed light on the selection of the lipid extraction technique. The chapter catalogs different microalgal cell disruption techniques including mechanical, chemical, and biological exploited to date along with lipid extraction methods such as conventional extraction lipid methods, green solvent-based extraction methods, and solvent-free extraction methods.

2 Algal Biomass

The composition of algal biomass constitutes three major chemical components, namely, carbohydrates, lipids, and proteins, as depicted in Fig. 12.1 (Chia et al. 2017). The abundance of any of the above depends largely on the type of strain and cultivation conditions, making it either carbohydrate- or lipid-accumulating (oleaginous) strain. Carbohydrate components provide an option for the formation of bioethanol and biobutanol (Gao et al. 2016). For example, *Chlorococcum littoral*, a carbohydrate-accumulating species, is reported to accumulate maximum amount of ~ 70% dry cell weight (Hu et al. 1998). The phenomenon of carbohydrate storage is mainly influenced by nutrient availability, stress conditions, light intensity, and CO_2 concentration (Chen et al. 2013). Other well-documented species having higher capacity to accumulate carbohydrate include *Chlamydomonas reinhardtii*, *Chlorella* sp., and *Scenedesmus obliquus* (Choi et al. 2010; Chia et al. 2017). Especially, *Chlorella* sp. are reported to store carbohydrate ranging from 18% to 51% of dry cell weight, under different nutrient stresses such as deprivation of nitrogen, sulfur, and phosphorus (Chia et al. 2017). *Chlorella vulgaris* FSP-E and *Chlorella vulgaris* P12

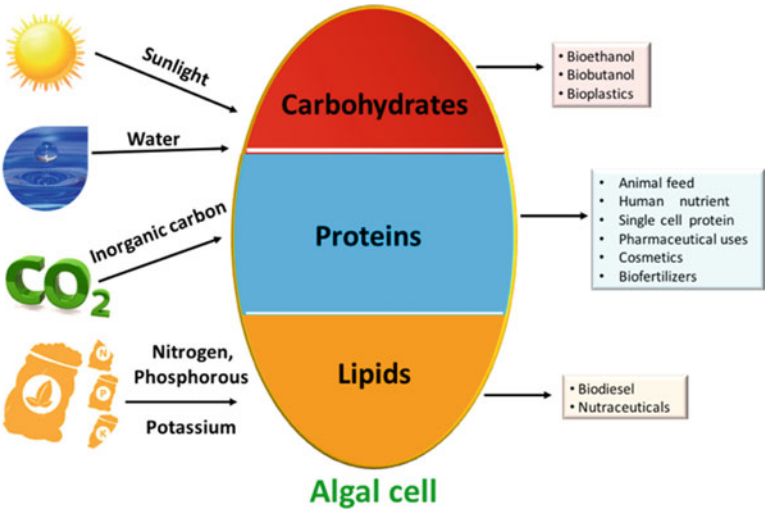

Fig. 12.1 Biochemical composition of algal cell

showed a significant increase in carbohydrate content upon nitrogen starvation from 5% to 41% and 51%, respectively, being 8–10 higher than control (Park et al. 2009; Ho et al. 2013).

The protein portion of microalgae is of great nutritional value as it can be used as animal feedstock, fertilizers, industrial enzymes, bioplastics, and surfactants. Historically, large-scale cultivation of algae as a source of protein ponds was first conceived by German scientists during World War II (Demirbas 2010). The biochemical composition of algae under normal condition has maximum proportion of proteins followed by carbohydrates, lipids, and nucleic acids (Toscano et al. 2013). Various microalgal species such as *Scenedesmus obliquus*, *Chlorella pyrenoidosa*, *Dunaliella bioculata*, and *Spirulina maxima* have been reported to constitute around 50–56%, 57%, 49%, and 60–71% protein of their dry cell weight, respectively (Demirbas 2009). *Chlorella* sp, and *Spirulina* sp,. Have good market due to their nutritional value (Pulz and Gross 2004). Commercially algal biomass products are available in the form of liquid, powder, capsules, and tablets in markets (Becker 2007). The algal proteins are composed of different amino acids such as isoleucine, leucine, valine, phenylalanine, tyrosine, methionine, threonine, alanine, arginine, etc. which are of high nutritional value and comparable to the protein-rich foods such as egg and soybean (Becker 2007). Algal proteins by *Chlorella vulgaris*, *Dunaliella bardawil*, *Spirulina platensis*, and *Arthrospira maxima* also meet the standards set by WHO and FAO (WHO/FAO 1973; Becker 2007).

On the other hand, the lipid content of microalgal cell is one of the most important components which has enormous potential in the production of biodiesel. Enhanced lipid production in microalgae is due to their adaptable metabolic response against any kind of change in their cultivation conditions (Chisti 2007). The triacylglycerol accumulation in algal cell is suitable for biodiesel formation after undergoing a simple transesterification process. Transesterification is a chemical process catalyzed by acid or alkali in the presence of alcohol especially methanol, under high temperature, which leads to the formation of methyl esters of fatty acids, which constitutes biodiesel and glycerol (Chisti 2007). The lipid content in various microalgae varies from 40% to 70% in oleaginous strains by implementing appropriate strategies like varying nutrient sources, using industrial wastewater, heavy metal stress, and hypersaline media (Takagi et al. 2006; Arora et al. 2017a, b; Arora et al. 2016). Few microalgal species, namely, *Botryococcus*, *Chlorella*, *Nannochloropsis*, *Scenedesmus*, *Dunaliella*, and *Phaeodactylum*, are well documented for their capability to be exploited for biodiesel production (Chia et al. 2017). For example, *Dunaliella teraticola* can accumulate up to 70–71% lipid content in media supplemented with 1.0 M NaCl, whereas *Chlorella vulgaris* was reported to have enhanced lipid content of up to 56% of the dry cell weight upon addition of iron to the media (Takagi et al. 2006; Liu et al. 2008). Variation in nitrogen and phosphorus content are the prime factors that can boost TAG accumulation in microalgae (Arora et al. 2016). Nitrogen deprivation in *Nannochloropsis* sp. and *Chlorella vulgaris* ESP-31 also triggered lipid content by 59% and 56%, respectively. However, *Scenedesmus obliquus* CNW-N, when grown under nitrogen stress for 5 days, accumulated an overall lipid content of 22.4% that contain a high proportion of

C16/C18 fatty acid, appropriate for biodiesel production. Further, marine microalgae, including *Crypthecodinium*, *Schizochytrium*, *Nannochloropsis*, *Nitzschia*, and *Phaeodactylum*, have been widely utilized for the production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Harwood and Guschina 2009; Chia et al. 2017).

3 Cell-Wall Characteristics

Algal cell walls are diverse in nature in terms of molecular component, linkages, and overall structure. The algal cell wall comprises of two important components, (i) an organized microfibrillar structure which serves as framework of the cell wall and (ii) the gel-like protein matrix within which the fibrillar component is fixed firmly, thus providing the structural integrity (Northcote et al. 1958; Yap et al. 2016). In addition to the cell wall, some microalgae have an external inorganic covering composed of silica frustules and calcium carbonate (Bolton et al. 2016) making it more resistant toward cell disruption. Interestingly, the cell wall of microalgae alters significantly under different environmental conditions such as nutrient depletion, light fluctuations, and salt and heavy metal stress, hampering the recovery of intracellular lipids (Praveenkumar et al. 2015; Yap et al. 2014). To date, various potential microalgal species have been reported to accumulate high lipid content intracellularly, but only a few commercially important species (*Chlamydomonas* sp., *Chlorella* sp., *Haematococcus* sp., and *Nannochloropsis* sp.) are the most extensively explored microalgae because of their prominent relevance in the field of biotechnology and bioenergy (Lee et al. 2017). The basic composition of algae cells comprises of cellulosic a polymer of β 1,4 linked D-glucose units in nature. However, the chlorophycean green algae have cell walls varying from cellulose pectin complexes to hydroxyproline-rich glycoproteins, respectively. Polysaccharides of algal cell wall comprise of different polymers such as hemicellulose (Domozych et al. 1980), chitin, pectins (Domozych et al. 2007), fucans (Berteau and Mulloy 2003), alginates (Michel et al. 2010), and carrageens (Michel et al. 2003) which make them distinct from each other.

The cell wall of the unicellular microalgae *Chlamydomonas reinhardtii* encompasses a network of fibrils and glycoproteins, mainly comprising of hydroxyproline (Hyp)-rich glycoproteins (HRGPs) arranged in five distinct layers, with extended oligosaccharides side chain on them (Arnold et al. 2015). Structural analysis of cell walls of *C. reinhardtii* and *C. gymnogama* elucidated arabinose, glucose, and galactose as the main sugar components bound to HRGPs (Bollig et al. 2007; Miller et al. 1974). These conserved Hyp-rich sequences in *Chlamydomonas* add to the strength of the cell wall, as these sequences allow the protein molecules to acquire the polyproline-dominant conformations resulting in more stable form when glycosylated (Homer et al. 1979; Ferris et al. 2001; Ferris et al. 2005; Van Holst and Fincher 1984). On the other hand, the cell wall of *Chlorella* consists of trilaminar layer having an outer covering of sporopollenin which is the main component leading

to its toughness (REF). Beneath the outer layer, heterogeneous secondary wall is rich in mannose and glucosamine (Burczyk and Hesse 1981; Kim et al. 2016). Interspecies variation has been reported in *Chlorella* depending upon the different growth conditions (Gerken et al. 2013; Lee et al. 2017). For example, an enhanced proportion of uronic acid and amino sugars associated with reduction in neutral sugars was reported in CO_2 enriched conditions (2% CO_2) (Cheng et al. 2015).

Additionally, the polysaccharides in cell wall of marine alga, such as *Nannochloropsis* sp., generally exist in sulfated form. A bilayer structure composed of an outer layer made up of hydrophobic algaenan, covering the inner cellulosic layer, contributes to the recalcitrant nature of the cell wall of *Nannochloropsis*. Characterization of the polysaccharides present in cell wall of *Nannochloropsis oculata* suggests that major portion of the polysaccharide is contributed by glucose (68%) followed by rhamnose, mannose, ribose, xylose, fructose, and galactose in small quantities (Brown 1991; Scholz et al. 2014). The cell wall of *Haematococcus pluvialis* encounters a series of chemical modifications and morphological variations during its development to a complete mature non-motile red colored cyst with highly resistant cell wall (Brennan and Owende 2010; Nogami et al. 2014; Praveenkumar et al. 2015; Kim et al. 2016). The three-layered cell wall consists of a trilaminar primary sheath (extracellular matrix made of algaenan with acetolysis-resistant material) with a thick amorphous layer made of mannose and cellulose and its heterogeneous arrangement (Damiani et al. 2006; Kim et al. 2016).

4 Cell Disruption Methods

Disrupting the cellular wall of algae allows for easier recovery of the intracellular lipids resulting in rapid and increased efficiencies in lipid extraction. The summary and comparison of cell disruption methods reported for lipid extraction from microalgae have been listed in Table 12.1 (Fig. 12.2). As noted in the previous section, due to the differences in cell-wall structure of various microalgae, the effectiveness of a particular cell disruption method varies from one microalgae to another. Lee et al. (2010) reported that for microalgae of *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp., the microwave treatment was the best cell disruption method. On the other hand, a study conducted by Halim et al. (2012) reported that grinding with liquid nitrogen increase the lipid extraction level in *Chlorella vulgaris*. Further, bead-beating method was also shown to extract higher lipid content from *Botryococcus braunii* than sonication, homogenization, French press, and lyophilization, respectively (Lee et al. 1998). Lee et al. (2010) reported bead beating as the most effective cell disruption method for microalgae. Chisti and Moo-Young (1986) reviewed various mechanical cell disruption methods and have reported that the choice and success of a particular cell disruption method depend on the

Table 12.1 Comparison of different cell disruption methods

Cell disruption method	Most efficient method	Microalgae	% Lipid extracted	References
Autoclaving	Microwaves	*Botryococcus* sp.	28.6	Lee et al. (2010)
		Chlorella vulgaris	11	
Bead beating		*Scenedesmus* sp.	11.5	
Microwaves				
Sonication				
Osmotic shock				
Sonication	Sonication	*Chlorella* sp.	20.1	Prabakaran and Ravindran (2011)
		Nostoc sp.	18.2	
Osmotic shock		*Tolypothrix* sp.	14	
Microwave				
Autoclave				
Bead beating				
Grinding	Grinding	*Chlorella vulgaris*	29	Zheng et al. (2011)
Sonication				
Bead milling				
Enzymatic lysis				
Microwaves				
Grinding	Osmotic shock	*Schizochytrium* sp. S31	48.7	Byreddy et al. (2015)
Bead vortexing				
Osmotic shock		*Thraustochytrium* sp. AMCQS5–5	29.1	
Water bath				
Sonication				
Shake mill				
Sonication	Sonication	*Schizochytrium*	34.5	Yel et al. (2017)
French press homogenization		sp. S31		
Microwave	Microwave	*Scenedesmus obliquus*	76–77	Subramanian et al. (2011)
Water bath				
Grinding	Ultrasonication	*Scenedesmus* sp.	90.8 (extraction efficiency rate)	Kim et al. (2017)
Ultrasonication				
Microwave				

microorganism on which one is working. Therefore, we cannot generalize the results obtained from one species to others (Zheng et al. 2011).

A number of laboratory-scale microalgae cell disruption methods are available. But only a few of the available methods scaled up for industrial purposes. For example, bead mill, high-pressure homogenizer, and Hughes press are used extensively at large scale (Shen et al. 2009; Schutte and Kula 1990). In a recent study, it has been reported that the osmotic shock is the best method cell disruption method that can be industrially scaled up (Sauer and Galinski 1998). Osmotic shock was

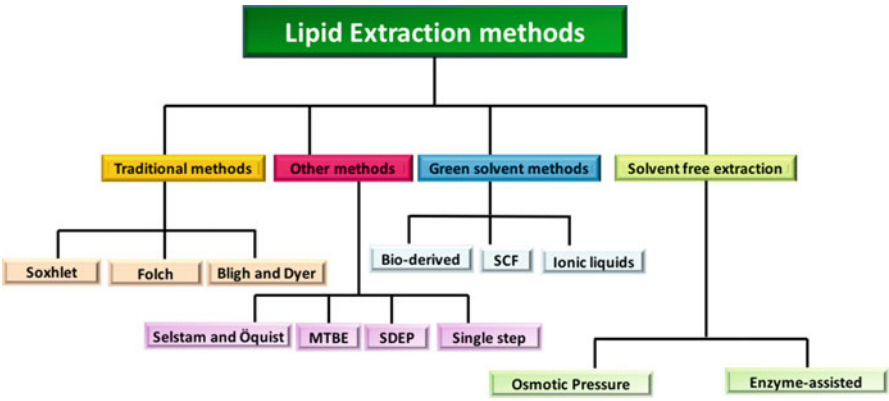

Fig. 12.2 An overview of different lipid extraction methods

successfully used in thraustochytrid cell disruption for lipid extraction (Chisti and Moo-Young 1986). The exact method has also been implemented at pilot scale for enhancing the release of lipid (Arnal et al. 2005). Some of the most efficient and commonly used methods are discussed in the sections below.

4.1 Grinding

Cell disruption using grinding involves mixing the freeze-dried biomass with liquid nitrogen. Further the sample is allowed to thaw. Finally with the help of a ceramic mortar and pestle, it is grinded for 2 min leading to the breakdown of the algal cell wall and the simultaneous release of intracellular lipids (Lee et al. 2010; Gouveia et al. 2007; Greenwell et al. 2010).

4.2 Bead Vortexing

This method leads microalgal cell wall breakage by grinding and agitating the cells on a solid surface of glass beads (Mercer and Armenta 2011). Here, the beads are excited in a bead mill generating a high shear force that destroys the microalgal cell walls (Munir et al. 2013). These beads are formed of zirconia-silica, zirconium oxide, or titanium carbide with an optimal diameter size of 0.3–0.5 mm to efficiently break the cell wall (Doucha and Livansky 2008; Hopkins 1991).

4.3 Osmotic Shock

The plasma membrane of algae allows a number of solute to pass through it when placed in solution. Thus, if algal cells are subjected to hypotonic solution, solutes from the algal cell will diffuse across the cell membrane into the water which can lead to cell destruction (Browne et al. 2009). This process is called osmotic shock treatment. The choice of solute is very important if osmotic shock is being used on a large scale, i.e., solute should be economical, easily available, and at the same time highly efficient (Subramani et al. 2015).

Algal cells in solution can be subjected to pressurized CO_2, leading the gas to dissolve into the solution and form the carbonate ion, CO_3^{2-}, and diatomic hydrogen, H_2 (Browne et al. 2009). These solutes will then make the solution hypertonic. Further the solutes will now have to be diluted (hypotonic solution) to create an osmotic shock for rupturing the algal cells. For this purpose, ultrasonic pulsation and throttling through a needle valve can be utilized. During ultrasonic pulsation, the carbonated solution is taken in a beaker and then placed into a water bath. The beaker is then subjected to ultrasonic waves leading to a rapid release of carbonate ions (Browne et al. 2009). The throttling technique involves atomizing algal cells by increasing velocity. This is done by slightly opening the throttling needle valve (Subramani et al. 2015).

4.4 Water Bath

Thermolysis of algal cells using a water bath is induced by heating the vessels containing algal cells to a temperature of 90 °C for 20–30 min which breaks the cell wall of the microalga (McMillan et al. 2013).

4.5 Ultrasonic-Assisted Extraction

Ultrasonic treatment involves using the energy of high-frequency acoustic waves for cell disruption. The introduction of these waves in the cell suspension incorporates high shear forces that break the cell (Chisti and Moo-Young 1986; Mendes Pinto et al. 2001). Ultrasonication is one of the most commonly used methods for the cell disruption of microorganisms. Ultrasound-assisted extraction was given by Adam et al. 2012. This method is solvent-free extraction method, and in ultrasound-assisted extraction, wet (95%) algal biomass was treated at low frequencies (20 kHz) with a 1000 W ultrasonic processor. An antioxidant, butylated hydroxy toluene (BHT), is added in the reactor at the time of lipid extraction. After this process, lipid was extracted in aqueous phase by saline solution (100 ml NaCl 0.9% m/w). Lipid

extracted by this method was only 0.21%. In comparison with other methods, ultrasonic method is the best method for microalgae cell disruption (Prabakaran and Ravindran 2011). Ultrasonic is more effective at low frequency (18–40 kHz) than at high frequency (400–800 kHz) (Cravotto et al. 2008). Equation for energy consumption in ultrasonic per unit of biomass is

$$\text{Energy consumption per unit mass of Biomass} = \frac{\text{Ultrasonic power} \times \text{Time}}{\text{Volume} \times \text{Concentration of Biomass}}$$

4.6 Expeller Press

It is an effective mechanical crushing method used for extracting oil from seeds which can also be employed to algal biomass (Demirbas 2009). It works on the principle of applying high mechanical pressure for crushing and breaking the cells that also squeezes out oil from the algal biomass. The pressure applied should be in a particular range because too much of pressure can lead to increased heat generation and choking problems (Ramesh 2013). On an average 70–75% oil can be recovered using this process. However, the method is expensive and time taking (Boldor et al. 2010).

4.7 Hydrothermal Liquefaction (HTL)

Algal-based biofuel is produced mainly by two methods, biodiesel via the lipid extraction and transesterification and bio-oil via pyrolysis/hydrothermal liquefaction. Hydrothermal liquefaction HTL is an oxygen-free thermochemical process which directly converts the wet biomass into biofuels, carried out at low temperatures (200–400 °C) and high pressure (6–15 MPa), respectively (Behrendt et al. 2008) (Fig. 12.3, Table 12.2). This process is very similar to pyrolysis and gasification, but is carried out at low temperatures (Tian et al. 2014). Various biomass feedstocks can be directly converted into biofuels without wasting energy in drying step, in turn enhancing the flexibility and improving the efficiency of the process (Demirbas 2011). In this process, cellulose, hemicellulose, and lignin are converted into four phases, namely, biocrude oil, aqueous phase, solid residue, and gaseous products (Kruse and Dinjus 2007). In hydrothermal liquefaction, wet microalgae water acts as a solvent and reaction medium (Goyal et al. 2008). The utilization of different types of homogeneous catalysts such as salts, acids, alkalis, and metal in the HTL of microalgae biomass has been reported to increase the yield of biocrude oil.

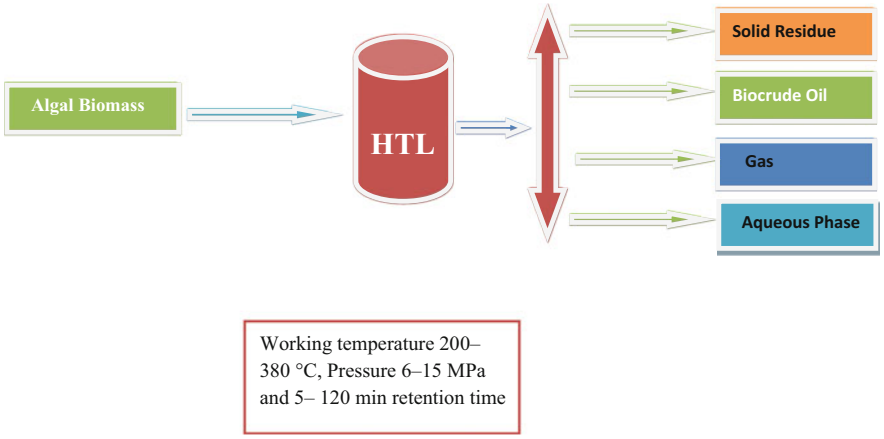

Fig. 12.3 A block diagram hydrothermal liquefaction (HTL) process

Table 12.2 HTL operational conditions and biocrude oil yield for algae biomass

Algae species	Temperature	Catalyst	Biocrude oil yield (%)	References
Spirulina	350 °C	$Fe(CO)_5$-S	78	Matsui et al. (1997)
Mixed microalgae	350 °C	Na_2CO_3	48	Ferrell and Sarisky-Reed (2010)
Chlorella pyrenoidosa	270 °C	–	39	Yu et al. (2011)
Chlorella vulgaris	350 °C	Na_2CO_3	20	Ross et al. (2010)
Chlorella sorokiniana	300 °C	–	28	Brown et al. (2010)
Chlorella sp.	220 °C	–	87	Chen et al. (2014)
Spirulina algae	300 °C	–	32.6	Vardon et al. (2011)
Chlorella pyrenoidosa		Al_2O_3		Xu et al. (2014)
Spirulina sp.	300 °C	Na_2CO_3	15.5	Ross et al. (2010)
Chlorella sp.	350 °C	KOH	22.4	Ross et al. (2010)
Nannochloropsis sp.	350 °C		43	Brown et al. (2010)
Dunaliella tertiolecta	360 °C	K_2CO_3	32	Qin et al. (2010)
Chlorella sp.	350 °C	CH_3COOH	20.4	Ross et al. (2010)
Spirulina sp.	350 °C	CH_3COOH	16.6	Ross et al. (2010)
Desmodesmus sp.	375 °C	–	49	Garcia et al. (2012)

The utilization of HTL to convert biomass in biofuels is not new and has been exploited since the 1970s (Appell et al. 1972). However, the conversion of algal biomass into biocrude oil is an emerging concept. The development of HTL of algal biomass has been divided into three periods, namely, pioneering time (1994–1999), silent time (2000–2008), and renaissance time (2009–present), respectively (Tian et al. 2014). Dote et al. (1994) first time reported the conversion of microalgae feedstock using HTL process (Dote et al. 1994). The key operational parameters include wet algal biomass, 5–50% total solid content, working temperature of 200–380 °C, and 5–120 min retention time, respectively, which is carried out in the presence of catalyst yielding 50–80% of biocrude oil (Matsui et al. 1997; Ikenaga et al. 2001; Duan and Savage 2011; Biller et al. 2011). The order of algal biomass conversion reported in the order lipid>proteins >carbohydrate (Biller et al. 2011). Besides the use of single microalgae in HTL, a mixture of microalgae can also be used in this process. The formula used for the calculation of the biocrude oil yield is given as follows:

$$\text{Biocrude oil } (\text{wt}\%) = \text{bio-oil/algal biomass} \times 100\%$$

The advantages of using HTL for producing biocrude oil from microalgae include:

Elimination of the drying process.
Low-temperature conditions required as compared to pyrolysis.
Utilization of the whole algal biomass.
Lipids, proteins, and carbohydrates can be converted to biocrude oil.
High lipid content in algae is not desirable.
Water is the reaction medium.
The process can be modified by changing in pressure or temperature.
The process may be catalytic or non-catalytic and high yield of biocrude oil (Tian et al. 2014).

4.8 Electroporation

In this method, algal cells in solution are treated with an external electric field supply, due to which formation of electropores takes place in algal cell wall and cell membrane (Barbosa Cánovas et al. 1999; Ho and Mittal 1996). The applied electric field is responsible for the size and number of the pores in algal cell (Rols et al. 1990; Tsong 1990).

4.9 Microwave Treatment

Algal cells can also be disrupted by exposing them to microwaves. The selective interaction of microwaves with polar molecules (e.g., water) causes local heating resulting in frictional forces from inter- and intramolecular movements (Amarni and Kadi 2010). This optimum treatment is done at 2450 MHz (Vasavada 1986), and the efficiency of the method depends on the free water concentration in cells. Water also reaches its boiling point faster when subjected to microwaves. This leads to cell wall expansion and an increase in the internal pressure (Chuanbin et al. 1998). Microwaves have also been used to extract vegetable oils and animal fats (Mahesar et al. 2008).

4.10 Algicidal Treatment

Algicidal bacteria are heterotrophic bacteria in the sea, which have been reported to attack and destroy target algae that release extracellular compounds like lipids. Chen et al. (2013) co-cultivated *Chlorella vulgaris* with the indigenous bacterium *Flammeovirga yaeyamensis* for energy-efficient microalgal oil extraction. They reported the presence of hydrolytic enzymes such as amylase, cellulase, and xylanase due to which high yield of lipid is obtained. But there are certain groups of algicidal bacteria that inhibit microalgal growth or break down the microalgal cells (Meyer et al. 2017). Lenneman et al. (2014) reported the 12-fold high lipid yield from microalgae with two algicidal bacteria *Pseudomonas pseudoalcaligenes* AD6 and *Aeromonas hydrophila* AD9.

5 Total Lipid Extraction Methods

Biodiesel from microalgae involves four steps, namely, cultivation, harvesting of microalgae, lipid extraction, and transesterification reaction (Amin 2009). Among these, lipid extraction process has been regarded as one of the most important steps which contributes (30–40%) to the cost of biodiesel production (Chisti 2007). This necessitates that the lipid extraction method should be sustainable and economical. To date, several methods that have been used by various researchers are briefly described in the sections below and in Fig. 12.2.

5.1 Soxhlet Method

Soxhlet extraction (Soxhlet 1879) method is mainly used for extraction of lipid from macroalgae. In brief, lipids are extracted from 100 g of dry microalgae for 8 h using 700 mL of n-hexane followed by 30 min solvent rinse and 30 min solvent evaporation (Kumar et al. 2018a). Cheung et al. (1998) reported that thermo-degradation of long-chain polyunsaturated fatty acids during Soxhlet extraction yields low content of unsaturated fatty acids. Advantages of Soxhlet extraction include:

Its simplicity.
Its less labor intensiveness.
Isolated lipid can be used directly for conversion.

However, the disadvantages of Soxhlet extraction include:

Time-consuming and laborious procedure.
Nonselective extraction.
Costly and pure solvent required.
High energy requirement.
Hazardous chemicals are used in the extraction process and thermo-degradation of long-chain polyunsaturated fatty acids (Cheung et al. 1998).

5.2 Folch Method

Folch et al. (1957) gave a simple method of lipid extraction from a tissue. According to this method use, the solvent chloroform/methanol in 2:1 V/V ratio yielded maximum lipid. Briefly first the lipids are extracted by chloroform/methanol from homogenized cells and filtering the homogenate followed by addition of distilled water at least fivefold to the solution containing lipids and then allowing the solution to separate into two layers and lipids to settle in the upper phase. This method is known as one of the oldest methods of lipid extraction and is a rapid and easy processing and is still used by researchers with some modification in the extraction of algal lipids (Kumar et al. 2015). The disadvantages of this technique include:

Less sensitivity.
Utilization of hazardous chemicals.
Costly procedure (Kumar et al. 2015).
Axelsson and Gentili (2014) reported that Folch method yields high lipid from *S. minutum* as compared to Bligh and Dyer (1959) and Selstam and Öquist (1985) method.

5.3 Bligh and Dyer Method

The Bligh and Dyer (1959) method is one of the oldest and widely used lipid extraction methods. It is very similar to the Folch method (1957) but mainly differs in the volume of solvent/solvent, solvent relation to the amount of tissue, and presence or absence of NaCl (Axelsson and Gentili 2014). Bligh and Dyer (1959) use 1:2 (v/v) chloroform/methanol solvent which is four times higher than the sample volume. Briefly chloroform/methanol is added to the homogenized cells of microalgae and blended for 30 s with addition of water and again blended for 30 s. Homogenate is then centrifuged to remove the solids. The chloroform layer contained the purified lipid. In order to improve Bligh and Dyer (1959) method, various researchers have done different modifications. The most common modification is the use of salt (NaCl, $MgCl_2$) instead of water (Kumar et al. 2018b, Kumar et al. 2017b). Some researchers modified the solvent/solvent ratio from 1:2 (v/v) to 2:1 (v/v) chloroform/methanol. Other solvent systems have been developed to replace the chloroform because of its toxic nature, but they are less sensitive (Halim et al. 2012; Sheng et al. 2011).

5.4 Other Methods

5.4.1 Selstam and Öquist Method

In this method microalgal biomass is heated at 80 °C for 10 min with isopropanol. Further 0.73% NaCl added to it in the ratio of 4:1 (v/v) and then homogenized. Lipid is then extracted from homogenate using chloroform:isopropanol:0.73% NaCl in 1:1:0.8 ratio (v/v/v). This method resulted in 17% lipid recovery from *S. minutum* (Axelsson and Gentili 2014).

5.4.2 Methyl-Tert-Butyl Ether (MTBE)

This method is modification of Folch, Bligh, and Dyer method which provides good recovery of almost all major types of lipids (Matyash et al. 2008). In brief, 1.5 ml methanol is added to the 200 ml sample, and the mixture is vortexed followed by the addition of 5 ml of MTBE. After 1 h incubation, 1.25 ml of water is added to the mixture which results in phase separation. After centrifugation to the upper phase, 2 ml of MTBE/methanol/water (10/3/2.5, v/v/v) is added for the extraction of total lipids. Matyash et al. (2008) reported the 67.3 ± 4.7% recovery of total lipid by Folch and 81.3 ± 8.1% by MTBE.

5.4.3 Single-Step Procedure

In a single-step method, microalgal paste is treated with 2:1 ratio of chloroform/methanol (v/v) solvent and then shaken vigorously (Axelsson and Gentili 2014). To the resultant mixture, 0.73% NaCl water solution is added to produce a 2:1:0.8 system of chloroform/methanol/water (v/v/v). Lipids are then easily separated in chloroform layer. Axelsson and Gentili (2014) reported that this method produce five times higher lipid as compared to Folch et al. (1957).

5.4.4 Simultaneous Distillation and Extraction Process (SDEP)

The SDEP method was applied for wet algal biomass (Tanzi et al. 2013). In this method, microalgal biomass paste is treated with terpene solvent (p-cymene, d-limonene, or a-pinene) in a round-bottomed flask. The mixture is then heated for 30 min, and after that water is added. Terpene solvent is then recovered from the water layer and lipid separated. Tanzi et al. (2013) reported that SDEP procedure with terpenes gives higher yield of lipids than the Soxhlet extraction and slightly lower than Bligh and Dyer (1959) method.

6 Green Solvent-Based Extraction Methods

Green solvents are environment-friendly solvents or biosolvents, derived either from naturally (water and CO_2) or processing of agricultural residues which have good solubilizing properties like conventional solvents (Kumar et al. 2017a).

6.1 Bio-derived Solvents for Solvent Extraction

Solvents are usually volatile organic compounds, mainly obtained from nonrenewable resources and hazardous in nature. Bio-derived and biodegradable solvents have become important for academic and industrial research due to the increasing health environmental concerns. Bio-derived solvents, also known as green solvent, satisfy the principles of green chemistry as they are less toxic, biodegradable, and renewable. In microalgae biofuel technology, solvent extraction is a key challenge. Sicaire et al. (2015) used 2-methyltetrahydrofuran (2-MeTHF) for the extraction of vegetable lipid for biofuel production. Mahmood et al. (2017) employed ethyl acetate (EtOAc), ethyl lactate (EtLac), and cyclopentyl methyl ether (CPME) for the extraction of lipid from microalgae *Chlorella vulgaris* and *Nannochloropsis* sp. and reported that 2-MeTHF and ethyl lactate provided two- to

threefold higher lipid yield as compared to conventional solvent.

6.2 Supercritical Fluids (SCF) Technology

Supercritical fluid extraction is an environment-friendly technology using carbon dioxide over other conventional methods. In SCE technology, supercritical fluid is used as solvent (Sahena et al. 2009). A substance is considered to be a supercritical fluid when it is above its critical temperature and pressure (Sahena et al. 2009). CO_2 is considered as supercritical fluid above 31.1 °C and 7.38 MPa, which makes it a best solvent for extracting thermally sensitive intracellular materials (Sahena et al. 2009). The characteristics feature of SCF includes its density, viscosity, diffusivity, heat capacity, and thermal conductivity (Dunford et al. 2003; Sahena et al. 2009). High density of supercritical fluids is responsible for high solubility of compounds, while low viscosities enable the penetration of solid surface. Supercritical CO_2 is a good solvent for the extraction of lipid as it eliminates the use of high temperatures and organic solvents for extraction. Santana et al. (2012) reported that by using supercritical carbon dioxide and Bligh and Dyer method, the same amount of lipid was obtained. The advantages of SCF technology include:

1. High penetration into porous solid materials as compared to other solvents resulting in extraction of higher lipid with reduced extraction times (ranges from hours to few minutes).
2. The solubility power of supercritical fluids can be manipulated by changing pressure and temperature.
3. CO_2 can be easily removed from lipid after extraction which can be easily recycled and reused.
4. No thermo-degradation of long-chain polyunsaturated fatty acids (Sahena et al. 2009).

However, this method also suffers from certain disadvantages, viz.:

1. The low solubility of polar lipids in SC–CO_2.
2. Very costly and complex equipment operating.
3. CO_2 is highly selective.
4. Clean supply of CO_2 is required and high power consumption (Hubbard et al. 2004).
5. To circumvent the extraction of polar lipids, researchers have used water with SC–CO_2 to increase the solubility of polar lipids (Dionisi et al. 1999).

6.3 Ionic Liquids

Ionic liquids are considered as green, nonaqueous salt solution, which contain both anions and cations. Ionic liquids are environmentally friendly alternative of

conventional volatile solvents, as they do not have the detectable vapor pressure which is causing no pollution (Jessop et al. 2003). Ionic liquids have been also used in catalysis, electrochemistry, spectroscopy, material science, etc. They are nonflammable and remain liquid at 0–140 °C. Ionic liquids can enhance the lipids yields in algal biomass due to their good solubility for lignocellulosic materials (Li et al. 2008). Choi et al. (2014) reported that ionic liquids enhancing the lipid yield from *Chlorella vulgaris*. The advantages of ionic liquids include:

Less harmful
Nonflammable
Remains in liquid state
Possesses both ions
Enables single-solvent extraction (Jeevan et al. 2017).

6.4 Emerging Green Solvents

6.4.1 Liquid Polymers

Low molecular weight polymers such as poly (ethylene glycol) (PEG), poly(propylene glycol) (PPG), poly(tetrahydrofuran) (PTHF), and poly (methylphenylsiloxane) (PMPS) are considered as nonvolatile class-based solvents (Heldebrant et al. 2006). Naughton and Drago (1995) for the first time used PEG a solvent for homogeneous catalysis. Kerton et al. (2009a) reported hydrophobic siloxane-based liquid polymers for yeast-catalyzed reaction polymer synthesis. These polymers are similar to various organic transformations (Vafaeezadeh and Hashemi 2015). The advantages of liquid polymers include:

Nonvolatile.
Nonflammable.
Nontoxic to humans, animals, and aquatic life.
Biodegradable by bacteria (Heldebrant et al. 2006).

6.4.2 Fluorous Solvents

The fluorous solvents are colorless, are free-flowing liquid, have low toxicity, and are less polar than that of chloroform and dichloromethane, mainly used fluorous reaction media (Reichardt 2003). Various types of fluorous solvents with wide range of boiling points are available, but mainly used are perfluorinated alkanes (Lemaire et al. 2004). Other types of fluorous solvents are perfluorinated dialkyl polyethers and perfluorinated trialkylamines. The advantages of fluorous solvents include not miscible with organic or aqueous solvents, low toxicity, wide selection of boiling

points, greater densities, easy recyclability, lower disposal concerns, low volatility, lesser the pollution, and inert in nature (Horvath 1998; Kerton 2009a, b).

7 Solvent-Free Extraction Methods for Algal Biomass

7.1 Osmotic Pressure

Osmotic pressure can breakdown the algal cell walls when salt concentration disturbs the osmotic pressure between algal cells (Lee et al. 2010). The damage can occur in algal cells by two types of stresses – hyper-osmotic and hypo-osmotic. When salt concentration is high outside, cells get shrink, and fluid present inside the cell diffuses outside, and damage occurs to the cell envelope (Yoo et al. 2012). In case of hypo-osmotic shock, salt concentration is high inside the cell fluid, and fluid flows into the cells, and cells get burst. This method is mainly used for the extraction of intracellular substances from biomass. Various authors (Lee et al. 2010; Yoo et al. 2012; Kim and Yoo 2013) reported the positive results using osmotic pressure. Donald et al. (2015) used osmotic pressure with CO_2 as solvent for the extraction of lipid from *Chlorella* sp. and reported good yield of lipid.

7.2 Enzyme-Assisted Extraction

Some enzymes have the potential to break down the cell wall of microalgae cells. The use of such enzymes for microalgal cell disruption enhances the extraction of lipid recovery (Taher et al. 2014). The highest lipid recovery was reported by Hong et al. (2012) using 27 different sonication-enzyme treatments at pH 4. Aqueous extraction processing (AEP) is an environment-friendly approach, but it has low lipid recovery (Rosenthal et al. 1996). Enzyme-assisted aqueous extraction processing (EAEP) increases the yield of lipid in soybean and sunflower seeds when used with sonication (Freitas et al. 1997; Sineiro et al. 1998). Enzyme-assisted aqueous extraction processing extracts and separates oil directly from wet algae biomass (Hong et al. 2012). This method is highly specific and rapid but effected by class of lipid and types of microalgae species (Liang et al. 2012). This method also requires low temperatures and highly specific and selective enzymes for high yield (Taher et al. 2014).

8 Conclusion and Future Avenues

Microalgal lipids have established their role for the production of biodiesel which can pave a path for sustainable alternatives fuels. Indeed, to develop a large-scale microalgal-based biodiesel biorefinery, a considerable amount of research is essential particularly involving the extraction of lipids and downstream conversion to biodiesel. The first step toward the efficient lipid extraction is cell disruption which relies on the inherent characteristics of microalgal cell wall. The microalgal cell wall composition is variable depending on the species and presence of recalcitrant biomolecules, necessitating the development of new methods which not only efficiently disrupt the microalgal cell but also are cost-effective, require less energy consumption, and ease the recovery of the lipids. Studies indicate that mechanical disruption techniques are optimal for large-scale lipid extraction but have high energy requirements. On the other hand, nonmechanical methods, though require less energy, have long treatment time and high cost. Indeed, integration of two or more cell disruption technologies involving chemical, mechanical, or biological could lead to overall reduction in cost and energy requirements.

Another crucial aspect is the development of economical and nontoxic lipid extraction techniques from microalgal biomass. To date, most of the studies utilize expensive and toxic inorganic chemicals which necessitates the development of green solvents or solvent-free systems for lipid extraction. In this respect, the utilization of supercritical CO_2 and osmotic and enzymatic treatments is being considered promising alternatives which improve the lipid extraction yield, eliminate the use of toxic chemicals, and ease the lipid recovery. However, these emerging techniques need further improvements to potentially reduce the cost and energy requirements. Clearly, the future studies should focus on understanding the alterations in the cell-wall structure of microalgae under various cultivation conditions, growth phase, and stress factors which will aid in the development of universal cell disruption techniques. Additionally, the development of a common online database summarizing effectiveness, energy requirements, and cost of different cell disruption and lipid extraction techniques will assist in the improvement of algal biodiesel production.

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Chapter 13
Biodiesel, Bioethanol, and Biobutanol Production from Microalgae

Abd El-Fatah Abomohra and Mostafa Elshobary

Abstract Due to depletion of fossil fuel supplies and hazardous impacts on the global climate, clean renewable and sustainable energy production is being critically demanded. First-generation biofuels are mainly produced from edible crops and oilseeds. Because of competition with human food, first-generation biofuels are restricted in their ability to accomplish the global biofuel need, climate change amendment, and economic growth. Consequently, second-generation and third-generation biofuels were developed from nonedible feedstocks including lignocellulosic biomasses and microalgae, respectively, to overcome these challenges. However, algae are considered as a superior feedstock for biofuel production because of their diversity. Some of the major benefits of algae are their extremely fast growth rate and the ability of sequestration of carbon dioxide with high oil and carbohydrate contents that can be easily transformed into biodiesel or other gasoline components such as butanol. Biodiesel has been receiving globally growing consideration due to the liquid fuel needs and its potential as a biodegradable nontoxic substitute to petroleum diesel. In addition, butanol has become an attractive biofuel as a by-product of algal biomass processing after lipid extraction for biodiesel, due to its higher energy content, lower vapor pressure, and less hygroscopy than ethanol. This chapter reviews the current status of microalgae for biodiesel and butanol production as eco-friendly alternatives for liquid fossil fuels.

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1 Introduction

Biofuel is defined as the fuel produced from, or by, living organisms. The energy in biofuel is derived from carbon fixation process in which CO_2 is fixed into sugar that is found only in living organisms and plants. Biofuels can be classified into four main generations (Demirbas 2011). "First-generation of biofuels" is produced directly from edible crops such as corn, wheat, and soybeans. These biofuels offer some CO_2 benefits and can help to resolve domestic energy security. But the most argumentative issue is that first-generation biofuels compete with human and animal food (Olabi 2013). Therefore, "second-generation of biofuels" was developed in order to overcome that issue. These are made from nonedible crops (lignocellulosic biomass) such as grass, wood, and other organic wastes (Gomez et al. 2008). However, the indigence of arable land is a drawback for several second-generation biofuels. This problem is avoided permanently by developing "third-generation of biofuels" that are derived mainly from algae (Singh et al. 2011), whereas the fourth-generation utilizes metabolic engineered algae for producing biofuels by enhanced photosynthesis (Lü et al. 2011). It is imperative to mention that despite the feedstock of biofuel changed significantly, insignificant changes in the biofuel characteristics between different "generations" can be recorded (Olabi 2010). Thence, microalgae as a feedstock for third-generation biofuels are recently discussed as a viable alternative energy feedstock that overcomes the major obstacles associated with previous generations, such as high volumetric productivity, nonedible feedstock, potential use of nonarable land and potential CO_2 mitigation, and use of wastewater or seawater (Norsker et al. 2011; Abomohra et al. 2017).

Microalgae are a group of photosynthetic organisms consisted mostly of carbohydrates, lipids, and proteins, which are produced in relatively large amounts in few days with simple growing requirements (light, CO_2, N, P, K, and other inorganic nutrients) (Huang et al. 2016). These components can be used for production of biofuels and other economically important co-products (Brennan and Owende 2010; Olabi 2013). There are different forms of algal biofuels including direct combustion (biomass), liquid fuels (bioethanol, biobutanol, bio-oil, and biodiesel), or gaseous fuels (biogas, biomethane, syngas, and biohydrogen). Different kinds of biofuels can be obtained from dried biomass by anaerobic digestion, fermentation, lipid transesterification, pyrolysis, gasification, and liquefaction (Abomohra et al. 2013; Chen et al. 2015; Ullah et al. 2015, Alam et al. 2017; Hu et al. 2017; Wang et al. 2018). Microalgae also can produce other biofuels such as biobutanol, bioethanol, and biomethane by fermentation of algal carbohydrates using different kinds of microbes under anaerobic conditions. There are many downstream processing steps which are required for liquid biofuels production from microalgae

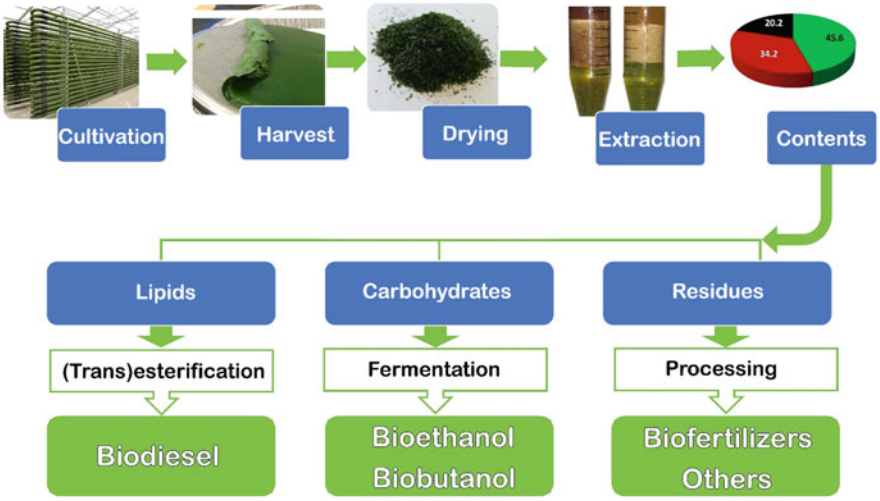

Fig. 13.1 Post-cultivation downstream processing of microalgae harvesting, drying, and extraction followed by production of different liquid biofuels from microalgal components

(Fig. 13.1). In addition to liquid biofuels, hydrogen gas can be formed directly by autotrophic microalgae under specific growth conditions (Drapcho et al. 2008). The present chapter will discuss the production of biodiesel, biobutanol, and bioethanol from microalgae and the consequent environmental impacts of large-scale production.

2 Urgent Need to Liquid Fuels

Development of industrial societies led to some related problems. The main problem is the depletion of oil resources (e.g., liquid fossil fuels are expected to completely exhausted by the middle of this century), land and water deterioration, air pollution, and greenhouse gases (GHGs) emissions. Crude oil reserves depletion and difficulties either in their extraction or refining and processing lead to significant continuous increase in its price (Laherrere 2006). This issue is mostly critical for transportation sector due to the absence of potential alternatives to fossil fuels. On the other hand, anthropogenic GHGs accumulation in our environment already surpassed the "dangerously high level" threshold of 450 ppm CO_2 and is still increasing (Schenk et al. 2008). The ignition of fossil fuels is estimated to release about six gigatons of CO_2 into the atmosphere per year (O'Reilly et al. 2012). The International Energy Outlook 2016 portends that CO_2 emissions will be increased by 10% in 2020 and 34% by the end of 2040. This increase is due to large-scale usage of fossil fuels for transport, electricity, and thermal energy generation. GHGs contribute not only to

global warming but also have other influences on the environment and human life. Oceans can capture approximately one-third only of the CO_2 emitted per year by human activities. By increasing CO_2 levels in the atmosphere, the amount dissolved in oceans will also increase, causing significant reduction in the water pH value. The high acidity of ocean and water bodies is harmful to marine ecosystem biodiversities such as coral reefs with massive implications in ocean life and consequently in earth life and human life (Ormerod et al. 2002). Therefore, the world faces huge challenges in order to develop regression techniques and adopt strategies to promote sustainable energy sources and clean replacements with high ability to sequester the increasingly atmospheric CO_2, to decrease the reliance on the limited fossil reserves, and also to pursue the economic sustainability and save the environment (Brennan and Owende 2010; Singh et al. 2010).

Many energy alternatives; such as solar, hydroelectric, geothermal, and wind energy and biofuels; are being studied and developed with various degrees of success, such as solar, hydroelectric, geothermal, and wind energy and biofuels (Dewulf and Van Langenhove 2006; Gilbert et al. 2007). Each alternative has its own advantages and disadvantages depending on the field of application. One of the most significant alternatives is liquid biofuel that can reach those goals, particularly in the short and medium term. Biofuels are sustainable and can be produced from green biomass products with the ability to minify the relying on fossil fuels. Biofuels also have, in principle, lower carbon liberation according to the short-term carbon cycle production, and their combustion returns back the CO_2 to the atmosphere that is required for plant growth. Hence, biofuel combustion was reported to be carbon neutral, in contrary to fossil fuel burning (Gomez et al. 2008). Therefore, there is growing interest to produce different liquid biofuels such as biobutanol and bioethanol which are produced by biomass fermentation, and biodiesel which is produced from lipids with animal or plant origin.

3 Biodiesel

Biodiesel is a mixture of fatty acid methyl/ethyl esters obtained by transesterification of algal oil, vegetable oil, or animal fats. These oil feedstocks optimally composed of triglycerides (90–98%) and small quantities of mono- and diglycerides, free fatty acids (FFAs, 1–5%), and trace residuals of other by-products such as carotenes, tocopherols, phosphatides, phospholipids, and sulfur compounds and traces of water (Bozbas 2008). Biodiesel is similar to traditional or "fossil" diesel, and, therefore, it is an alternative fuel. Many feedstocks can be used for biodiesel production such as lipids of vegetable seeds, organic wastes, and marine biomass. However, the current most reported promising feedstock for suitable oil is microalgae. In order to overcome first-generation and second-generation biofuel problems, the biofuel feedstock should not compete with human and animal food products and resources. Moreover, the feedstock should be easily available in large quantities and sustainable without negative environmental impacts.

Biodiesel is considered one of the most potential alternatives for fossil diesel fuel, because its physical and chemical properties are similar to those of petroleum diesel. Biodiesel acts as regular fossil diesel with relatively lower emissions. It is more heavier compared to fossil diesel (Mathur and Sharma 2010), while its viscosity can be easily controlled within the acceptable international standards levels. Therefore, the viscosity proposed is comparable with fossil diesel viscosity (Mathur and Sharma 2010). The flash point is another important parameter and is defined as the temperature at which fuel will sparkle when exposed to a flame or a spark. Biodiesel flash point was reported to be higher than that of fossil diesel. Thus, safety to store biodiesel and its blends is higher than fossil diesel (Mathur and Sharma 2010).

Biodiesel has several environmental benefits, such as it is highly biodegradable and has lower emissions of toxic and carcinogenic gases (Sheehan et al. 1998). It also has a favorable effect on the operation of the diesel engine. Biodiesel burns more efficiently and results in significant reduction in emissions of carbon monoxide, unburned hydrocarbons, and particulate matter such as smut and sludge (Kumar 2014). Thus, biodiesel is widely discussed as alternative clean fuel because it has only about 10% oxygen content and no sulfur nor aroma, which results in full burning. Biodiesel has ideal lubrication properties due to the small amounts of glycerin (Bošnjaković 2013). It can be stored without any specific additional infrastructure, just like diesel fuel. It has higher cetane number (CN) which increases the quality of ignition of biodiesel as well as blended with fossil diesel (Mathur and Sharma 2010). The main procedures for microalgae application would be lipid extraction to later produce biodiesel by transesterification process. There are different techniques to accomplish the lipid extraction that can be summarized into two main categories: mechanical and chemical methods (Halim et al. 2012). The methods used for chemical lipid extraction include Soxhlet, accelerated solvent, and supercritical fluid extraction. However, the mechanical extraction methods include oil expeller, ultrasonic-assisted extraction, and microwave-assisted extraction (Halim et al. 2011; Khoo et al. 2011; Abomohra et al. 2016a). In addition, the entire microalgal cells might be processed directly in the whole culture (wet processing) to produce different bioliquids, reducing dewatering procedures and overall production costs (Rashid et al. 2014). After lipid extraction, lipids can be converted into biodiesel using different transformation methods.

3.1 Reduction of Biodiesel Viscosity

Currently, biodiesel cannot be used directly in the modern diesel engines because of its high viscosity; thus, it requires pretreatment to decrease the oil viscosity to outfit the transport vehicle engines and to meet the international standards. Some technologies might be used to decrease the viscosity, such as dilution and microemulsification with solvents or alcohols (Zhang et al. 2010). Oil dilution can be done using solvents, such as ethanol, to accomplish as diesel fuels. Dilution also decreases the density and viscosity of oils. Ethanol can be added (4% w/v) to diesel

fuel in order to improve the efficiency of biodiesel (Bilgin et al. 2002). In addition, microemulsion formation is one of the possible solutions for reducing the viscosity of vegetable oil. Microemulsions are the thermodynamically stable and transparent colloids; the diameter of its droplets ranges from 100 to 1000 Å. Microemulsion may be consisted of vegetable oils, alcohol, a surfactant, and a cetane improver, in the presence or absence of diesel fuels. Butanol, octanol, and hexanol may be used to reduce oil viscosity to meet the preferable requirement for diesel fuel (Kumar 2014).

3.2 *Methods Used for Biodiesel Production*

There are different types of oils from different origins which can be converted to biodiesel basically by transesterification or esterification reactions. Figure 13.2 represents a schematic diagram of a typical unit operation for biodiesel production. The feedstock quality is very critical issue, as depending on its quality, either esterification (acid catalytic) or transesterification (alkali catalytic) reactions can be used for production of biodiesel. Both esterification and transesterification processes are used either in a continuous or batch process. However, the latter is more suitable to small pilot-scale plants that produce $\leq 10 \times 10^5$ gallons per year and provide

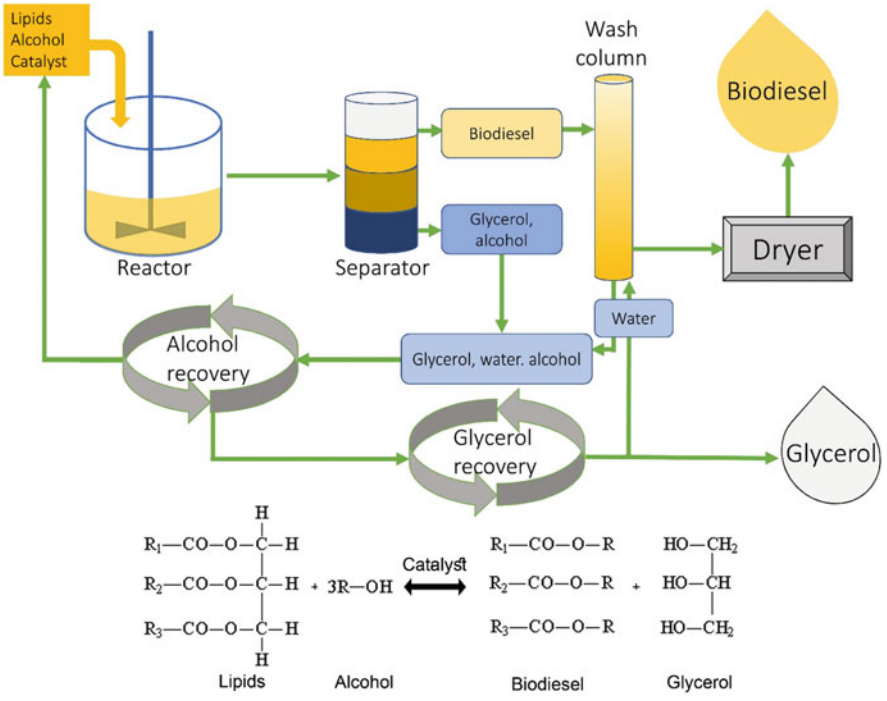

Fig. 13.2 Typical flow diagram and reaction of catalytic biodiesel production from lipids

operational flexibility (FAPC-150 BC). Continuous reaction permits usage of high-volume systems for separation that increases throughput (FAPC-150 BC). In addition, enzymatic and non-catalytic conversion processes can be used. In general, methanol is the most commonly used alcohol for oil conversion into biodiesel, which requires proper handling due to its high flammability.

3.2.1 Transesterification

Most of the current biodiesel production operation systems use alkali catalysis (transesterification) for oil conversion. The purpose why there is a great concern in the alkali process is it is more efficacious and less corrosive than the acid process, making it a favorable catalyst to be used in commercial production. Usually, potassium hydroxide (KOH), sodium hydroxide (NaOH), or sodium methoxide (CH_3ONa) are the most common catalysts used with alcohol (methanol or ethanol) and any kinds of oils. This method works well if the FFAs and moisture contents of the oil are <0.1% and phosphorous content is <10 ppm (FAPC-150 BC). However, base catalysts are very sensitive to FFAs content and moisture. As the oil feedstock with high content of FFAs results in soap formation, which has countereffects on downstream processing and reduces the biodiesel yield. Moreover, NaOH and KOH also lead to water formation, which reduces the reaction rate and causes soap formation. Therefore, sodium methylate (sodium methoxide) is more efficient than NaOH or KOH as a catalyst, but it is more costly. Sodium methoxide is marketed as 30–50% solution in methanol for easier handling, and added in amount of 0.3–0.5% of the weight of the oil. However, higher concentration of base as KOH or NaOH (0.5–1.5% of the weight of the oil) is required when used as a catalyst (FAPC-150 BC). Transesterification is a reversible reaction, i.e., excess methanol is necessary to direct the equilibrium favorably.

According to Barnwal and Sharma (2005), the catalyst concentration required to the reactor varies from 0.5% to 1% of oil (*w/w*). Another critical variable during transesterification reaction is the reaction temperature. Although the standard temperature for the reaction is 60 °C, different temperatures could be applied depending on the type of catalyst with different conversion rates, and for that reason, the range of temperature should be within 25–120 °C (Barnwal and Sharma 2005; Marchetti et al. 2007).

3.2.2 Esterification

If the content of FFAs in the feedstock is >1% of the oil content, alkali catalysis is not the good choice. Prior to transesterification, FFAs can be removed by chemical neutralization using a base such as NaOH or physical deacidification using vacuum. However, some oil is lost during this pretreatment, and, therefore, it is not recommended. Fats and oils with high FFAs content can be used to produce biodiesel using acid esterification process. In this case, soap formation is not a problem because there are no alkali metals in the reaction medium. In addition to

FFAs, acid catalysts can be used to transesterify triglycerides, but the reaction might take several days to complete which is not applicable for industrial processing. However, the esterification of FFAs to esterified alcohol is relatively rapid; the whole reaction would take about 1 h at 60 °C. To improve reaction rates, the formed water needs to be continuously removed from the reaction medium by phase separation (FAPC-150 BC).

In acid esterification reaction, large amount of acid (5–25%) and a high alcohol: FFAs molar ratio (20:1 - 40:1) are required. Similar to the alkali esterification, excess of alcohol improves the conversion of triglycerides; but, the recovering of glycerol becomes more difficult. Accordingly, relation between alcohol and raw material should be kept in the optimal ratio, and it should be determined by preliminary experiment (Marchetti et al. 2007). Phosphoric acid and sulfuric acid are the most prevalent acid catalysts. Generally, one mol% of sulfuric acid is an efficient to final conversion of 99% in about 50 h (Marchetti et al. 2007), with a temperature range from 55 to 80 °C. The moment that the conversion of the fatty acids to alcohol esters has achieved the equilibrium, the alcohol, water, and acid mixture is eliminated by settling or centrifugation. Fresh alcohol and base catalyst are added to the remaining transesterification reaction. Consequently, esterification process should be followed by transesterification for better results and complete conversion of oil.

3.2.3 Enzymatic Conversion

Recently, lipase has been taken a great interest for enzymatic catalysis of oils for biodiesel production in the form of solution or immobilized onto some support materials. Lipases are a group of enzymes which are commonly used to catalyze some reactions such as hydrolysis, acidolysis, and alcoholysis, but it was documented that lipases catalyze the transesterification as well as esterification reactions too (Marchetti et al. 2007). The reaction can be performed at 35–45 °C with a time range 4–40 h. Although reuse of the immobilized enzymes keeps the reactive flow and relatively reduces the cost, there is still a huge gap between the cost of available techniques and the industrial application. In addition, there is no uniform enzyme that can be used with different feedstocks. Due to the high cost of enzymes, this process is not economically favorable for biodiesel production from microalgae.

3.2.4 Non-catalytic Conversion

Non-catalytic conversion may be used for enhancing the reaction of oil with alcohol or to increase miscibility of the oil alcohol phase and to avoid the disadvantages of the previous methods. Non-catalytic methods used include supercritical conversion, microwave-assisted conversion, or ultrasound-assisted conversion (Bharathiraja et al. 2014).

3.2.4.1 Supercritical Alcohol

Although this is a relatively new advanced and more relevant technique (Warabi et al. 2004), there is a doubt regarding whether transesterification and esterification are more efficient and faster processes for oil conversion into biodiesel (Marchetti et al. 2007). This process is very simple, and the reaction was found to be completed in a very short time within 2–4 mins. Further, since no requirement for a catalyst, the purification of biodiesel is much simpler, easier, hassle-free, and environment-friendly (Demirbas 2005). Several conditions are usually applied to identify the optimum conditions for the conversion such as time and temperature of reaction, catalyst loading, stirring rate, and alcohol/oil molar ratio (Meher et al. 2006).

3.2.4.2 Microwave-Assisted Conversion

Microwave-assisted conversion is performed under microwaves, where the reaction is done in a short time with extreme reduction in the by-product quantity and fast separation process (Hernando et al. 2007). Moreover, high quantity and quality of the products are reached within a relatively short time (Nüchter et al. 2000), which results in significant reduction in the production cost.

3.2.4.3 Ultrasound-Assisted Conversion

Ultrasound-assisted conversion provides an effective mixing method to achieve a maximal mixing and enhance liquid-liquid mass transfer (Ji et al. 2006) to increase the interaction surface area between alcohol and oil (Stavarache et al. 2006). Ultrasound was reported to increase the mass and heat transfer in the solution which increases the reaction rate and product yields (Adewuyi 2001); thus, it supplies the required activation energy for initiating the reaction.

3.3 After Conversion

After reaction completion, biodiesel will be in a mixture of excess methanol, catalyst, and glycerin. As a rule of thumb difference, specific gravity in the mixture of compounds results in self-phase separation. As can be presented in Fig. 13.3, gravity separation is favorable to recover biodiesel from the by-products (glycerin and methanol). However, feedstock impurities may induce emulsion formation, which makes the separation process more difficult. To overcome this problem, either saturated salt (NaCl) or centrifugation breaks the emulsion and accelerates the phase separation. In addition, methanol concentration used for the reaction has to be minimized for good phase separation, although a good conversion reaction requires excess of methanol. Using distillation, glycerin and methanol can be further

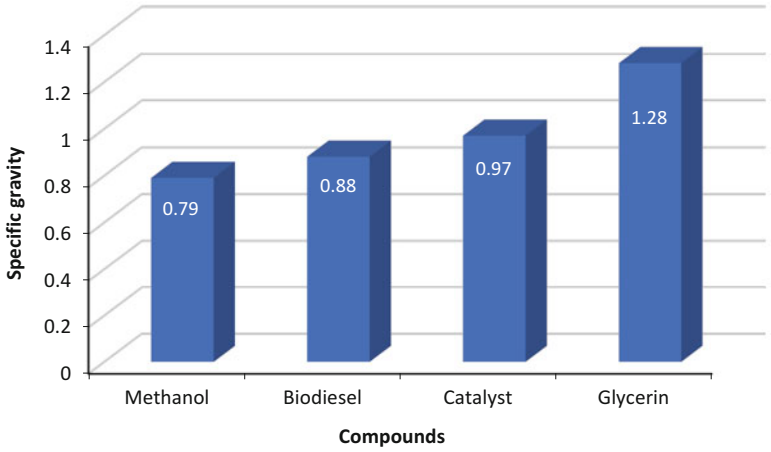

Fig. 13.3 Specific gravity of the compounds used and produced through biodiesel production

purified. After phase separation, the residual methanol in the produced biodiesel should be further removed by evaporation. The biodiesel quality depends mainly on the biodiesel conversion rate and acid value, whereas the best quality biodiesel is indicated by the lower acid value.

Finally, microalgal biomass residue after lipid extraction for biodiesel production may be reused to produce other liquid biofuels such as biobutanol, bioethanol, or bio-oil (Gouveia and Oliveira 2009; Miranda et al. 2012) and/or gaseous biofuels such as biomethane, biohydrogen, and syngas (Ferreira et al. 2013), which significantly reduces the overall cost.

4 Biobutanol

Since lipid-based microalgae biodiesel production has been broadly discussed, carbohydrate-based microalgae and microalgae residues obtained from the biodiesel production should be taken into consideration. Some studies were done on microalgae-based carbohydrate fermentation and microalgal biodiesel residue to other biofuels such as ethanol (Brennan and Owende 2010; Daroch et al. 2013), butanol (Cheng et al. 2015; Wang et al. 2016), as well as biogas (Lakaniemi et al. 2013). The first fermentation process to form butanol was achieved by Louis Pasteur in 1861. Since then, the fermentation had been modified to produce acetone and ethanol plus butanol (Jones and Woods 1986). So, the production of these solvents biologically is well known as acetone-butanol-ethanol or ABE fermentation.

Biobutanol is biomass-derived butanol; it may be utilized as a fuel in an internal gasoline combustion engine. Due to it being a totally nonpolar, long hydrocarbon, it is a similar to gasoline which makes it suitable to work in gasoline vehicles without upgrading or mechanism alterations (Hönig et al. 2014). Moreover, the

heat of vaporization of butanol is slightly greater than that of gasoline (Hönig et al. 2014). Thence, vaporization rate of butanol is similar to gasoline, so the butanol-blended gasoline didn't show cold start problem and can be used as 100% biobutanol fuel instead of gasoline (Pospíšil et al. 2014). Biobutanol is characterized by high miscibility, low volatility, high energy contents from 33.07 MJ Kg^{-1} (Klass 1998) to 36.1 MJ Kg^{-1} (Laza and Bereczky 2011), and density of 810 Kg m^{-3} (Pfromm et al. 2010).

Different from biodiesel, the main feedstock of biobutanol is carbohydrates. Thus, carbohydrates might be used efficiently as a source for hydrocarbon production (the main constituent of fossil fuels). Carbohydrates include monosaccharides, disaccharides, polysaccharides, or oligosaccharides based on the length and configuration of carbon in the biomass feedstock (Gloria et al. 2013). The carbohydrate contents of microalgae are generally higher than lipids because lipid accumulation requires severe stress conditions, whereas carbohydrate production is basically achieved by normal photosynthesis through Calvin cycle (Radakovits et al. 2010). Carbohydrate content of algae varies significantly according to the algal species. Several algae, including chlorophytes, have carbohydrates in the cell wall that composed mainly of cellulose and soluble polysaccharides (Domozych et al. 2012) and in plastids mainly in the form of starch, glycogen, and other polyglucans depending on the algal taxa (Rismani-Yazdi et al. 2011; Domozych et al. 2012). Starch or glycogen is the main reserved carbohydrates in most green algae and cyanobacteria (Singh and Olsen 2011; Chen et al. 2013). *Chlorella* sp., *Dunaliella* sp., *Chlamydomonas* sp., and *Scenedesmus* sp. have reported as industrially relevant species of green microalgae. Those algae contain higher than 50% dw digestible starch, cellulose, and glycogen (Singh and Olsen 2011). Unlike lignocellulosic biomass of the second-generation biofuel, mannose, xylose, and galactose are found only in tiny amounts and fermented less efficiently (Foley et al. 2011).

4.1 Algae Pretreatment for Biobutanol Production

Proper pretreatment of biomass is a critical step as it breaks down polymer crystalline structure such as cellulose and starch to form simple fermentable sugars which results in faster hydrolysis and higher yields (Mosier et al. 2005). In this context, it was observed that using intact algal cells produces poor biobutanol productivity due to lower conversion rates (Wang et al. 2016). The selection of most suitable pretreatment process can also reduce the releasing of inhibitors to the subsequent hydrolysis and fermentation (Sun and Cheng 2002). Practically, the starch content of microalgal biomass can be converted directly into biofuel under dark and anaerobic conditions, but the biofuel production rate and yield are much lower (Ueno et al. 1998). Consequently, algal biomass requires pretreatment to increase the production yield. The ABE fermentation process is accomplished in three main steps: (1) pretreatment of biomass, (2) fermentation, and (3) recovery (Fig. 13.4).

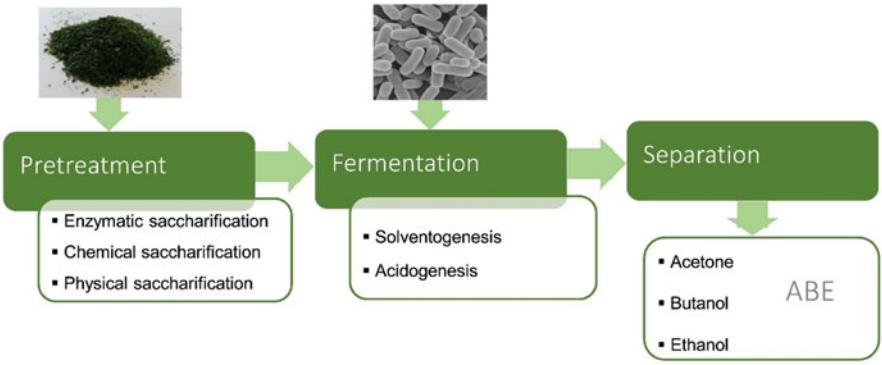

Fig. 13.4 Flowchart showing the main steps of acetone-butanol-ethanol (ABE) production from microalgae

Generally, the cost of pretreatment process of feedstock for butanol production is very influential, and it estimates from 40% to 70% of the selling prices (Srirangan et al. 2012). Thus, the selection of a suitable method for microalgae saccharification pretreatment needs to take the cost into consideration. The pretreatment process can be performed using different three methods including hydrolysis/saccharification, nourishment, or sterilization (Hemming 2011). However, saccharification is the most common and efficient method to convert carbohydrate polymers into simple fermentable monomers.

4.1.1 Hydrolysis/Saccharification

Saccharification is usually the main pretreatment step in fermentation of lignocellulosic materials or cellulosic microalgal biomass. This method can be used to achieve saccharification of raw feedstocks using enzyme digestion, alkaline, thermolysis, and acid hydrolysis either diluted or concentrated. Those methods can be assorted into three major groups, namely, enzymatic saccharification, physical saccharification, and chemical saccharification. These pretreatment saccharification methods have a certain economic cost that depends on several parameters including (i) alkaline or acid reagent, (ii) electricity cost, (iii) time of thermal pretreatment and working temperature, (iv) surfactant loading during enzymatic hydrolysis, (v) type of hydrolytic enzymes used, and (vi) type of feedstock used (Hernández et al. 2015). Regarding pretreatment costs, different methods may be sorted, from high to low costs, as (i) enzymatic pretreatments (using amylases and cellulases), (ii) chemical pretreatment (alkaline and acid), and (iii) physical pretreatment (microwaving, sonication, high-pressure homogenization, and heat) (Eggeman and Elander 2005; Talebnia et al. 2010; Tao et al. 2011).

4.1.1.1 Enzymatic Saccharification

These processes include the utilization of hydrolytic enzymes such as cellulases, amylases, and glucoamylases. Microalgae cell wall composed mainly of cellulose and very low amount of hemicellulose, while lignin is completely absent. Thus, lignin degradation enzymes are not necessary in case of algal enzymatic saccharification. Moreover, the complicated additional pretreatment methods, such as alkaline or acidic pretreatment or steam explosion, are also not required making it easier, simpler, and cheaper than lignocellulosic feedstocks. Microalgae-based cellulose and starch hydrolysis depend on the cleavage of β-1,4-glycosidic linkages between the hydroglucose subunits in cellulose molecules into cellobiose and cellodextrin which are degraded to glucose by β-glucosidase (Soni et al. 2010). Also, cleavage of α-1,4-glycosidic linkages in starch into dextrin further hydrolyzes dextrin into glucose and oligosaccharides using glucoamylase (Van Der Maarel et al. 2002). Enzymatic saccharification has many advantages such as lower costs of equipment according to mild conditions of enzymatic hydrolysis with higher glucose yields, without toxic by-products or sugar degradation products (Cara et al. 2007). In addition, enzymatic saccharification saves energy due to its lower temperature and higher selectivity of components present in microalgae, where an enzyme selectively degrades a specific chemical linkage (Mubarak et al. 2015). Ellis et al. (2012) demonstrated that fermentation of enzymatic pretreatment using xylanase and cellulase produces 9.74 g L^{-1} of total ABE as compared with acid−/alkali-pretreated algae that produces only 2.74 g L^{-1} of total ABE.

However, the enzymatic digestion is a costly process that decreases the large-scale application of ABE production (Kumar and Murthy 2013). Furthermore, the expensive enzymatic digestion for the conversion of polymers into fermentable sugars is not required for some microalgae such as *Clostridium saccharoperbutylacetonicum* which is an amylolytic microorganism that can perform this process (Thang et al. 2010).

4.1.1.2 Chemical Saccharification

The chemical saccharification process is distinguished by its short reaction time but usually requires harsh reaction conditions of higher temperature, pressure, and addition of acid (e.g., H_2SO_4, HCl, and HNO_3) or base (NaOH, KOH, and Na_2CO_3). Besides, it produces inhibitors such as furfural and 5-hydroxymethylfurfural, which may suppress fermentative reaction and also needs costly removal of the waste (Mussatto et al. 2010). To avoid the production of those inhibitors and to improve saccharification efficiency, the suitable reaction conditions should be maintained including temperature, residence time, and moisture content (Okuda et al. 2008). Acid/alkaline hydrolysis is preferable for sugar production from microalgae, due to the fast hydrolysis reaction and the negligible content of lignin in microalgae, which makes it easier to hydrolyze and not require violent condition when compared with chemical hydrolysis of lignocelluloses (Park

et al. 2012; Ho et al. 2013; Harun et al. 2014). The efficiency of chemical hydrolysis of cellulosic feedstock depends on the substrate, acid/alkali concentration, temperature, and retention time (Wang et al. 2011). By optimization of these parameters, the yield of fermentable sugars will be increased (Castro 2014). Castro (2014) demonstrated that sugar yield of 166.1 g kg^{-1} of dry algae from indole and notably butanol-producing bacteria *Clostridium saccharoperbutylacetonicum* N1–4 was obtained, while it was treated for 120 min by 1.0 M sulfuric acid at 80–90 °C. Besides acid hydrolysis, the use of base has also been proved for hydrolysis of microalgae carbohydrates. Harun et al. (2011) recorded the maximum glucose yield of 0.35 g g^{-1} biomass from *Chlorococcum infusionum* using 0.75% (*w/v*) NaOH at 120 °C for 30 min. They concluded that NaOH efficiently destroys the microalgal cell wall. Then, carbohydrates were further hydrolyzed into simple fermentable monomers of glucose by NaOH. These results showed the advantages of using microalgae-based carbohydrates for biobutanol production, as the fermentable sugars from microalgae can be achieved more efficiently in much easier, simpler, and cheaper chemical saccharification processes, without energy-intensive pretreatment or enzymatic hydrolysis during microalgal biomass saccharification. However, the sugar yield of acid/alkali saccharification may still be lower than that of enzymatic saccharification (Choi et al. 2010). However, the combination of acid hydrolysis and enzymatic digestion obtained high yield (Park et al. 2012; Castro 2014).

4.1.1.3 Physical Saccharification

Physical pretreatment usually refers to the application of physical force to enhance the hydrolysis and fermentation process of carbohydrates (Talebnia et al. 2010; Tao et al. 2011). Till now, this pretreatment method is still little being examined for microalgae biomass except macroalgae or seaweeds or lignocellulosic biomasses (Laghari et al. 2014). However, several studies used most efficient physical pretreatment such as microwave and sonication. Microwave application takes more interest in various biomass transformation processes than conduction/convection heating. Microwave is a more direct, rapid, and steady method as it allows the heated substrates to directly interact with an electromagnetic field and generate heat (Macquarrie et al. 2012). Additionally, ultra-sonication of algal feedstock enhances the rate of hydrolysis to simple fermentable sugar by increasing the surface area, sugar solubility, and substrate digestibility and weakens the cell wall for enzymes to be more accessible (Zhao et al. 2013). It was found also that ultrasonic-assisted extraction method could enhance glucose extraction from *Chlorella* sp. biomass than traditional solvent extraction.

4.2 ABE Fermentation Metabolic Pathway

The ABE fermentation is carried out using saccharolytic butyric acid-producing microorganisms such as bacterium *Clostridium acetobutylicum*, also known as "Weizmann organism." Unlike yeast, clostridia are capable of producing alcohols from a greater variety of carbohydrates which mainly use hexoses or pentose as carbon source (Yoshida et al. 2012). Moreover, disaccharides (sucrose, mannose) and polysaccharides (starch) also have been reported as fermentable sugars by clostridia (Groot et al. 1992; Campos et al. 2002). A variety of different microorganisms can be incorporated into biobutanol fermentation, including *Granulobacter saccharobutyricum*, *Amylobacter butylicus*, and *Bacillus orthobutylicus* (Dürre 2007). However, it is well characterized that most of *Clostridium* sp. perform biobutanol fermentation such as *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* (Gao 2016). The butanol is a by-product of this fermentation. Furthermore, a recoverable amount of H_2 is released, in addition to a number of other by-products such as ethanol, acetic acid, isopropanol, and lactic propionic acid. However, some *Clostridium* sp. shift or modify the by-product including *C. acetobutylicum* (which produces isopropanol instead of acetone), *C. aurantibutyricum* (which produces butanol besides isopropanol and acetone; George and Chen 1983), and *C. tetanomorphum* (which produces equimolar quantity of butanol and ethanol; Gottwald et al. 1984). It is important to mention that despite microorganisms incorporated in the butanol fermentation can be changed, they have common amylolytic properties toward starch-based polymers. Since *Clostridium* sp. have been dominant species for ABE fermentation, several efforts have been made on screening new natural strains and modifying wild strains through mutagenesis (chemically or physically), evolutionary engineering, and metabolic engineering strategies to improve their butanol production and decrease the cost (Xue et al. 2013).

ABE fermentation is an anaerobic bacterial metabolism of standard butyric acid fermentation that consists of two distinct processes, acidogenesis and solventogenesis. Acidogenesis takes place during exponential phase of bacterial growth. At acidogenesis stage, the clostridial cells show significant morphology alternation as the rod-shaped cells become swollen and look like a cigar, where carbohydrates are accumulated in the form of granulose inside the cells, which is mainly composed of α-1,4-linked polyglucan (Shaheen et al. 2000). In the acidogenesis phase, monosaccharides are immediately reassembled to form organic acids, mainly acetate and butyrate, about twice as much butyrate is produced compared to acetate (Fig. 13.5). Those acids present in associated form, which are lipophilic and can easily diffuse across the cell membrane (Skřivanová and Marounek 2007). The release of these organic acids in the medium reduces the pH value, which further induces the onset of the solventogenesis step (Gheshlaghi et al. 2009; Li et al. 2011). The switch from acidogenesis to solventogenesis was reported to start only at pH lower than 5.1 (Millat et al. 2013).

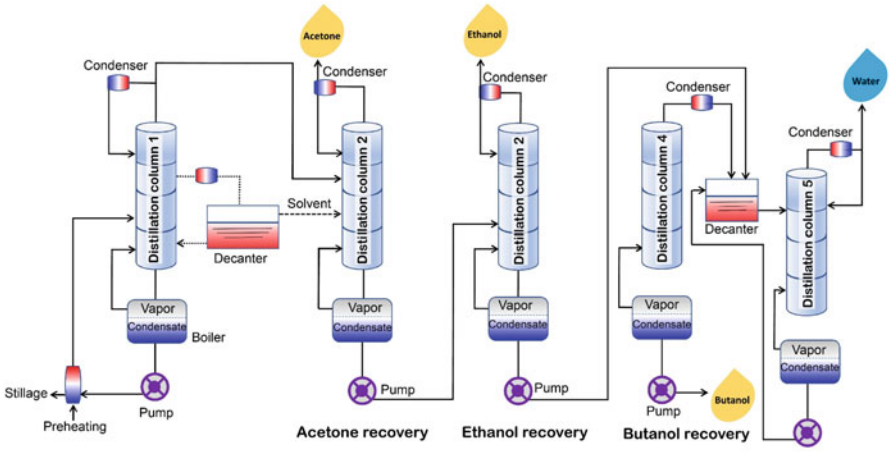

Fig. 13.5 The overall detailed process of acetone-butanol-ethanol (ABE) production by fermentation (Modified from Xue et al. 2013)

On the other hand, solventogenesis occurs at the end of exponential phase to the early stationary phase of growth inside the cytoplasm, where a major metabolism of acid production has been slowed down and converted the excreted acetate and butyrate into the acetone and butanol (approximately 2 butanol:1 acetone) (Fig. 13.5). This process is coupled with H^+ gradient which is very important to many vital cellular activities (Mitchell 2011). Acetone, butanol, and ethanol were obtained in the ratios of 3:6:1, respectively, as by-products (Qureshi et al. 2006). However, butanol concentration of around 1–2% can prohibit the cell growth through disruption of the cell membrane (Jin et al. 2011). In the meantime, the cells start synthesizing endospores by consuming the previously stored carbohydrate granules for survival. The endospores are resistant to various stress conditions such as UV light, heat, drought, or frost. When provided with preferable conditions, the spores will germinate and start the cell cycle again (Wang et al. 2014); thus, ABE fermentation performance is further restricted. In spite of all these restrictions that existed, advanced metabolic engineering and butanol removal techniques may improve the competitiveness of ABE fermentation.

Butanol production differs from ethanol, mainly in the substrate fermentation and slight changes due to distillation step. In addition, production of butanol from agricultural by-products and microalgal biomass could be more efficient than methanol or ethanol production. In addition, butanol is a superior fuel for transportation over ethanol according to its greater energy content with a value closer to gasoline, immiscible properties, lower volatility, lower corrodibility, and lower hygroscopicity (Srirangan et al. 2012). Moreover, it is less corrosive to distribution and storage infrastructures (Dürre 2007). Also, it has a better energy density and performance than ethanol and can be derived from a wide range of inexpensive sustainable feedstocks (Gao and Rehmann 2014; Gao et al. 2014). Current progress has been done in the characterization of biobutanol and other liquid biofuels from whole wastewater grown microalgae (Ellis

et al. 2012) and inexpensive lipid-free microalgae (Cheng et al. 2015). Therefore, Green (2011) projected that biobutanol has the potency to substitute both ethanol and biodiesel in the market estimate to worth $247 billion by 2020.

5 Bioethanol

At present, bioethanol is the most utilized alcoholic biofuel and is the major biofuel in the global market (Chia et al. 2017). Ethanol is considered as an octane booster for gasoline; and blending with up to 40% ethanol can reduce gasoline consumption by 3.0–4.4%, enhance the efficiency of internal combustion engines, and decrease CO_2 emissions by 19–35 metric tons year^{-1}. Bioethanol also can be produced from carbohydrate-based microalgae like biobutanol. Bioethanol can be manufactured from microalgae via three methods: hydrolysis followed by fermentation of biomass, dark fermentation, and photofermentation (de Farias Silva and Bertucco 2016). Each pathway or process has its own features, as it is shown in the following sections (Fig. 13.6).

5.1 Hydrolysis and Fermentation

This method is based on the production of microalgae biomass in photobioreactors (PBRs) after pretreatment to break down the cell structure and hydrolysis of the biomass chemically or using enzymes as previously discussed in Sect. 4.1. Hydrolyzed biomass is then fermented using different microorganisms such as yeasts (*Saccharomyces* sp.) or bacteria (*Zymomonas* sp.) to obtain bioethanol. Fermentation using carbohydrate-based microalgae as bioethanol feedstock can be classified into two main routes, namely, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). During SHF, both hydrolysis and fermentation are done individually and successively using two different reactors (Xiros et al. 2013). The priority of this approach is that two processes can be performed separately at their own particular optimum conditions (Danquah et al. 2011; Xiros et al. 2013). However, this technique will face some restrictions such as additional neutralizing and purification steps (acid or alkali hydrolysis) and reduction of hydrolysis rate by inactivation of cellulases after glucose and cellobiose accumulation during enzymatic hydrolysis (Tengborg et al. 2001). In contrast, hydrolysis and fermentation during SSF occur simultaneously at the same time in the same reactor. The inhibition of cellulases by glucose and cellobiose could be decreased because both compounds are directly fermented by the yeast to bioethanol (Xiros et al. 2013). Moreover, SSF would also need lower enzyme loading and gives higher bioethanol yield compared to SHF (Lin and Tanaka 2006). Other advantages of SSF are eradicating the demand for separate reactors to perform hydrolysis and

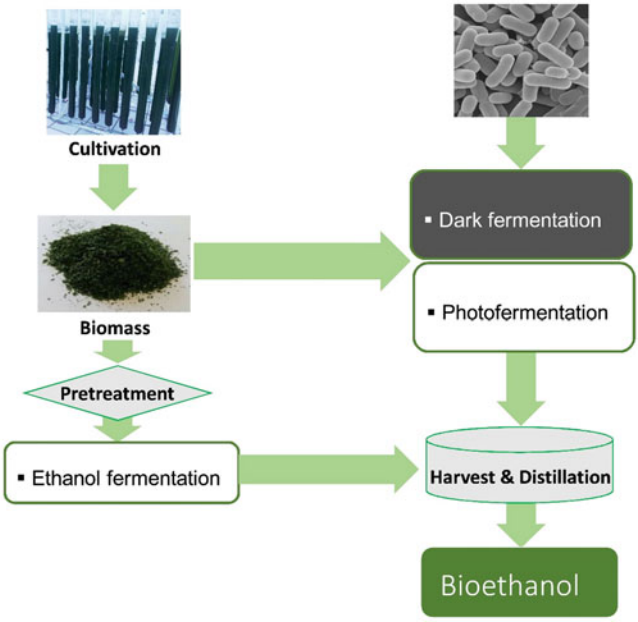

Fig. 13.6 Different routes of microalgae conversion into bioethanol

fermentation successively, fast fermentation, and lower contamination risk by external microflora (Lin and Tanaka 2006). However, the main downstream of SSF is the need to adjust operating conditions optimal for both hydrolysis and fermentation (Gupta and Demirbas 2010) and difficulty in process control. In addition, recycling of enzymes and yeast is complicated in SSF, which makes the process more challenging when considering scale-up for large-scale industrial purposes (Olofsson et al. 2008). Several factors affect the hydrolysis efficiency including hydrolysis strategies (enzymatic or acidic), hydrolytic enzyme composition, hydrolysis time, alkali/acid concentration, and loading of microalgal biomass (Ho et al. 2013). The conversion efficiency of the hydrolyzed microalgae to bioethanol is also influenced by using SHF or SSF processes (Ho et al. 2013). The hydrolysis of microalgal sugars releases fermentable, simple monomers including glucose (as a predominant), mannose, galactose, xylose, rhamnose, and arabinose based on the algal species and types of carbohydrate contents. These sugars are fermented via mild treatments by ethanol-producing microorganisms into bioethanol (Ho et al. 2013).

This method is very competitive for algal biomass due to the high fermentation efficiency of yeasts under mild conditions which results in high yield of bioethanol produced. On contrary, the main disadvantages of this method are the complexity and high cost due to using of costly enzymes and yeasts for fermentation (de Farias Silva and Bertucco 2016). The highest yield of ethanol from algae was 0.26 g g^{-1} algae using *Chlorococcum infusionum* biomass and the fermentable yeast *Zymomonas mobilis* (Harun et al. 2011). The carbohydrate content of the algal

biomass was about 33% of algal dry weight and was pretreated by an alkaline method using 0.75% (*w/v*) NaOH at 120 °C for 30 min. Biomass of *Chlamydomonas reinhardtii* containing close to 60% carbohydrates also produces a comparable yield of 0.24 g ethanol g^{-1} biomass (Choi et al. 2010).

5.1.1 Dark Fermentation

Dark fermentation means the fermentation of organic substrates into a mixture of low molecular weight organic acids (such as acetic acid) and alcohols (ethanol; de Farias Silva and Bertucco 2016). Several microalgae including *Chlorella reinhardtii*, *Chlamydomonas moewusii*, *C. vulgaris*, and *Chlorococcum littorale* can produce ethanol via cell wall by means of dark intracellular process (Ueno et al. 1998; Deng and Coleman 1999). Microalgae may be obligated to naturally synthesize ethanol through metabolism by fermentation of their carbohydrates and lipids when switching the growth to dark conditions (Beer et al. 2009; Abo-Hashesh et al. 2011). However, microalgal dark fermentation is not a significant process for the production of bioethanol (de Farias Silva and Bertucco 2016).

5.1.2 Photofermentation

Photofermentative or photanol process is a natural pathway where sunlight energy is converted into fermentable organic products which is a highly efficient metabolic mechanism (Hellingwerf and De Mattos 2009). In this method, microalgae might be preferentially chosen instead of yeast as a potential biological system to produce bioethanol. Photanol is not only restricted to bioethanol production, but it produces huge amount of natural products through glycolysis-based fermentation. Hemschemeier and Happe (2005) reported that under sulfur depletion, *Chlamydomonas reinhardtii* reorganizes the whole metabolism including stop growth and start starch accumulation. Hence, anaerobiosis is established generating hydrogen and ethanol, where pyruvate formate lyase (PFL) splits pyruvate into acetyl-CoA, which can be reduced further to acetaldehyde by the action of acetaldehyde dehydrogenase. The latter is converted into ethanol by the action of alcohol dehydrogenase (ADH). Costa et al. (2015) suggested that ethanol can be synthesized from metabolism of *C. reinhardtii* using a minimal growth medium containing the mixotrophic carbon source. Recently, Costa et al. (2015) succeeded to produce ethanol from *C. reinhardtii* through normal metabolic pathways at 25 °C in a light/dark cycle using tris-acetate phosphate (TAP) with sulfur starvation and organic carbon sources as a growth medium.

5.2 *Bioethanol Recovery*

Product recovery process is a crucial step in the commercial production of bioethanol. The bioethanol can be recovered by distillation process, where the bioethanol distillate is concentrated (about 37%) under the azeotrope condition in the distillation column. The lower product is discharged to stripping column to eliminate excess water, and the remaining bioethanol is drained and reduced to fluid form that is used as a supplement or substitute for gasoline in cars (Patil et al. 2008). The recovery of bioethanol might reach to 99.6%. On the other hand, solid wastes are eliminated by centrifugation, followed by drying in rotary dryer. Then, it is recovered in an evaporator as concentrated syrup and clean condensate (15–20% of total solid weight).

6 Current Status of Microalgal Biofuel Industry

In principle, microalgae can produce all kinds of biofuels through conversion of lipids into biodiesel and further hydrolysis of carbohydrates followed by fermentation to bioethanol and biobutanol. However, many challenges that encumber the marketing and scaling up of microalgal biofuel manufacturing processes are still existing. Microalgae cultivation as a promising feedstock for biomass production still needs a great deal of expensive nutrients (Alam et al. 2017), water resources, and post-cultivation processing. The advantages and obstacles of utilizing microalgae for biofuel production are summarized in Table 13.1. There is a desperate need to find an alternative non-expensive medium to grow microalgae. Wastewater is abundant and an enriched source of nutrients and, therefore, has many benefits for algal cultivation. Application of wastewater as growth medium for microalgae reduces the production cost and provides sustainable source for large-scale microalgae-based biofuel production (Cheah et al. 2016, Han et al. 2016, Abomohra et al. 2016b). In addition, marine microalgae can be used for biomass production by growing on seawater (Abomohra et al. 2017).

7 Economic Feasibility and Environmental Impacts

In comparison with seaweeds and higher plants, microalgae have a much faster growth rate under optimum conditions which results in higher biomass and products productivity. Moreover, they modify their metabolic pathways to accumulate oils or carbohydrates as storage compounds under stress conditions. These advantages add to their increased demand for application in biofuel production. In addition, lipids show high conversion efficiency of more than 95% biodiesel from the oil extracted from *Schizochytrium limacinum* containing 57% oil contents (Johnson and Wen

Table 13.1 Advantages and obstacles of microalgae utilization for biofuel production

Advantages	Obstacles
1. Microalgae can be grown around the year; therefore, oil yield of microalgae exceeds that of the best oilseed crops (Schenk et al. 2008)	1. Species selection should meet the requirements of biofuel production and other valuable by-products (Ono and Cuello 2006)
2. Reducing the freshwater resources than higher plants (Dismukes et al. 2008)	2. Achieving higher photosynthetic rate needs further development of the cultivation systems (Pulz and Scheibenbogen 1998)
3. Microalgae can be grown in marine water using nonarable lands minimizing the associated negative environmental impacts (Searchinger et al. 2008)	3. More research and development of techniques for unialgal cultivation, reduction of evaporation, and control of CO_2 and O_2 diffusion (Ugwu et al. 2008)
4. Microalgae have a rapid growth rate with high lipid content (20–50% dw); they can double their biomass in few hours at exponential growth rates giving higher biomass productivity (Spolaore et al. 2006; Chisti 2007)	4. Negative energy balance after accounting the energy consumption for CO_2 transfer, water pumping, harvest and postprocessing (Hirano et al. 1998)
5. Improving air quality by biofixation of CO_2 (1 kg of dry algal biomass utilize about 1.83 kg of CO_2) (Chisti 2007)	5. There is a shortage of data for large-scale cultivation (Pulz 2001)
6. Wastewater can be used as nutrient source, and, therefore, microalgae have high capacity to treat different wastewater effluents (Cantrell et al. 2008)	6. Incorporating flue gases is inappropriate at high concentrations due to the presence of toxic compounds (Brown 1996).
7. Algae growth does not require any pesticides or herbicide supplement (Rodolfi et al. 2009)	
8. Many valuable by-products can be produced, and the residual biomass after lipid extraction can be used as feed or biofertilizers (Spolaore et al. 2006)	
9. The biochemical composition can be modulated by varying different factors and techniques; therefore, the oil yield may be significantly enhanced (Qin 2005)	

2009). Stephenson et al. (2010) confirmed that *C. vulgaris* has a total biodiesel areal productivity of 8.2 tons ha^{-1} y^{-1}. However, Abomohra et al. (2014) recorded about 12 tons ha^{-1} y^{-1} biodiesel productivity of *S. obliquus* cultivated outdoor in transparent plastic bags. In order to achieve an economic balance, lipid-free biomass can be converted into methane by anaerobic digestion, bioethanol and biobutanol by fermentation, and fish feed or can be re-used as nutrients for microalgae (Abomohra et al. 2018) which can recycle back phosphorus and nitrogen. In a previous study, using 0.4 g L^{-1} of residual lipid-free algal biomass enhanced the average fresh weight and survival of *Artemia* by 24% and 86%, respectively, over the control (Abomohra et al. 2014). In addition, using of 15% of lipid-free biomass mixed with 10 g L^{-1} waste glycerol enhanced the biomass and lipid productivity of *S. obliquus* by 30.5 and 59.7%, respectively (Abomohra et al. 2018). Moreover, waste glycerol

can also be recovered and used for many pharmaceutical products or fermented to produce biogas or bioethanol.

From the commercial point of view, microalgal biofuel must be competitive with fossil fuel which is, at present, low cost. The total outcome of microalgae-based biofuel production technology varies by different algal species, different cultivation systems, and different harvesting methods and algal biorefineries (Frank et al. 2013; Abomohra et al. 2016b). However, the competitiveness of biofuel with conventional fuel depends mainly on the cost of algal biomass production. In order to approach the competitiveness, the highest price of microalgal biomass should be estimated, which can be further compared with the current production price. In the case of biodiesel as example, Chisti (2008) reported that the amount of algal biomass (M, tons) equal to the energy of a crude petroleum barrel can be determined by the following equation:

$$M = E_{\text{petroleum}} / \left[q(1-w)\, E_{\text{biogas}} + yw\, E_{\text{biodiesel}} \right]$$

where $E_{\text{petroleum}}$ represents the energy contained in a barrel of crude petroleum (~6100 MJ), q represents biogas volume produced by anaerobic digestion of the residual algal biomass (~400 m^3 ton^{-1}), w represents the oil content of the biomass in percent by dry weight, E_{biogas} represents the energy content of the generated biogas (~23.4 MJ m^{-3}), y represents the yield of produced biodiesel, and $E_{\text{biodiesel}}$ represents the average energy content of the produced biodiesel (~37,800 MJ per ton). According to Chisti (2008), the maximum acceptable price of microalgal biomass should be equal to the price of a crude petroleum barrel ($P_{\text{petroleum}}$, $); thus:

$$\text{Acceptable price of biomass } (\$/\text{ton}) = P_{\text{petroleum}}/M$$

Using the previous equations, the expected acceptable price of microalgal biomass could be calculated. Satyanarayana et al. (2011) evaluated the cost of US$ 2.8 for 1 L of biofuel produced from algal biomass. In order to potentially replace petroleum, the production cost of microalgal oils needs to be related to the current price of petroleum diesel ($P_{\text{petroleum}}$, $/barrel) according to the following equation (Abomohra et al. 2016b):

$$\text{AC}_{\text{algae}}(\$ \text{ per liter of algae oil}) = P_{\text{petroleum}} \times 85.22 \times 10^{-4}$$

However, the previous equation neglects the possible additional profits from biomass residues and other products from microalgae. For example, at the cost of US$ 124 for a barrel of petroleum diesel, acceptable maximum cost of microalgal oil should be US$ 1.057 L^{-1}. At the present price of petroleum diesel (US$ 61.7 per barrel, March 2016), microalgal oil cost should not exceed US$ 0.526 L^{-1} (Abomohra et al. 2016b).

Reduction of the greenhouse effect through CO_2 sequestration and abundant oxygen production are the most important positive environmental impacts of microalgae cultivation. In addition, various other micropollutants such as

pharmaceutical contaminants lead to environmental pollution which results in corruption of human resources (Xiong et al. 2017). Water and land use are also two important environmental factors that need to be considered. Interestingly, microalgae can be grown in arid lands where no other crops can survive. In addition, microalgae need relatively lower amount of water as water can be recycled after harvest. Consequently, large-scale cultivation of microalgae will not only have economic benefits through biofuel and valuable compounds production but also will help to save our planet.

8 Summary

Commercial cultivation of autotrophic microalgae for food production dates back to the 1950s. At present, autotrophic microalgae were discussed as a source for liquid biofuels such as biodiesel, biobutanol, and bioethanol. Biodiesel is produced from microalgal lipids, while biobutanol and bioethanol are produced from carbohydrates. As yet, there is no commercial production of such biofuels due to the high production costs and technical issues concerning post-cultivation processing. Recently, advances in biorefinery open many chances to develop integrated and sustainable productions of varied liquid fuels from microalgal biomass in a cost-effective approach in the next future decades. It is expected that the prospects for liquid biofuel production from autotrophic microalgae will much improve in the near future, especially using genetically modified microalgae.

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Chapter 14
Progress and Challenges in Biodiesel Production from Microalgae Feedstock

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Abstract Increasing energy demand and limited fossil fuel sources have developed the interest of researchers toward biofuel, as it is regarded as the promising approach for continuous source of energy. Microalgae are considered as a desirable feedstock for biodiesel production due to its inherent capacity to synthesize large amount of oil. The key steps in microalgae biofuel synthesis are cell culture, cell recovery, lipid removal, and fatty acid methyl ester (FAME) production. The high cost of biodiesel production is the major bottleneck in the microalga biofuel technology. Among the four steps, harvesting and lipid extraction count more than 50% of the total cost of biodiesel production. Recently, nanoparticle engineering-based methods have been applied as a powerful tool in algae system to overcome the technical problems. Another problem is the mass cultivation of microalgae, which carries major importance because massive biomass is required for viable production of biodiesel. Closed cultivation system (photobioreactor) and open cultivation system (open raceway ponds) are emerged as a solution for mass cultivation of microalgae, but there is a need to understand the design and principle of cultivation system. In this chapter, a pragmatic and critical discussion is tried to put forward with the ongoing research on microalgae with future trends.

1 Introduction

At present, microalgal biofuel has been regarded as one of the most promising and sustainable energy resource to meet current global energy crises (Chisti 2007). Microalgae are unicellular, oxygenic, and photosynthetic microbes, able to convert atmospheric CO_2 into biomass (Mandal and Mallick 2009). Apart from this, microalgae have immense potential to depollute the water by consuming urea, released by the animals, and at the same time, the rate of CO_2 conversion into biomass has been increased. Hence, the algal biomass can easily be converted into

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various kinds of biofuels by utilizing some thermochemical techniques like liquefaction, pyrolysis, gasification, extraction, transesterification, etc. (Mata et al. 2010).

Microalgae are photosynthetic microorganism, and its simple cultivation process requires light, sugar CO_2, N, P, and K, and they can produce some valuable metabolites like carbohydrates, proteins, and lipids in a large quantity even in smaller time period. These bioproducts can be treated for biofuels as well as useful chemicals. Generally, the microalgal strains have extensively been utilized for low-cost biodiesel production with the help of efficient cultivation conditions.

Microalgal growth has been undertaken by varieties of cultivation strategies included as photoautotrophic, heterotrophic, and photoheterotrophic mode. In the current scenario, among all these cultivation conditions, researchers have strongly focused on heterotrophic and photoheterotrophic culture conditions that trigger the accumulation of biomass and lipid productivities (Kanaga et al. 2015). However, the major bottleneck of using these cultivation strategies is expensive organic carbon sources' supplementation (glucose, fructose, sucrose, etc.) to the culture medium (Rai et al. 2013). Therefore, recently, the studies are centered for the supplementation of organic carbon-rich waste resources toward inexpensive biodiesel production.

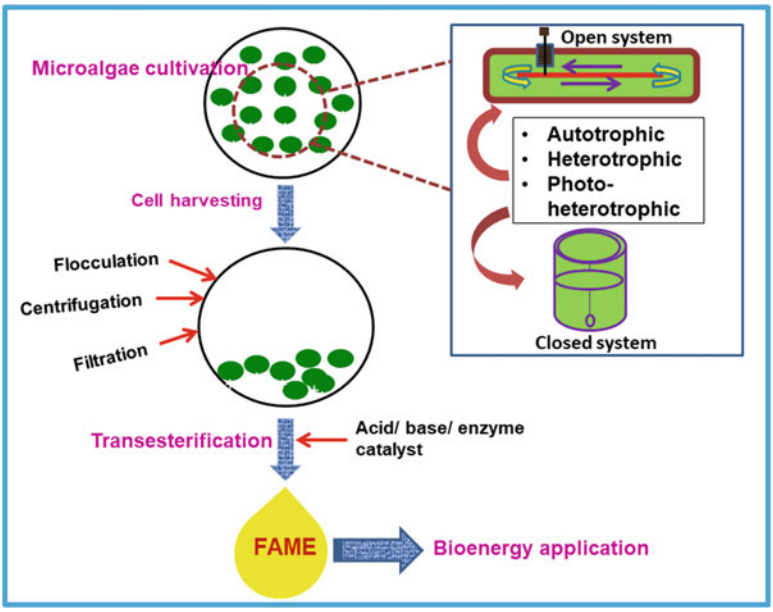
In the microalgae-based biofuel generation, cell lysis is another key step that helps in lipid extraction. Algal cell wall generally consists of multilayered rigid cell wall, which confines the complete extraction of intracellular compounds (Surendhiran and Vijay 2014; Zheng et al. 2011; Rodrigues and da Silva Bon 2011). Therefore, the cell wall needs to be disrupted by using pretreatment methods with the help of organic solvents or other physical methods. The microalgal oil or lipid is converted into fatty acid methyl esters by transesterification that is a thermochemical process to produce the raw material of biodiesel, implementing acid, base, or enzyme catalysts. The overall scheme of microalgae-based biodiesel production has been entailed below (Scheme 14.1).

2 Microalgae and Culture Techniques

Microalgae cultivation system depends on the capacity for oil production by varying different culture modes. The characteristics of cell proliferation and biochemical characteristics of microalgae are recognized to depend on such cultivation conditions. The four types of culture modes are described in the following sections:

2.1 Photoautotrophic Cultivation

The cultivation mode which represents the utilization of solar radiation as energy source and inorganic carbon (CO_2) as the carbon source to produce chemical energy through photosynthesis is known as photoautotrophic cultivation system. This is one



Scheme 14.1 Stepwise process of biodiesel (FAME) production from microalgae

of the most commonly used cultivation condition for microalgal growth. The lipid production efficiency of microalgae exists in the range of 5–68% under photoautotrophic mode (Chen et al. 2011).

The major advantages of photoautotrophic cultivation are given below:

- Consumption of CO_2 as carbon source for biomass as well as lipid production.
- Contamination problem is reduced by using this culture mode, in contrast with others; hence, outdoor mass cultivation techniques are commonly functioned by adopting photoautotrophic mode.
- Microalgae consume the solar energy and atmospheric CO_2 to produce valuable metabolites and hence lead to reduce global CO_2 level.

Apart from the advantages, this culture condition also demonstrates some disadvantages from the aspect of higher biomass production. For example:

- Less light penetration to the cell.
- Mutual shading of the cells, causing light inadequacy.
- Less biomass production enhancing biomass harvesting cost.

2.2 Heterotrophic Cultivation

The culture condition where microalgal strains require the utilization of organic carbon supplementation as energy source is referred as heterotrophic culture

technique (Chojnacka and Marquez-Rocha 2004). This culture condition could escape the complications concomitant with restricted light that hinder the biomass volume in large-scale photobioreactors during photoautotrophic cultivation (Huang et al. 2010). Microalgae lipid enhancement was reported under heterotrophic metabolism, as about 40% upsurge was achieved in *Chlorella protothecoides* by altering the culture mode from photoautotrophic toward heterotrophic (Xu et al. 2006)**.** Microalgae have the enormous capability of accumulating a number of different organic carbons like glucose, acetate, glycerol, fructose, sucrose, etc. for their proliferation (Liang et al. 2009). Literatures depicted that the inexpensive organic carbon substrates like corn powder hydrolysate were used by replacing technical grade glucose, attributing to the increase in biomass and lipid productivity of 2 g/L/d and 932 mg/L/d, respectively (Xu et al. 2006). The maximum lipid yield (3700 mg/L/d) was also described with (Xiong et al. 2008) a 5 L fermenter worked by an upgraded fed-batch cultivation condition. Heterotrophic growth provides higher cellular lipid biosynthesis, as compared to photoautotrophic cultivation. However, the heterotrophic arrangement often contains some difficulties associated with contamination.

2.3 Mixotrophic Cultivation

Mixotrophic culture technique is a way of microalgae growth where cells are able to utilize both organic and inorganic carbons via photosynthesis. The CO_2, expelled through cell respiration will be confined and recycled under photoautotrophic system (Mata et al. 2010). In contrast with photoautotrophic and heterotrophic condition, mixotrophic cultivation is recently utilized as one of the most suitable modes for microalgal oil production.

2.4 Photoheterotrophic Cultivation

Photoheterotrophic cultivation is regarded as the utilization of light as well as organic carbon. The main dissimilarity between mixotrophic and photoheterotrophic metabolism is that the second involves light radiation as the energy source, whereas mixotrophic culture can utilize organic complexes to assist this persistence (Chojnacka and Marquez-Rocha 2004). Moreover, along with the lipid productivity, some of the light-mediated valuable products can be boosted in photoheterotrophic cultivation (Ogbonna and Tanaka 1998). Hence, it is the most acceptable cultivation technique in the present scenario.

3 Cultivation System

Microalgae cultures are usually classified into open cultivation systems and closed cultivation systems using different photobioreactors. Among them, open or raceway pond was attempted firstly to scale up for microalgae cultivation.

3.1 Open System

Open system like raceway pond contains some of the major advantages as well as disadvantages also. The advantages are listed below:

- Nominal capital and operational costs.
- Less energy requisite for culture mixing.
- Open systems generally involve huge areas for scale-up.

  The major disadvantage of raceway pond includes:
- Prone to contamination problem with other microalgal strains, even with bacterial loads and grazers.
- Furthermore, it is a challenging issue to get rid of growth factors including evaporation, culture temperature, etc. (Oyler 2009; Mata et al. 2010).

3.2 Closed Cultivation Systems

Closed cultivation system is mainly regarded as photobioreactors (PBRs) which are well-organized and more proficient in contrast with open system. The operating performance and quality has been maintained properly by using advanced controlled conditions. The construction of PBRs can be performed in accordance with the strain of choice, and further, it can be optimized as well as validated also.

A number of advantages remain in PBR system like:

- It employs comparatively tiny space while enhancing the light convenience.
- Efficiently reducing the risk of contamination.

Despite of the advantages, PBRs also contain some bottlenecks, such as:

- Excess heating.
- Benthic algal proliferation.
- Sterilized condition.
- Development of dissolved oxygen ensuing for growth reduction.
- Maximum capital costs involving for constructing and functioning (Chisti 2007) (Table 14.1).

Table 14.1 Biomass productivities of various microalgal strains based on different photobioreactors

Photobioreactor	Volume (L)	Strain	Culture condition	Biomass yield (g/L/d)	References
Bubble column	**0.4**	*Tetraselmis* sp.	Photoautotrophic	8.6	Kim et al. (2017)
Flat plane airlift	2	*Chlorella vulgaris*	Photoautotrophic	0.086	Benavente-Valdés et al. (2017)
Stirred tank	**2**	*Chlorella vulgaris*	Photoautotrophic	0.181	Benavente-Valdés et al. (2017)
Vertical tubular	**2**	*Chlorella kessleri*	Photoautotrophic	0.087	De-Moris and Costa (2007)
Bubble column	**0.5**	*Chlorella vulgaris*	Photoautotrophic	0.004	Shin et al. (2018)

3.3 Operating Considerations for Bioreactors Based on Modifying Batch and Continuous Process

(a) *Shear stress*

Shear stress can be defined as the constituent of stress, which is coplanar with a material cross-sectional area. It can be denoted as τ and co-related with the following formula:

$$\tau = F/A \tag{14.1}$$

wherein F is represented as applied force and A is material cross-sectional area.

Generally, shear stress occurs from the vector component which remains parallel to the cross-sectional area of material.

(b) *Chemostat with recycle*

Cell recycle is able to increase the conversion rate (productivity) and enhances the constancy of system by lessening process perturbation effect. A material balance on cell biomass concentration around the fermenter yields the following equation:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = FX_0 + \alpha\, FCX_1 - (1+\alpha)\, FX_1 + V\mu_{\text{net}}X_1/V \tag{14.2}$$

where α represents the recycle ratio based on volumetric flow rates, C denotes the concentration factor, F implies nutrient flow rate, V exhibits culture volume, and X_0 and X_1 are cell concentration in feed and recycle streams (Figs. 14.1 and 14.2).

(c) *Fed-batch operation*

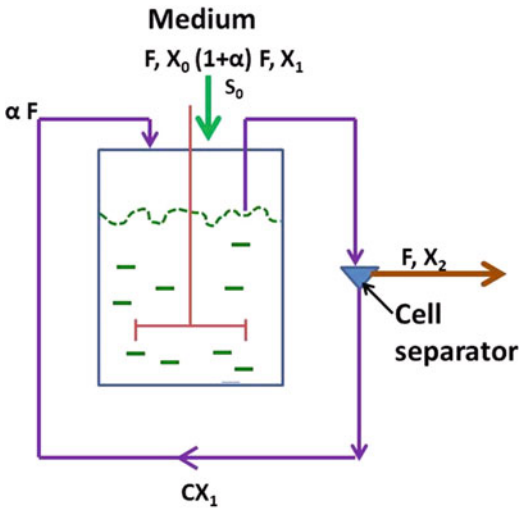


Fig. 14.1 Chemostat with cell recycle via providing cell separator

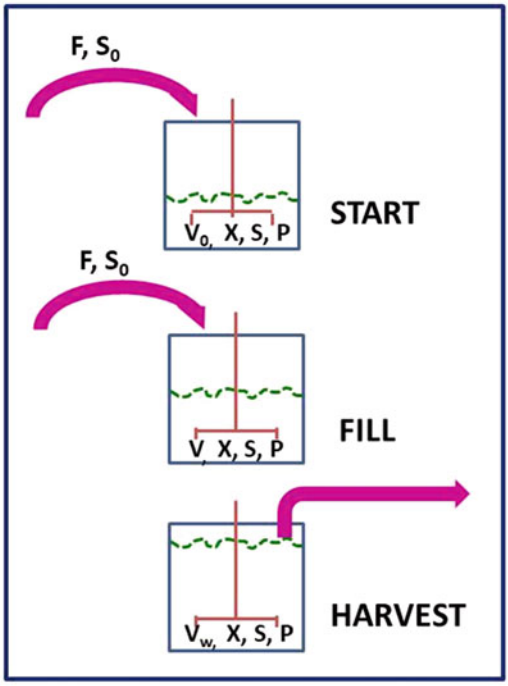


Fig. 14.2 Schematic diagram of fed-batch culture

In fed-batch mode, the substrates are intermittently supplied, and product is removed at the end of the process. The rate of change of biomass concentration is determined below:

Table 14.2 Biomass and lipid productivities of microalgal strains based on culture mode

Algal strains	Culture mode	Biomass productivity (g/L/d)	Lipid productivity (g/L/d)	References
Chlorella vulgaris	Batch	0.2341	0.074	Deng et al. (2018)
Chlorella zofingiensis	Batch	0.1714	0.036	Mao et al. (2018)
Chlorococcum sp.	Semicontinuous	0.1066	0.0238	Zhou et al. (2013)
Cholestrella sp.	Batch	0.0316	0.0075	Karpagam et al. (2015)
Nannochloropsisoculata	Batch	0.1357	0.0171	Polishchuk et al. (2015)
Nannochloropsis gaditana	Continuous	0.49	0.051	San Pedro et al. (2013)

$$\frac{\mathrm{d}x}{\mathrm{d}t} = V\left(\mathrm{d}x^{\mathrm{t}}/\mathrm{d}t\right) - X^{\mathrm{t}}(\mathrm{d}v/\mathrm{d}t)/V^2 \tag{14.3}$$

The following table describes the different culture modes of microalgal strains toward biomass and lipid yield (Table 14.2).

4 Biomass Harvesting

Algae harvesting comprises detachment of algae from the media supporting the growth of algae and in other words thickening the biomass of algae (Cooney et al. 2009; Alam et al. 2016). The principle of harvesting technology depends on the characteristics of algae and the state of constancy in which they thrive (Chen et al. 2011). Commonly for engendering the algal biomass, suitable cultivating environment is required that helps in the process of retrieval of microalgal biomass (Chen et al. 2014). Recovery of biomass from the media is the major bottleneck in the microalgal-mediated bioproducts because of its smaller size 5–50 μm along with the negative charge on the surface of microalgae and the density of media for the growth of algae is similar to that of algal cell density (Garzon-Sanabria et al. 2012; Milledge and Heaven 2013). Researcher has investigated that 30% of the total cost is involved in harvesting of algal biomass, hence it is the most perplexing issue for microalgal biorefinement process (Georgiana and mayfield 2012; Mata et al., 2010). Harvesting process for algae biomass plays a significant role, and an efficient, appropriate, and cost-effective technique is yet to develop.

Table 14.3 Different conventional harvesting techniques

Harvesting techniques	Advantages	Disadvantages	References
Centrifugation	Quick process	Expensive technique	Heasman et al. (2000)
	Efficiencies of recovery is generally high	High capital cost	
		Generation of shear force results in cell damage	
Filtration	Biomass recovery is high	Suitable for larger cells	Christenson and Sims (2011); Show and Lee 2014)
		Clogging is an issue	
	Variety of filters are available	Costly technique	
		Membrane replacement and cleaning are required	
Sedimentation	Simple and low-cost method	Separation is slow in specific microalgal cells but mostly suited to non-motile cells	Gupta et al. (2017), Wiley et al. (2011)
Flotation	Cost-effective method	Not suitable for marine microalgae	Barros et al. (2015)
	Suitable for large-scale process		

4.1 Conventional Techniques for Algal Biomass Harvesting

Centrifugation, filtration, flotation, and flocculation are the various harvesting techniques for algal biomass. All these cell harvesting methods have been documented in tabular form (Table 14.3).

4.1.1 Centrifugation

It is the fastest harvesting process but also considered as the most expensive technique, as high operational cost is required, which confines its application to high-value products, such as highly unsaturated fatty acids, pharmaceuticals, and other commodities (Rawat et al. 2011; Christenson and Sims 2011; Zhou et al. 2013). This process involves generation of a centrifugal force which increases the separation of particle (Hitttab et al. 2015). Centrifugation can be used to recover microalgae from the liquid broth, the test was conducted in laboratory on pond effluent at 500–1000 × g, and the result showed 80–90% recovery of microalgal cells in 2–5 mins (wenku.baidu.com). Recovery from centrifugation is rapid but also energy demanding. However, for the recovery of microalgal cells, centrifugation is the most desired method, specifically for producing prolonged shelf-life concentrates for aquaculture. In a sedimenting centrifuge, the biomass recovery depends on few characteristics like settling time of the cell, settling depth, and the residence time cell in centrifuge (Molina Grima et al. 2003; Show and Lee 2014).

4.1.2 Filtration

Filtration technique is used for separation of algae from culture medium where filter membrane permits water through it and allows cells to deposit over the membrane (Bhatt et al. 2014; Zhou et al. 2013). The method takes place constantly till filters have a dense biomass of algae. Various forms of filtration are membrane filtration, microfiltration, vacuum filtration, dead-end filtration, and tangential flow filtration (TFF). For the large-scale process, the membrane filtration is not frequently applied (Harun et al. 2010). Micro- or ultrafiltration membrane is generally expensive and requires energy along with regular membrane replacements (Rawat et al. 2011). Microfiltration having pore size from 0.1 to10 mm is appropriate for harvesting fragile and small algal cells, whereas large algal cells can be successfully collected by vacuum filtration when used with filter aid (Uduman et al. 2010; Rawat et al. 2011); hence, both are expensive as driving of biomass requirement marks them as energy intensive (Pragya et al. 2013). Another process for efficient retrieval of cells is dead-end and tangential flow filtration (TFF) method. The larger microalgal cells having the diameter of over 70 mm are suitable for dead-end filtration, while for the recovery of small algae cells, TFF is much suitable because of slight clogging issue (Danquah et al. 2009; Christenson and Sims 2011).

4.1.3 Gravity Sedimentation

Gravity sedimentation technique is used for concentrating the algal biomass and treatment of wastewater (Wiley et al. 2011). The disadvantage of this method includes poor compaction and slow settling velocities as this process yields a wet and voluminous biomass. Settling rates of 0.1–2.6 cm cause low settlement that results to the destruction of the algal biomass throughout the process, Hence it limits successive harvesting by this process (Christenson and Sims 2011; Chen et al. 2011). To fasten the microalgal settling rate, some prior steps can be applied to gravity sedimentation like a flocculation (Chen et al. 2011). Cells having high concentration and larger dimensions can effectively be detached by this technique like *Spirulina*. Induced sedimentation velocity is one of the factors for better sedimentation rate (Munoz and Guieysse 2006; Shelef et al. 1984).

4.1.4 Flotation

Floatation is considered as the much efficient and promising than any other method. The principle is based on separating the microalgal cells through gravity, where collision take place between particle and bubble (Shelef et al. 1984; Pragya et al. 2013). In case of flotation, the algae moves upward, whereas in sedimentation, it moves downward; hence this favors floatation, as high flow rate is required in mass cultivation (Rawat et al. 2011), and along with it, the microalgal cells having the radius of 1000 mm can also be caught in this process (Pragya et al. 2013).

Currently, three main flotation techniques are known: (1) dissolved air flotation, (2) electrolytic flotation, and (3) ozonation-dispersed flotation (ODF) (Chen et al. 2011). At a high pressure, the air is dissolved in water, which makes the solution supersaturated, resulting in bubble nucleation. It is essential to upsurge the size of the algal cells, so that flocculants can easily bind to cells and settle down, and this increases the effectiveness of this method (Milledge and Heaven 2013).

The principle of the electroflotation is based on the development of hydrogen by bubbles via electrolysis. The process involves the interaction of negatively charged microalgal cells to hydrogen bubbles (Barros et al. 2015). Advantage of this method includes ecological affinity, protection, and adaptability. Requirement of high power, because of the fouling of cathodes, leads to the disadvantage of this method (Chen et al. 2011).

Ozonation-dispersed flotation is an alternative technique of generating bubbles which are charged. *Chlorella vulgaris* was harvested by using this method; the results indicated an elevation in lipid/FAME profile from 31% to 55%. This process also helps in the formation of biopolymers via breaking of cells hence increases the technique efficacy through providing better separation. In open ponds, the contamination is the biggest disadvantage of ozonation-dispersed flotation (Barros et al. 2015; Rawat et al. 2011).

4.2 Novel Nanomaterial for Cell Harvesting

There is lack of suitable technique for microalgae harvesting, and a cost-effective method needs to be developed (Lee et al. 2015). Lately, integrated approaches are suggested to overcome the limitations of biodiesel production from microalgae. Instantaneous harvesting and cell disruption of microalgal cells using nanoparticles (NPs) have been stated as the economical process for harvesting (Lee et al. 2014a). Nanoparticle engineering is a process of producing artificial nanoparticle on the scale of nanometer. Table 14.4 has been demonstrated for nanomaterial-based cell harvesting.

4.2.1 Magnetic Nanoparticle

Magnetic separation is recognized as the magnetic nanoparticle-based separation technique that has the benefit of mild processing and fast and ease of scale-up (Borlido et al. 2013). For example, Fe_3O_4, a metal-oxide nanoparticle, surrounded by hydroxyl ions, has an opposite surface charge. The surface ionic interaction occurs by gaining or losing protons that develop negative charge above the isoelectric point and vice versa. The mechanism is based on the electrostatic attraction between negative zeta potential of microalgae magnetic separation which takes place in microalgae.

Table 14.4 Nanomaterial-mediated cell harvesting

Microalgae	Nanomaterial used	Nanoparticle type	Harvesting efficiency (%)	References
Botryococcus braunii and *Chlorella ellipsoidea*	Fe_3O_4	Magnetic	95	Hu et al. (2013), Xu et al. (2011)
Chlorella sp.	Cationic metal aminoclays (Al3+, Ca2+, Mg2+)	Aminoclays	97	Lee et al. (2013)
Chlorella sp.	Fe_3O_4-covered (PDDA)	Hybrid	99	Lim et al. (2012)
Nannochloropsis maritima	Fe_3O_4	Magnetic	95	Hu et al. (2013), Xu et al. (2011)
Chlorella sp.	Aminoclay-coated nZVI	Hybrid	100	Lee et al. (2014)
Chlorella sp.	Fe_3O_4	Magnetic	95	Toh et al. (2014)
Nannochloropsis sp.	Nanochitosan	Chitosan	97	Farid et al. (2013)
Chlorella KR-1	Fe_3O_4	Magnetic	>95	Lee et al. (2014b)
Chlorella sp.	Aminoclay-coated TiO_2 composite	Hybrid	85	Lee et al. (2014b)

Microalgae *Botryococcus braunii*, *Chlorella ellipsoidea*, and *Nannochloropsis maritime* are harvested by nano-sized (Fe_3O_4) magnetic nanoparticle. Nine-five percent of harvesting efficiency was achieved in 5 min (Hu et al. 2013; Xu et al. 2011). Single Fe_3O_4 nanoparticle was used for the of *Chlorella* sp., with cell density of 0.3×10^{7} cells/ml; results reported the harvesting efficiency of 95% was achieved (Toh et al. 2014). When the same nanoparticle was used for *Chlorella* KR-1, the harvesting efficiency more than 95% was reported in less than 1 min with pH of 9.0 (Lee et al. 2014b).

Generally, detachment of microalgae through magnetic nanoparticle marks the cell surface structure attachment to nanoparticle. Magnetophoretic harvesting technology offers an economical and reliable means of recycling magnetic flocculants (Lee et al. 2015). Few efforts are made to recover magnetic nanoparticle (Xu et al. 2011). Prochazkova et al. (2013) documented 100% retrieval of de-attachment of magnetic NPs from microalgae cells using 10% H_2SO_4 (% vol) with ultrasound and heating at 40 °C conditions.

4.2.2 Chitosan and Aminoclay Nanoparticles

Chitosan is cationic in nature and used as a bioflocculant in microalgae harvesting process.

It works on the principle of neutralization of charge and bridging mechanism (Beach et al. 2012). The method called ionic gelation is used, resulting in the formation of chitosan nanopolymer, which is used for the harvesting of marine microalgae *Nannochloropsis* sp. (Farid et al. 2013). The results showed the 9% increase in biomass when nanochitosan is used instead of bulk chitosan, and dosage was also reduced to 40%.

Due to its high cost, the use of chitosan is restricted for microalgae harvesting system. For harvesting of oleaginous *Chlorella* sp., Al3+, Ca2+, and Mg2+, which are the various cationic metal aminoclays along with some materials such as AI-APTES and Mg-APTES 3-[2-(2-aminoethylamino)ethylamino] propyltrimethoxysilane are used (Lee et al. 2013). The results indicated the harvesting efficiencies of 97% within 30 min, however retaining a pH of 7 in the solution. The aggregation and precipitation of negatively charged microalgal cells with nanoparticle are easy and effective processes (Lee et al. 2015).

4.2.3 Hybrid Nanoparticles

Hybrid nanoparticle is reported as the high efficiency tool regardless of ecological changes such as variety of species, pH of solution, media, hybrization of nanoparticle is reported for example the hybridization of metal oxide nanoparticle bearing some functional coating (Lee et al. 2015).

For magnetophoretic separation of *Chlorella* sp., the rod-shaped nanoparticle and Fe_3O_4 spherical nanoparticle covered with poly(diallyldimethylammonium chloride) (PDDA) were used. Ninety-nine percent of harvesting efficacy was achieved when rod-shaped nanoparticle was used. On the other hand, when using the spherical ones, 80% of harvesting efficiency was achieved. The reason may be in spherical shape nanoparticle and the formation of constant magnetic moment from shape which tends to form large aggregates (Lim et al. 2012). The recycling viability of aminoclay was stated, using combination with magnetic particles (Lee et al. 2014). The highly positive charge surface and the small aggregation size of 100 nm were achieved when aminoclay-coated nZVI composite was used in the ratio of 1.0 aminoclay/nZVI. Using the nanoparticle composite (>20 g/L), 100% of the *Chlorella* sp. was harvested within 3 min. Harvesting efficiency of 85% was achieved for *Chlorella* sp. within 30 min, having the pH of 6.5, when aminoclay coated with TiO_2 composite was used (Lee et al. 2014b).

5 Lipid Extraction Techniques

The extraction of lipid from microalgae is classified as mechanical and chemical methods (Halim et al. 2012). Oil expeller, microwave-assisted extraction, and ultrasonic-assisted extraction are categorized as the mechanical methods, whereas

the chemical methods include Soxhlet extraction, supercritical fluid extraction, and accelerated solvent extraction (Halim et al. 2011; Khoo et al. 2011; Balasubramanian et al. 2011).

The chemical process requires organic solvents like n-hexane, ethanol, etc. These chemicals are being toxic and inflammable, affecting the human health and environment. Using solvents such as chloroform, methanol, and n-hexane, the effectiveness of oil extraction can be enhanced.

For the extraction of lipid, both the solvents having polar and nonpolar properties can be used, and its selection is based on surface charge of the target species. In addition, better yield can be obtained if polar component will be extracted using polar solvents (Balasubramanian et al. 2011).

5.1 Dry Biomass

5.1.1 Expeller or Press

This technique of mechanical press is based on breaking of cells, and the technique allows to extract about 75% of oil by compressing the oil from the dry biomass (Harun et al. 2010). Topare et al. 2011, reported 75% of lipid from filamentous algae by a screw expeller press, whereas some amount of lipid existing in cake form was removed using solvent extraction technique. Slow and the requirements of large quantity of biomass are the disadvantage of this method (Harun et al. 2010).

5.1.2 Ultrasound-Assisted Extraction of Oil

The methods like solvent extraction and mechanical press result in the less lipid extraction because of the thick cell walls of microalgae as it blocks the release of intralipid (Neto et al. 2013). The ultrasound-assisted extraction method is based on the extreme sonication in liquid which results in the formation of sound waves which travel in liquid media and causes the high-pressure and low-pressure cycles. In the low-pressure cycle, the small vacuum bubbles are produced during the high-pressure cycle, the bubbles collapse violently, and the phenomenon called cavitation is caused (Mubarak et al. 2015).

During high-pressure cycle, the small vacuum bubbles, which are produced in the low-pressure cycle, collapse violently and result in a phenomenon called cavitation. During cavitation, the shearing forces are formed around the algal cells by high-pressure and high-speed liquid, result in breaking the structure of the cell mechanically, and develop better material transfer process by supporting the extraction of lipids (Suali and Sarbatly 2012).

5.1.3 Microwave-Assisted Extraction of Oil

In microwave-assisted extraction, electromagnetic radiation of frequency in the range of 0.3–300 GHz is applied. A noncontact heat source is applied for microwave-assisted method; the heat source can enter into biomaterials and results in the interaction with polar molecules such as water in the biomass and causes the heating in the sample (Mubarak et al. 2015).

Iqbal and Theegala (2013) compare, the potential of solvent, biodiesel (methyl soyate) containing 20% (BD20) and 40% (BD40) biodiesel combined with ethanol at three various temperatures of 80 °C, 100 °C, and 120 °C for lipid extraction using microwave-assisted method. The yield of MAE using chloroform methanol was compared with Soxhlet extraction method. The extraction of lipid with BD40 showed better yield instead of other solvent. Seventy-seven percent of the overall lipid content was obtained at 95 °C in 30 min, with hexane along with processing system of microwave having continuous resonant which was used for *Scenedesmus obliquus*. The main advantages of microwave-assisted extraction (MAE) majorly include superior quality of oil with reduction in extraction time.

5.1.4 Chemical Methods for Extraction of Oil

The chemistry theory of "like dissolves like" is the principle for extraction of lipid using solvents from microalgae. An idyllic solvent demands for high levels of specificity for lipids particularly acylglycerols, and to confirm low-energy distillation, the solvent must be volatile enough to distinguish the lipid from solvents. n-Hexane, DBU (1,8-diazabicyclo-[5.4.0]-undec-7-ene), n-hexane/isopropanol, n-hexane/2-propanol, DBU/ethanol, DBU/octanol, acetone/dichloromethane, and methylene chloride/methanol are the solvents used for extraction of lipid. The most widely used organic solvent among these is chloroform/methanol (1/2 v/v) for extraction of lipid from microalgae because of the better yield and less time required for extraction of lipid (Mubarak et al. 2015; Neto et al. 2013).

A mixture of chloroform/methanol in the ratio of 2:1 was used for extraction of lipid from animal tissue and named it as Folch method (Wang and Wang 2011) (Folch and Stanley 1956). Another method is the Bligh and Dyer widely used for extraction of total lipid, and purification is done with solvents as methanol and chloroform and water which act as cosolvent. The main drawback of solvent extraction includes toxicity of chemicals and volatile nature (Aresta et al. 2005).

5.2 Wet Biomass

Ninety percent of the total cost is involved in the dry-based extraction of lipid from microalgae for biodiesel production, and also the process is energy extensive.

Authors have stated extraction of oil from wet microalgae which results of 25% reduction in the energy can be achieved by using wet biomass of algae; hence this process does not require drying (Lardon et al. 2009).

Liquid dimethyl ether (DME) is used as solvent for extraction of lipids along with hydrocarbons from wet *B. braunii*, and results showed the equivalent yield as Soxhlet extraction using solvent as hexane for dried biomass of algae (Kanda et al. 2012). They discussed that extraction process using DME provides better cell disruption and saves energy.

Yoo et al. (2012) investigated the novel method of lipid extraction from wet *Chlamydomonas reinhardtii* with osmotic treatment. The results show the twofold increase in the lipid recovery.

Sathish and Sims (2012) reported a lipid extraction method for marine wet algae *Nannochloropsis* sp. with 90% water; the process is described as single-step supercritical method for instantaneous extraction. Using acid and base hydrolyses of wet mixed cultures of *Chlorella* and *Scenedesmus* sp. containing 84% moisture, 79% of lipid was extracted.

5.3 Novel Approaches for Lipid Extraction

With the progression of study, nanoparticle engineering has occurred as a new arena, and it has the potential to overcome many challenges in a microalgae biodiesel process. Nanomaterial has proven its potential for increasing the yield of biodiesel.

For increasing the lipid content of *Chlorella vulgaris*, the microalgae was cultivated in magnesium aminoclay; it is water soluble and cationic charged (Farooq et al. 2016). The results documented that the cell size seems to be increased from 3.524 μm to 4.175 μm and FAME (fatty acid methyl ester) content was also improved by 266, 289, and 275 mg/g at different concentrations of magnesium aminoclay 0.02, 0.05, and1.0 g/l. In addition, when reused water of magnesium aminoclay was used, *Chlorella vulgaris* was seen to be grown proficiently.

MgAC nanoparticle was used for increasing the lipid content of *Chlorella* sp. KR-1 and also to reduce the bacterial population (Kim et al. 2016). The result indicated that at 0.01–0.1 g/l concentration of MgAC, the lipid productivity was increased to 410 mg FAME/l//d. It was seen that the increased FAME content was 25% higher than the control which is 327 mg FAME.

Cellulose nanofibrils have emerged as the cost-effective and approachable nanomaterial for increasing the lipid content in microalgal cells. CNF delivers the machine-driven stress to microalgal cells causing decrease in cell size and development, hence increasing the lipid synthesis (Yu et al. 2016).

6 Transesterification

6.1 Homogeneous Base-Catalyzed Transesterification

Presently, for the production of biodiesel, homogeneous based catalysts are more desirable, such as hydroxides of potassium or sodium (KOH, NaOH), potassium methoxide ($KOCH_3$), sodium methoxide ($NaOCH_3$), and sodium ethoxide ($NaOCH_2CH_3$) (Lam et al. 2010; Narasimharao et al. 2007; Atadashi et al. 2013). Generally, for comparing the biodiesel yield, sodium methoxide ($NaOCH_3$) and potassium methoxide ($KOCH_3$) are effective catalyst instead of sodium hydroxide (NaOH) and potassium hydroxide (KOH) because of their capability to break into CH_3O and Na + and CH_3O and K+, respectively (Atadashi et al. 2013). In industries, these catalyst are commonly used due to various reasons (Lotero et al. 2005):

I. Capable of catalyzing the reaction under low atmospheric pressure and low reaction temperature.
II. Less time is required for achieving the high conversion.
III. Commonly available and cost-effective.

It is investigated that base catalyst can endure large amount of free fatty acid (FFA). Though, the reactions for base catalyzed the amount of free fatty acid should be least as possible (Lam et al. 2010).

6.2 Homogeneous Acid-Catalyzed Transesterification

Mostly, the common catalysts used for homogeneous reaction are H_2SO_4 and (HCl) and sulfonic acid (Atadashi et al. 2013; Lam et al. 2010). Acid-catalyzed transesterification is unresponsive to the occurrence of free fatty acid, which marks the advantage for the acid-based transesterification (Kulkarni and Dalai 2006). Sagiroglu et al. (2011) documented, when HCL was used for acid-catalyzed transesterification of sunflower oil, along with 100 °C reaction temperature and 1.85 wt% catalyst concentration, 95.2% of biodiesel yield was achieved from *Chlorella pyrenoidosa*, when Another catalyst H_2SO_4 with 0.5 wt% catalyst loading was used (Cao et al. 2013). For commercial-based application, acid catalysts are not a standard choice, because they offer slow reaction rate and their molar ratio of alcohol to oil is high.

6.3 Heterogeneous Base-Catalyzed Transesterification

Basic zeolites, alkaline earth metal oxides, and hydrotalcites are the solid base catalysts that have been developed for biodiesel production. On the other hand,

CaO, an alkaline earth metal oxide, has gained attention due to its high strength, higher activity, and moderate reaction condition required that can be produced from low-cost source like CaO and $CaCO_3$ (Zabeti et al. 2009).

Heterogeneous catalysts are generally more vigorous than heterogeneous catalyst (Arzamendi et al. 2007; Kouzu et al. 2008). The reaction rate of biodiesel production can be slow down by using CaO as catalyst (Chew and Bhatia 2008), although when CaO is used with glycerol, calcium diglyceroxide is removed during the process (Kouzu et al. 2008, 2009).

6.4 Heterogeneous Acid-Catalyzed Transesterification

The main characteristics require for solid acid catalyst reaction are mainly the amount of acid catalyst used and hydrophobic surface of species (Kulkarni and Dalai 2006).

Presently, the research for biodiesel is more focused on novel and sustainable solid acid catalysts for transesterification reaction (Jacobson et al. 2008). For the production of biodiesel, some solid catalysts have been discovered including zirconium oxide (ZrO_2), titanium oxide (TiO_2), tin oxide (SnO_2), zeolites, etc. Jacobson et al. (2008) reported 65% low ester yield was achieved, at lower temperature of 200 °C, with short span of reaction time of 10 h, and the catalyst was produced by infusing 10 wt. % of WO_3 onto ZrO_2-Al2O_3. The catalytic activity of $SO_4$2−/TiO_2 and SO_4 2−/ZrO_2 was assessed for transesterification of cotton seed oil. The results indicated that the catalyst activity was directly proportional to its definite surface area; a higher yield of 90% was achieved for $SO_4$2−/TiO_2 having the surface area of 99.5 m^2/g, whereas 85% of yield was achieved with a specific surface area of 91.5 m^2 /g in case of SO_4^{2}−/ZrO_2.

7 Conclusion and Future Prospect

Production of biodiesel from microalgae is a cost-effective process in comparison with conventional vegetable oil and animal fat transesterification. The increasing demand of fossil fuel can be replaced by algal biodiesel production by using the large-scale photobioreactors to provide better and large quantity of algal biomass. The complete steps of microalgae biodiesel, including culture techniques, cultivating system, biomass harvesting, lipid extraction techniques, and transesterification, are thoroughly discussed in this chapter. To scale up the biomass harvesting and lipid extraction method, in the biodiesel production process, various nanoparticle and advanced material-based methods have been investigated. As a result, increment in cell size, pigment, lipid, and processing time for cell separation was seen. Hence, recent advancement in nanoparticle engineering, development like metal oxides, magnetic, chitosan and aminoclay, hybrid nanoparticle were also discussed. Further

investigation is required for the environmental safety of nanoparticle and its influence on overall process of biodiesel production. Moreover, there is further need of exploring waste stream for cost-effective algae biomass development in bioenergy application.

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Chapter 15
Biogas Upgrading by Microalgae: Strategies and Future Perspectives

Dillirani Nagarajan, Duu-Jong Lee, and Jo-Shu Chang

Abstract Microalgae are being increasingly considered as a potential biomass feedstock for various biofuels, biodiesel in particular. Microalgal biomass for biofuel production purposes can be derived by cultivation using several waste resources, such as wastewater or flue gases, due mainly to the absence of the stringent regulations usually applied for food grade health supplements from microalgae. Anaerobic digestion and dark fermentation, the two highly used biomass digestion processes, generate biogas (a mixture of CH_4, CO_2 and other gases) and a COD (chemical oxygen demand)-rich effluent with leftover organic acids from the fermentation process. Microalgae can utilize the CO_2 present in the biogas stream, thus increasing the methane content and improving the fuel properties of biogas. Several reports indicate that certain microalgae are highly tolerant to the high concentrations of methane present in the biogas stream and can effectively utilize the CO_2 in photoautotrophic/mixotrophic mode of cultivation to obtain microalgal biomass. The organic acids of the effluent can also be used as a carbon source for mixotrophic/heterotrophic mode of microalgal cultivation, thus providing a cleanup of both the liquid and gaseous effluents of the fermentation process. This chapter describes in detail the capability of microalgae for carbon capture from biogas and their efficiency in the utilization of organic acids from various effluent streams. A

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biorefinery concept, integrating anaerobic digestion and microalgal cultivation is proposed, and the future perspectives are discussed.

1 Introduction

Renewable energy in the form of biofuels is steadily gaining research momentum and finding its way into the energy mix for consumption. This invigorating change is driven by the necessity to replace fast depleting fossil fuel resources, improve energy security, and combat the environmental effects caused by the imprudent use of fossil fuels. It has been predicted that renewable energy might become prominent in the energy mix, mainly due to the advent of new technologies and State support (Annual Energy Outlook 2018, EIA). Substantial research has focused on liquid biofuels, of which biodiesel and bioethanol dominate the renewable energy market. Biohydrogen and biomethane are the most promising gaseous biofuel candidates. Biomethane (defined as >97% of methane of biological origin) is currently being viewed as an important alternative energy source and has potential applications in the transport sector (Åhman 2010), or it can be converted to electricity or heat via combined heat and power stations (Weiland 2010). Biogas is a prominent source of biomethane, which is derived from the Anaerobic Digestion (AD) of organic matter. AD occurs in nature under anaerobic conditions in ocean sediments, ruminant intestines, and anthropogenic methane emissions in sites like landfills and livestock agriculture, contributing to an annual release of 0.55–1.3 billion tons of CH_4 to the atmosphere (Braun 2007). Despite the fact that the GHG reduction potential of other biofuels is questionable based on the feedstock, energy consumption, and emissions profile (Haberl et al. 2012), biogas production by AD can markedly contribute to reduction in GHG emissions, with negative GHG emissions when used as a fuel in particular (Tilche and Galatola 2008; Uusitalo et al. 2014). Atmospheric methane concentrations due to anthropogenic emissions are projected to increase to a staggering 405 Tg (terragram) CH_4 per year by 2030 (Abbasi et al. 2012), and biogas production by AD is an effective way of capturing the released CH_4, since CH_4 is almost 25 times more potent than CO_2 as a GHG.

The major components in the biogas include CH_4 and CO_2, along with numerous other compounds like H_2S, NH_3, water vapor, and certain trace elements. The effective composition of biogas is influenced by the nature of feedstock used and the reaction conditions applied for efficient digestion of the feedstock. Commercial biogas production plants generally operate at wastewater treatment plants for the AD of sewage sludge, at landfill sites for degradation of garbage, and at animal husbandry sites for the AD of manure, and also separate digesters can be set up for AD of agricultural biomass. Of the 18,000 AD plants in Europe, around 12,000 installations operate on agricultural feedstock (European Biogas Association Statistical report, 2017), while about 1200 of the 2200 AD plants in the USA are located in wastewater treatment plants (American Biogas Council). The composition of biogas varies depending on the feedstock used, along with the presence of other impurities, as summarized in Table 15.1. The major energy carrier of biogas is methane, and the

Table 15.1 Composition of biogas based on the feedstock used and the European biofuel standard for biogas as a transportation fuel

Component	Effect of the component (Ryckebosch et al. 2011)	Biogas from Wastewater treatment plants (Toledo-Cervantes et al. 2017a)	Biogas from AD of organic matter (Surendra et al. 2014)	Biogas from landfill (Muñoz et al. 2015)	European biofuel standard (Toledo-Cervantes et al. 2017a)
Methane CH_4	Energy carrier	55–70%	50–75%	35–65%	>95%
Carbon dioxide CO_2	Reduces the heating value	30–45%	25–50%	5–50%	<2.5–4%
Nitrogen N_2	Reduces the heating value	0–1%	0–5%	5–40%	
Oxygen O_2	Explosion risk due to high concentration of O_2	0–0.5%		0–5%	<0.001–1%
Water H_2O	Corrosive, particularly in combination with the SO_x and NO_x form acids, condensation might lead to freezing	5–10%	1–5%	0–5%	
Hydrogen sulphide H_2S	Corrosive, generates SOx upon combustion which forms acids with water	0–10,000 ppm_v	0–5000 ppm	0–100 ppm	<5 mg Nm^{-3}
Siloxanes	Generates SiO_2 and quartz upon combustion, could block engine parts	2–41 mg Nm^{-3}		0–50 mg Sim^{-3}	<10 mg Nm^{-3}
Benzene, toluene, and xylene BTX	Corrosive	<0.1–5 mg Nm^{-3}		–	<500 mg Nm^{-3}
Ammonia NH_3	Corrosive, in combination with water	0–100 ppm_v	0–500 ppm	0–5 ppm	<10 mg Nm^{-3}
Halogenated compounds	Corrosive in combustion engines	<0.1 mg Nm^{-3}		20–200 ppm	
Hydrocarbons	Corrosive in combustion engines	0–200 mg Nm^{-3}			
Carbon monoxide CO	Corrosive, in combination with water	–		0–3%	
Hydrogen H_2		–		0–3%	

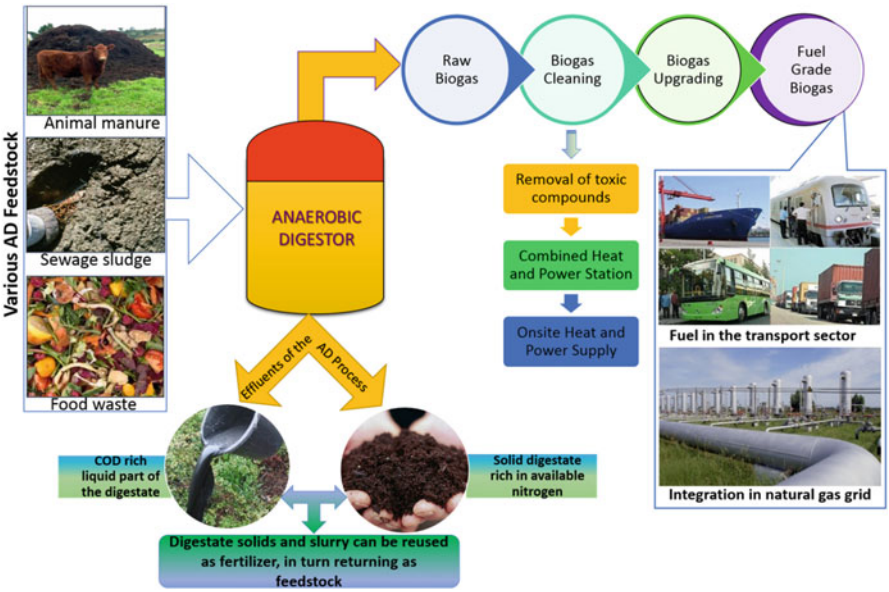

Fig. 15.1 A schematic illustration of the operation of an AD plant (Adapted from American biogas council)

other substances are regarded as impurities. Based on the end use application, additional components in biogas needs to be removed and the methane content in biogas enhanced. The biogas generated in a fermenter in an AD plant can be used in a CHP station with a desulfurizing step, and the CHP station that generated heat and electricity could be used directly within the plant or supplied elsewhere (Patterson et al. 2011). However, when it comes to the use of biogas as a transportation fuel, stringent regulations are applied as the various extraneous impurities present in raw biogas can impede the performance of combustion engines. The European standard for transportation grade biogas is presented in Table 15.1**,** and usually the biogas needs upgrading of its methane content to increase the fuel performance.

The schematic of an anaerobic digestion plant for the production of biogas is illustrated in Fig. 15.1. The feedstock, like organic matter, animal manure, sewage sludge, microalgae, macroalgae, or even food waste, is treated in an appropriate manner to enhance the methane generation potential and fed into the digester. Raw biogas in generally cleaned up of the toxic compounds like H_2S and siloxanes, which could then be used in a CHP station for the generation of heat and electricity for onsite use. Further, the biogas can be upgraded for its methane content and purified of all impurities to be used as a fuel. It can then be integrated with the natural gas grid or be used as a transportation fuel (American Biogas Council). The leftover digestate from the fermenter is then separated as solid and liquid fractions, which can then be further reused as fertilizers. The prospect of utilization of liquid digestate from AD as a nutrient source is discussed in detail in Sect. 3. Biogas upgrading can be performed by various physical, chemical, and biological methods, and detailed information

regarding these have been reviewed earlier in detail (Muñoz et al. 2015; Kadam and Panwar 2017; Angelidaki et al. 2018). Successful physical/chemical processes applied in commercial biogas plants include water scrubbing by physical adsorption, chemical absorption with amine solutions, pressure swing adsorption, membrane separation, cryogenic processes, and scrubbing with organic physical scrubbers (Angelidaki et al. 2018). The methane recovery with the physical/chemical processes is over 96%, and the upgraded biogas usually has a methane content of 95–97% meeting the fuel standard specifications. Biological processes for biogas upgrading include (a) chemoautotrophic conversion of CO_2 to CH_4 using H_2 as electron donor, (b) photosynthetic CO_2 capture by microalgae or cyanobacteria, (c) microbial conversion of CO_2 into valuable liquid products like ethanol, and (d) microbial electrochemical conversion of CO_2 to CH_4. Of these, this chapter deals with the upgrading of biogas by microalgal carbon capture. Biogas upgrading by microalgae is an eco-friendly, zero waste, and green technology that could simultaneously remove CO_2 from biogas and the organic nutrients present in the liquid AD digestate (Chen et al. 2018). This chapter presents the basic principles of biogas production by AD and the carbon capture potential of microalgae. The utilization of organic acids by microalgae via mixotrophic metabolism is discussed in detail, and an integrated biorefinery for AD and microalgal cultivation is proposed.

2 Anaerobic Digestion and Biogas Production

Anaerobic digestion is the fermentation of complex organic matter in the absence of oxygen, resulting in the decomposition of organic matter to CH_4, CO_2, H_2, and some volatile fatty acids. AD is a multi-step process, and it occurs in a sequential order, as defined by the dominant microbial population in the digester. The four major stages of AD are hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and the major activities in these stages are illustrated in Fig. 15.2. Hydrolysis is the first step of AD and results in the dissolution or disintegration of the complex organic matter to simple monomers, increasing their bioavailability to the fermentative bacteria. The predominant bacterial species in this phase are generally found to be strict or facultative anaerobes of the genera *Clostridium*, *Bacteroides*, *Butyrivibrio*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, and members of the *Enterobacteriaceae* family (Amani et al. 2010; Merlin Christy et al. 2014). These organisms are endowed with an array of hydrolytic enzymes like amylase, cellulase, cellobioase, protease, and lipase which act on carbohydrates, proteins, and lipids, eventually degrading them into monosaccharides, long-chain fatty acids, and amino acids. The feedstock for AD is highly versatile (animal manure, food waste, sewage sludge, lignocellulosic biomass, microalgae, macroalgae), and hydrolysis is essential for the liquefaction and subsequent solubilization of the solid organic matter. Hydrolysis of all the compounds present is crucial, since certain materials are highly recalcitrant, or they cannot be hydrolyzed by bacterial depolymerases (lignocellulose in particular), and hence it is often dubbed as the "rate-limiting" step (Park et al. 2005). A pretreatment step can greatly enhance hydrolysis efficiency and improve the methane generation

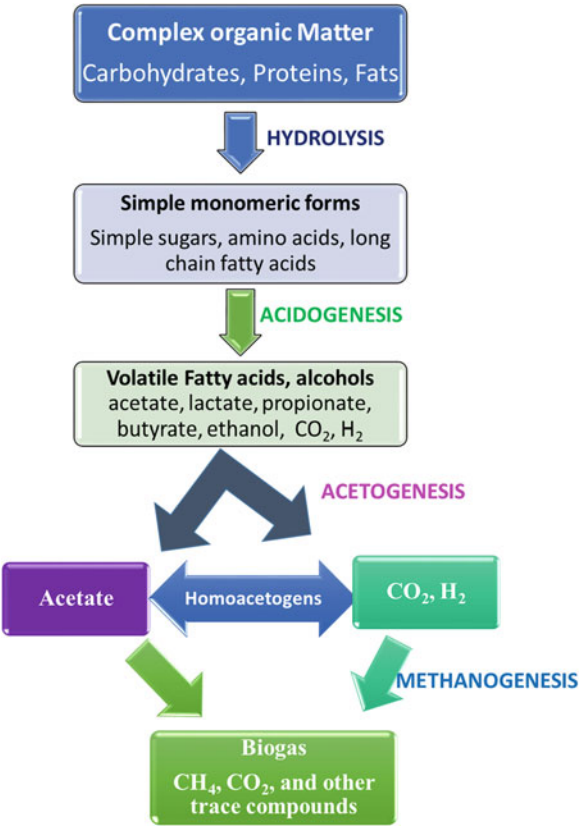

Fig. 15.2 A schematic illustration of the various stages of anaerobic digestion (AD)

potential of the applied feedstock. The pretreatment step is chosen based on the feedstock used, energy requirements, and the feasibility for use in large-scale applications (Carrere et al. 2016).

Acidogenesis is the principal phase of the conversion of monomers to higher organic acids, alcohols, aldehydes, and gaseous products. Fermentative bacteria (both obligate and facultative) use the monosaccharides derived from sugars and convert them to organic acids like lactate, propionate, butyrate, propionate, and acetate, along with alcohols like ethanol or methanol, accompanied by the evolution of CO_2 and H_2. Fatty acids and amino acids arising from lipids and proteins can be utilized as carbon sources by anaerobic bacteria, further converting them into simpler compounds. The major bacterial species present in this stage are from the genera *Bacillus*, *Clostridium*, *Micrococcus*, *Pseudomonas*, *Lactobacillus*, *Salmonella*, *Corynebacterium*, *Eubacterium*, *Escherichia coli*, *Desulfobacter*, *Desulfomonas*, and *Desulfovibrio* (Merlin Christy et al. 2014). Acidogenic bacteria are generally the fast growing in the reactor, with an operational pH value of about 4.5–5.5 as defined by the production of acids in the medium. Of the organic acids produced in the acidogenesis phase, acetate and butyrate are preferred for methane

generation. Acidogenic and hydrolytic microbes are linked closely to each other based on their growth rate and pH requirements, and together they are the fastest-growing organisms in the reactor, completing the hydrolysis and acidogenesis within 10–15 days (Cirne et al. 2007).

The next phase, acetogenesis, is characterized by the conversion of the higher organic acids to acetate and hydrogen by acetogenic bacteria. Acetogenic bacteria are slow-growing obligate anaerobes, and an optimal pH of around 6 is preferred (Merlin Christy et al. 2014). The growth rate is lower for these bacteria, with prolonged lag periods required for the adjustment to their immediate environments. Hydrogen evolution in acidogenesis phase is accompanied by the accumulation of electron sinks in the form of higher acids and alcohols, and acetogenic bacteria catalyze the conversion of these electron sinks to acetate, CO_2 and H_2 (Merlin Christy et al. 2014). The major acetogenic bacteria are the following: *Syntrophomonas wolfeii*, *Syntrophobacter wolinii*, *S. fumaroxidans*, *Pelotomaculum* sp., *Smithella* sp., and *Clostridium aceticum* (Amani et al. 2010). The hydrogen evolved during acetogenesis is toxic for acetogenic bacteria, and a low partial pressure of hydrogen is preferred. A syntrophic association exists between hydrogen-evolving acetogenic bacteria and hydrogen-consuming methanogenic bacteria, and this relationship in combination with the efficient conversion of the organics to acetate determines the efficiency of biogas production (Weiland 2010). Higher hydrogen concentration favors methane formation, while lower hydrogen concentrations favor acetate formation from CO_2 and H_2 by homoacetogenic bacteria. Notable homoacetogenic bacteria include *Acetobacterium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus*, and *Sporomusa* (Saady 2013). However, homoacetogens can outgrow methanogens in an AD process at low temperature and other thermodynamically unfavorable conditions (Ye et al. 2014).

The final phase is the methane-generating phase, defined as methanogenesis. Archaea dominate the methanogenesis phase due to their unusual metabolic capability of utilizing acetate, CO_2/H_2, formate, or other methylated carbons as a source of energy and carbon, evolving methane in the process (Enzmann et al. 2018). Methanogenic organisms in AD can be acetoclastic methanogens or hydrogenotrophic methanogens. Acetoclastic methanogens generate methane by acetate decarboxylation and produce methane and CO_2. Very few species are capable of acetoclastic methanogenesis including *Methanosarcina barkeri*, *Methanococcus mazei*, *Methanotrix soehngenii* (Weiland 2010), *Methanosaeta concilii*, *and Methanosarcina acetivorans* (Amani et al. 2010). Hydrogenotrophic methanogens generate methane via the reduction of CO_2/H_2, and most methanogens are capable of this function including species of the genera *Methanospirillum*, *Methanococcus*, *Methanobrevibacter*, *Methanococcus*, *Methanoculleus*, and so on (Amani et al. 2010). The efficiency of the AD process is determined by the methanogens and their ability to outcompete homoacetogens and methanotrophs in a bacterial consortia,; hence, it is essential to control the process parameters in AD favoring methanogens. At the end of AD, the resultant products are biogas (a mixture of CO_2 and CH_4) and the residual digestate. The digestate can be further divided into solid and liquid fractions. The solid digestate is easy to handle with higher bioavailable nitrogen for plants and is usually applied as a bio fertilizer

(Möller and Müller 2012). The liquid part is particularly rich in the leftover organic acids from the fermentation and other macronutrients like NH_3 and phosphorus. The amount of total nitrogen (N), total phosphorus (P), and chemical oxygen demand (COD) levels in liquid anaerobic digestate can range from 139 to 3496 mg/L (65–98% of ammonia nitrogen), 7–381 mg/L (82–95% phosphate), and 210–6900 mg/L, respectively (Xia and Murphy 2016a). This liquid digestate can be used as a carbon source for the cultivation of microalgae, since microalgae can assimilate organic carbon in the presence/absence of light under mixotrophic/heterotrophic conditions, respectively. The nitrogen and phosphorus are used for growth and biomass accumulation as well. Hence, after microalgal treatment, the liquid digestate has relatively low concentrations of N, P, and COD aiding in subsequent environmental release without the fear of eutrophication of surrounding water bodies.

Dark fermentation (DF) for biohydrogen production is another most commonly used anaerobic fermentation process, with hydrogen as the principal product and COD-rich leftover fermentation liquor as a by-product. The basic biochemical pathway for dark fermentation is similar to the first three stages of AD, accomplished by both obligate and facultative anaerobic bacteria. Methanogenesis is usually inhibited in such processes by careful control of the reaction parameters like temperature and pH (Ghimire et al. 2015). The organic acids present in the fermentation liquor, particularly acetate and butyrate can be assimilated by microalgae in mixotrophic mode of cultivation (Liu et al. 2013a). AD digestate and DF liquor are both needed to be processed further to enhance the energy recovery in each process.

3 Microalgae and Carbon Capture

Microalgae is an umbrella term for the countless unicellular/simple multicellular, prokaryotic and eukaryotic organisms that can fix the atmospheric CO_2 via photosynthesis into organic biomass. The estimated number of classified algal species were around 75,000 in 2012 (Guiry 2012) and is currently at 150,000 species as described by Algaebase (http://www.algaebase.org/). All these include properly named and characterized species, and still numerous algal species could be isolated and characterized. This huge number explains the diversity that can be seen in algae related to their habitats, morphology, physiology, phylogeny, and carbon metabolism. Microalgae are now considered as the third-generation feedstock for the production of biofuels, because of their higher photosynthetic efficiency. The theoretical maximum for photosynthetic efficiency (PE) of a green plant in bright sunlight is estimated to be 13% and a practical PE around 8–9% is attainable under optimal conditions (Bolton and Hall 2008), while reported global average PE for terrestrial plants is around 1–2%. Microalgae can have higher PE, anywhere between 1% and 21% based on various reports (Brennan and Owende 2010). Higher PE results in higher oil productivity close to 136,900 L oil/ha year in high oil microalgae. A biodiesel productivity of 121,104 kg biodiesel/ha year can be achieved with high oil microalgae, whereas it is very low in traditional oil crops

like jatropha and soybean (Mata et al. 2010). These two traits set microalgae apart from other potential biofuel resources, and additionally microalgal cultivation requires minimal nutrients, atmospheric CO_2 as carbon source, minimal requirements for land and water, noninterference with local agriculture, and no land use changes. Microalgae fixes atmospheric carbon via a series of reactions in the presence of sunlight in the light and dark reactions of photosynthesis. An estimated 180 tons of CO_2 is required for the production of about 100 tons of microalgal biomass (Chisti 2008) and other than atmospheric carbon dioxide (which is currently at 407 ppm), various relatively inexpensive gases rich in CO_2 can be used for microalgal cultivation.

Carbon capture by microalgae is an economically viable option for biological carbon mitigation, and microalgae can be cultivated in CO_2-rich gases like industrial flue gases (cement industries, coal fired power plants) and CO_2 emissions from ethanol industries. Certain microalgae can tolerate very high concentrations of CO_2, as high as 50–70%, previously reported for *Chlorella* species (Maeda et al. 1995; Sung et al. 1999; Yue and Chen 2005). The CO_2-rich off-gas from ethanol fermentation has been used for the cultivation of *Arthrospira platensis* (Bezerra et al. 2013) and *Chlorella vulgaris* (Zhang et al. 2017a). The fermentation CO_2 from acetone-butanol-ethanol fermentation for biobutanol production has been used successfully for the cultivation of capnophilic *E. coli*-based succinic acid production, with a maximum succinic acid concentration and productivity of 65.7 g/L and 0.76 g/l/h, respectively. The CO_2 capture from this fermentation off-gas has enriched the hydrogen content of the gas to up to 92.7% (He et al. 2016). The CO_2 released during an integrated dark-photo fermentation for hydrogen production has been used for the cultivation of *C. vulgaris*, and microalgal biomass rich in proteins (48.6% by weigh of biomass) was obtained (Lo et al. 2010). The VFA-rich fermentation effluents from a dark fermentation reaction and the CO_2 rich off-gas were both used as a carbon source for the mixotrophic cultivation of *Chlorella vulgaris* ESP6, and CO_2 content of the off-gas was reduced from 34% to 5% with complete consumption of acetate and butyrate in the liquid effluent. The resultant carbohydrate-rich microalgal biomass was used for biohydrogen production, thus enhancing the energy recovery from the initial energy input (Liu et al. 2013b).

While the CO_2 released during fermentation reactions is relatively pure and can be directly used for the cultivation of microalgae (Xu et al. 2010), the composition of CO_2-rich industrial flue gases vary depending upon the source, and an additional 142 compounds are known to be present with around 3–25% by volume of CO_2 (Van Den Hende et al. 2012). The most important compounds present include SOx, NOx, unburned carbohydrates, CO, water vapor, O_2, chlorine, fluorine, heavy metals, and other related compounds. The SO_x and NO_x can dissolve in culture medium leading to a drop in medium pH, and other impurities might be lethal to microalgae. Selection of a microalgal strain resistant to high CO_2, fluctuations in medium pH, robust growth characteristics, and simple pretreatment of flue gases can help attain high biomass productivities when using flue gas as a carbon source for growth (Cheah et al. 2016). Life cycle analysis and design parameters for microalgae-based carbon capture indicates that microalgal biodiesel production

with flue gas capture can be profitable based on the microalgal strain chosen and fuel production pathway (Gebreslassie Berhane et al. 2013; Gong and You 2014; Hernández-Calderón et al. 2016; Gutiérrez-Arriaga et al. 2014). Flue gas has been successfully used for the cultivation of *Chlorella* sp. (Kao et al. 2014), *Desmodesmus* sp. (Aslam et al. 2017), and *Desmodesmus abundans* (Lara-Gil et al. 2016). Biogas contains even higher concentrations of gaseous CO_2, in the range of 20–50% depending on the AD feedstock used. Biogas does not contain many toxic compounds like flue gas, and it is the product of anaerobic fermentation; hence, it is available at ambient temperature alleviating the need for thermotolerant strains. The utilization of biogas CO_2 by microalgae for biogas upgrading is discussed in detail in Sect. 5.

4 Utilization of Volatile Fatty Acids from Fermentation Effluents by Microalgae

The acid fermentation pathways of anaerobic bacteria lead to the breakdown of the input carbon source into organic acids in the acidogenic and acetogenic phase, which is then converted to methane by the archaeal methanogens. The major volatile fatty acids from the typical mixed acid fermentations of anaerobic bacteria include formate, acetate, lactate, butyrate, propionate, valerate, and isovalerate. Alcohols like ethanol, methanol, propanol, and isopropanol can also be found in smaller quantities based on the fermentative organism and fermentation conditions (Zhou et al. 2018). Microalgae are capable of assimilating these volatile fatty acids via the central carbon metabolic pathway, similar to bacteria and higher eukaryotes. Microalgae are endowed with certain transporters for the effective transport of VFAs at the expense of energy, and inside the cell, these VFAs enter carbon catabolic pathways.

The principal VFA in majority of effluents is acetate, and it is also the most commonly used carbon source for the mixotrophic/heterotrophic cultivation of microalgae. Acetate enters the cell via a monocarboxylic carbon/proton transport protein under aerobic conditions. The transporter is not specific for acetate but a general transporter for monocarboxylic acids (Perez-Garcia et al. 2011). In the cytoplasm, acetate is converted to acetyl coenzyme A (acetyl CoA) by acetyl CoA synthetase at the expense of an ATP molecule. Acetyl CoA can be further metabolized via the glyoxylate cycle for the formation of C4 metabolites, or it can enter the tricarboxylic acid (TCA) cycle for the generation of ATP and carbon skeletons for anabolism and reducing equivalents (Perez-Garcia et al. 2011). Acetyl CoA is also the major precursor for fatty acid synthesis in microalgae; hence, acetate availability is the rate-limiting step for lipid accumulation in microalgae (Ramanan et al. 2013). Under nitrogen deprivation, cells reduce protein synthesis due to the unavailability of nitrogen-arresting cell division. Nitrogen limitation also activates certain deaminases that act on AMP; hence AMP concentration declines leading to the reduced

activity of isocitrate dehydrogenase of TCA cycle, resulting in the accumulation of citrate which is then converted to acetyl CoA. Thus, funneling of available carbon as acetyl CoA under nitrogen deprivation helps in increased lipid accumulation in eukaryotic oleaginous microalgae (Ratledge 2004). In *Chlamydomonas reinhardtii*, starchless mutants exhibit higher oil accumulation, but the wild-type strains depend totally on external acetate availability for enhanced lipid accumulation. Acetate addition, seven times higher than the standard conditions, resulted in a steady increase in lipid accumulation (Fan et al. 2012). *Chlorella sorokiniana* could outcompete aerobic and anaerobic bacteria for acetate consumption in heterotrophic growth with unsterilized dark fermentation effluents, with a 55% carbon yield on acetate (Turon et al. 2015a). Acetate addition is also known to stimulate carotenogenesis in *Haematococcus pluvialis*, triggering the conversion to heterologous cysts compared to that of non-acetate-based growth (Kobayashi et al. 1991). Acetate concentrations as high as 10 g/L has been used for the cultivation of *Chlorella vulgaris*, but low acetate concentration aids in the use of acetate as a sole carbon source by microalgae. This may be due to the fact that higher acetate concentrations (particularly the sodium or potassium salts) can cause an increase in culture pH upon acetate consumption and a pH- stat culture is required (Perez-Garcia et al. 2011).

Butyrate can be consumed by microalgae in the same manner as acetate, via the monocarboxylate/proton transporter. Butyrate is believed to be converted to acetyl CoA via crotonyl CoA, but the mechanism of conversion is not clearly understood (Baroukh et al. 2017). It could be possible that butyrate is metabolized by the beta-oxidation pathway of fatty acids in the peroxisomes as previously reported for the yeast *Candida tropicalis* (Kurihara et al. 1992). Once converted to acetyl CoA, it can be further processed by the glyoxylate cycle or tricarboxylic acid cycle. Butyrate is not a preferred carbon source for microalgae, and clear diauxic pattern of growth has been observed in the presence of acetate in *Chlorella sorokiniana (*Baroukh et al. 2017*)*. Butyrate above a concentration of 0.1 g/L is inhibitory for microalgal growth (Liu et al. 2013a), while a concentration of 0.3 g/L can be tolerated in mixotrophic growth (Baroukh et al. 2017). However, butyrate inhibition is believed to be relieved in the presence of acetate, owing to initial biomass accumulation by acetate consumption and secondary consumption of butyrate for energy generation. A higher acetate, butyrate ration, could enable butyrate consumption in microalgae, as high as 8:1 as reported for *Chlorella protothecoides* (Fei et al. 2015). An increase in substrate to microorganism ratio is also believed to overcome butyrate inhibition. A food to microorganism ratio of 4.5 was optimal for total VFA assimilation (Liu et al. 2013b), while a ratio of 1.1 was observed to be optimal for the use of butyrate as a sole carbon source for *Chlorella vulgaris* ESP6 (Liu et al. 2013a).

Propionate is the third major VFA to be produced during the acidogenesis phase. Mechanism of propionate utilization in microalgae is unknown, but in certain photosynthetic bacteria, it is assimilated by conversion to propionyl-CoA which is then carboxylated to methylmalonyl-CoA. A molecular rearrangement of methylmalonyl-CoA leads to succinyl-CoA, which then enters TCA cycle (Neilson and Lewin 1974). Propionate as a sole carbon source (at a concentration of 10 g/L

carbon equivalent to glucose) did not support biomass production and lipid accumulation in *Scenedesmus sp*. R-16, but the high concentration of this acid used could also inhibit microalgal growth. The effect of propionate concentration on microalgal growth was not shown (Ren et al. 2013). Propionate at a concentration of 3000 mg/L did not support biomass and lipid accumulation of a microalgal consortium (Venkata Mohan and Prathima Devi 2012). Propionate could be consumed by microalgae at very low concentrations in a diauxic pattern in the presence of acetate. An acetate-butyrate-propionate ratio of 8:1:1 works well for both *Chlamydomonas reinhardtii* (Moon et al. 2013) and *Chlorella protothecoides* (Fei et al. 2015). Lactate was inhibitory for the growth of *Chlorella vulgaris* ESP6 at a concentration of above 0.5 g/L (Liu et al. 2013a), and this could happen due to the acidification of the intracellular environment upon import of this acidic metabolite. However it has been shown that lactate was never transported inside the cell or utilized for growth in *Chlorella vulgaris* (Liu et al. 2013a), *Chlorella sorokiniana*, and *Auxenochlorella protothecoides* (Turon et al. 2015b). Valeric and isovaleric acids have been utilized by *Chlorella vulgaris* and *Scenedesmus sp*. R16 (Cho et al. 2015; Ren et al. 2014), while isovalerate was reported to be the second best carbon source next to acetate for *C. protothecoides* FACHB-3 (Wen et al. 2013).

5 Microalgae-Based Biogas Upgrading and Biomass Production

Microalgae have been used for effective nutrient removal from wastewaters of domestic and industrial origin in high rate algal ponds for over a century. The nature of the AD slurry and the role of microalgae in bioremediation of the AD slurry before further processing are described in detail in Sect. 2. Microalgae-based biogas upgrading of real biogas (raw/desulfurized) and synthetic or simulated biogas are summarized in Tables 15.2 and 15.3, respectively. The nature of the organic acids present in most wastewaters are not presented in detail, and still the high COD is contributed primarily by the presence of VFAs as discussed previously. The removal and utilization of COD by microalgae represents the organic acid fraction that is utilized. Real biogas from AD plants have all the impurities and other toxic components of unknown nature that could influence microalgal growth; hence they were summarized separately (Table 15.2). Studies on biogas upgrading of simulated biogas (which mainly consists of CH_4, CO_2 and sometimes H_2S) for evaluating their effect and tolerance levels in microalgae provide valuable insight into the metabolism of these compounds by microalgae (Table 15.3).

The chief component of biogas is methane (CH_4) present in about 40–70% *v/v*, and generally most microalgae chosen for biogas upgrading are tolerant to the levels of CH_4 seen in biogas. Biological consumption of CH_4 by microalgae has been shown in some reports, but the mechanism of such consumption is unknown (Prandini et al. 2016; Lebrero et al. 2016). Since the axenic status of these cultures

Table 15.2 Biogas upgrading by microalgae with real biogas fed from AD process

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Scenedesmus obliquus FACHB 416 with fungus *Ganoderma lucidum* 5.765	Cylindrical PBR, initial inoculum 120 mg/L, 25 °C, 200 μmol/m^2/s, mixed LED light red: blue as 5:5, 14 h:10 h light/dark cycle	79.92% RE	0.353 g/L/day	85.82% COD, 83.81% TN, 84.26% TP	>90% CH_4	Microalgae co-culture with fungi proved to be better than mono algal culture or co-culture with activated sludge	Wang et al. (2017)
	Desulfurized biogas: 67% CH_4, 29% CO_2, 2.95% H_2O, 0.21% O_2, <0.005% H_2S						
	Filtered and UV sterilized biogas slurry from a WWTP plant						
Chlorella vulgaris and nitrifying-denitrifying activated sludge	Cylindrical PBR, initial inoculum 113 mg/L, 25 °C, 200 μmol/m^2/s, mixed LED light red: blue as 5:5, 16 h:8 h light/dark cycle	66.93–88.27% RE	0.136 g/L/day	72.39% COD, 75.48% TN, 73.67% TP	92.58% CH_4	*Chlorella vulgaris* seem to perform well with activated sludge for biogas upgrading, while algal- fungal culture performed well in nutrient removal	Zhang et al. (2017b)
	Desulfurized biogas: 64% CH_4, 31% CO_2, 3.15% H_2O, 0.54% O_2, <0.005% H_2S						
	Filtered and UV sterilized biogas slurry from a WWTP plant						

(continued)

Table 15.2 (continued)

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Indigenous microalgae that naturally grew in the reactor	Cylindrical PBR, 25 °C, 130 µmol/m^2/s, light/dark cycle of 43.2 Ks, pH maintained at 8 with CO_2 injection	91.8% CO_2 assimilation rate	1.27 mg Chl. a dm^{-3}	–	–	–	Takabe et al. (2017)
	Membrane separated CO_2 from biogas with 983 dm^3 m^{-3} CO_2, 9.02 dm^3 m^{-3} CH_4, <0.2 cm^3 m^{-3} H_2S and 0.2–0.38 cm^3 m^{-3} NH_3						
	Raw digestate from a WWTP plant						
Chlorella sorokiniana	50 L open PBR, M-8a medium, ambient temperature, light intensity of µmol/m^2/s, light dark cycle of 12 h:12 h, continuous culture with dilution rate 0.1 day^{-1}	89–93% RE	0.06 g/L/day	–	>90% CH_4, 2–4.5% CO_2	Lower temperatures during dark/night enhances CO_2 solubility, facilitating continuous biogas feeding and enhanced growth rates	Meier et al. (2017)
	Raw biogas from in-house diluted wine treating anaerobic reactor with 65% CH_4, 32% CO_2, passed to microalgal culture via a mass transfer column at a volumetric gas load of 1 L/day per L algal culture						

Leptolyngbya sp. CChF1	250 mL glass flasks with Zarrouk's medium, 27.1 °C, 920 lux, 120 rpm, 10 days, aerated with biogas	93.48% RE, 98.19% CO_2 capture efficiency	0.090	–	–	Lipid rich biomass (164.8 mg/g DW)	Choix et al. (2017)
	Raw biogas from a tequila vinasse treating plant with 74% CH_4, 25% CO_2 and 1% other gases						
Chlorella vulgaris FAHCB31 and *Ganoderma lucidum*	16.8 L cylindrical PBR, synthetic domestic sewage with influent C/N ratio of 5/1, 25 ° C, 200 μmol/m^2/s, 160 rpm, 7 days	65–84% RE	0.44 g/L/day	81.92% COD, 81.66% TN, 81.52%TP	93.25% CH_4	–	Xu et al. (2017)
	Desulfurized biogas from a farm AD plant with 67.6% CH_4, 28.4% CO_2, 3.54% H_2O, 0.47% O_2, <0.005% H_2S						
Scenedesmus sp.	16.9 L open cylindrical glass PBR with 8.9 L diluted slurry in distilled water, initial biomass 30% v/v, 22 ± 2 °C, red light at 630 nm, 148.5 μmol/m^2/s, light/dark cycle 12 h:12 h	126.1 mg/L/day CO_2 assimilation rate	89.4 mg/L/day		1.2% CO_2 50.4% CH_4 21.6% O_2 0.4 ppmv H_2S	Resistance and complete removal of up to 3000ppmv H_2S, up to 18% v/v methane biologically consumed	Prandini et al. (2016)
	Raw untreated swine water-based biogas slurry						

(continued)

Table 15.2 (continued)

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
	Raw biogas from WWTP with 70.7% CH_4, 26.1% CO_2, 0.23% O_2, 1550ppmv H_2S						
	Same as above, except 24 h illumination	219.4 mg/L/day CO_2 assimilation rate	141.8 mg/L/day		7.5% CO_2 64.7% CH_4 17.8% O_2 5 ppmv H_2S		Prandini et al. (2016)
Chlorella vulgaris FAHCB 31	Transparent polyethylene bag as PBR, initial biomass of 0.068 g/L, 25 ° C, light intensity 150 μmol/m^2/s, light dark cycle as 14 h:10 h, mixing by shaking the bag thrice a day	–	0.139 mg/L/day	64.76% COD, 55.67% TN, 53.84%TP	83.46% CH_4		Wang et al. (2016)
	Filtered and UV sterilized AD slurry						
	Desulfurized biogas from AD treating piggery wastewater with 61.38% CH_4, 32.57% CO_2, 5.52% H_2O, 0.54% O_2, <0.005% H_2S						

S. obliquus FAHCB 416	Same as above	–	0.151 mg/L/day	60.39% COD, 56.71% TN, 52.97%TP	84.28% CH_4		Wang et al. (2016)
Nitzschia palea FAHCB 211	Same as above	–	0.107 mg/L/day	68.11% COD, 59.08% TN, 60.03%TP	84.21% CH_4		Wang et al. (2016)
Scenedesmus obliquus FACHB 31	Transparent polyethylene bag as PBR, initial biomass of 153 mg/L, 25 ° C, light intensity 200 μmol/m^2/s, light dark cycle as 12 h:12 h, mixing by shaking the bag thrice a day	78.81% RE	311 mg/L/day	75.29% COD, 74.63% TN, 81.73% TP	88.25% CH_4		Xu et al. (2015)
	AD treated piggery wastewater, autoclaved and COD adjusted to 1600 mg/L by dilution with distilled water						
	Desulfurized biogas from AD treating piggery wastewater with 58.67% CH_4, 37.54% CO_2, 3% H_2O, 0.79% O_2, <50 ppm H_2S						

(continued)

Table 15.2 (continued)

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Chlorella sp. and aerobic activated sludge	Transparent PVC column PBR, continuously illuminated at 230 µmol/m^2/s, ambient temperature, mixing by biogas sparging	pH7: CO_2 fixation rate 285 mg/L/day	155 mg/L/day	–	–		Lebrero et al. (2016)
		23% RE					
	MSM medium at pH 7	pH8.1: 62% RE					
	Raw biogas from pilot scale UASB anaerobic digestors treating vinasse with 58.9–81.8% CH_4, 11.6–38.1% CO_2, 0.4–0.8% H_2S						
Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 877.68 mg dry weight, 25 °C, light intensity 800 µmol/m^2/s, light dark cycle as 12 h:12 h, mixed LED light with red:blue at 5:5, mixing by shaking the bag thrice a day	57.7% RE	494.23 mg/L	78.91% COD, 73.05% TN, 67.54% TP	93.68% CH_4, 1.57% CO_2, 3.8% H_2O, 0.99% O_2, <50 ppm H_2S		Yan and Zheng (2014)
	Filtered and UV sterilized biogas slurry						

	Desulfurized raw biogas with 64.21% CH_4, 31.38% CO_2, 3.79% H_2O, 0.68% O_2, <50 ppm H_2S						
Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 1.8 g, 25 °C, light intensity 2000 μmol/m^2/s, light dark cycle as 12 h:12 h, red LED light, mixing by shaking the bag thrice a day	51.28% RE	560 mg/L	85.35% COD, 77.98% TN, 73.03% TP	92.74% CH_4	High-intensity red LED light showed better performance than blue or white light	Zhao et al. (2013)
	Filtered and UV sterilized biogas slurry						
	Desulfurized raw biogas with 67.35% CH_4, 28.41% CO_2, 3.48% H_2O, 0.73% O_2, <50 ppm H_2S						
Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 64.52 mg/L, 25 °C, light intensity 250 μmol/m^2/s, light dark cycle as 12 h:12 h, mixing by shaking the bag four times a day	86.15% RE	615.84 mg/L	88.74% COD, 83.94% TN, 80.43% TP	92.16% CH_4		
	Filtered and UV sterilized biogas slurry						

(continued)

Table 15.2 (continued)

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
	Desulfurized raw biogas with 70.65% CH_4, 26.14% CO_2, 3.11% H_2O, 0.23% O_2, <0.005% H_2S						
Chlorella sp. MB-9	Outdoor vertical column glass PBR, mixing by aeration with desulfurized biogas for 30 min, followed by aeration with air for 30 min for 8 h a day, biogas flow rate at 0.05 vvm	86.3% RE	0.32 g/L/day	–	91.1% CH_4	Mutant *Chlorella* resistant to CH_4 and H_2S, on-site biogas upgrading in outdoor PBRs, lipid productivity of biomass not affected	Kao et al. (2012b)
	Modifies f/2 medium in artificial sea water at pH 7.4–7.6						
	Desulfurized biogas from a swine farm AD plant with 69% CH_4, 20% CO_2 and H_2S<50 ppm						
Chlorella sp. MM-2	Outdoor vertical column glass PBR, mixing by aeration with desulfurized biogas for 30 min, followed by aeration with air for 30 min for 8 h a day, biogas flow	70% capture efficiency on cloudy days, 80% on sunny days	0.276 g/L/day		84% CH_4 on cloudy days, 87% CH_4 on sunny days	Mutant *Chlorella* resistant to CH_4 and H_2S, on-site biogas upgrading in outdoor PBRs	Kao et al. (2012a)

	rate at 0.1 vvm, initial biomass 1.2 g/L						
	Modifies f/2 medium in artificial sea water at pH 7.4–7.6						
	Desulfurized biogas from a swine farm AD plant with 70% CH_4, 20% CO_2, 8% N2, and H_2S<100 ppm.						
Chlorella vulgaris p13	Bubble column glass PBR, continuously illuminated at 1000 µmol/m^2/s	–	0.11–0.16 g/L/h growth rate	–	–		Doušková et al. (2010)
	Defined medium with alkalized ammonia-rich digestate liquor used for CO_2 capture and nitrogen source						
	Direct biogas from in house AD plant treating corn stillage with 30–80% CH_4, 20–56% CO_2, 0.2% H_2S						
Chlorella vulgaris SAG 211-11b	Indoor spiral PBR, modified Bold's basal medium, pH 5.5, 25 °C, light intensity 100 µmol/m^2/s	97% RE			53.3% CH_4, 2.5% CO_2, 1% O_2 and 438 ppm H_2S		Mann et al. (2009)
	Raw biogas with 57.5% CH_4, 41% CO_2, 1% O_2 and 438 ppm H_2S						

(continued)

Table 15.2 (continued)

Microalgal species	Cultivation method	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Arthrospira platensis UTEX 1926	1 L glass PBR, defined medium with pH 9.5, 30 °C, light intensity 35.6 µmol/m^2/s, fed-batch	95% carbon utilization efficiency	0.035 g DW/L/day	–	–		Converti et al. (2009)
	Supplied with real biogas with 70–72% CH_4 and 17–19% CO_2						

PBR photobioreactor, *RE* CO_2 removal efficiency, *WWTP* wastewater treatment plant, *DW* dry weight, *Chl.a* Chlorophyll a, *COD* Chemical oxygen demand, *TN* total nitrogen, *TP* total phosphorus, *HRAP*-high rate algal pond

Table 15.3 Biogas upgrading by microalgae with synthetic biogas

Microalgal species	Cultivation conditions	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Microalgal consortium	Outdoor HRAP, year-round study with alkalized centrate as nutrient source	–	22.5 g/m^2/day in august	–	99.6% CH_4 in August, <5 mg m^{-3} H_2S		Marín et al. (2018)
	Simulated biogas $CH_4/CO_2/H_2S$ = 70:29.5:0.5						
Scenedesmus sp.	0.5 L glass bottle PBR, defined medium, continuous illumination at 5.5 Klux, 150 rpm mixing	>96% RE	4.4 g/L	–	>98% CH_4	Lipid rich biomass with 34% lipids by dry weight	Srinuanpan et al. (2018a)
	Simulated biogas CH_4/CO_2 = 60:40						
Chlorella sp.	Outdoor pilot scale HRAP with an absorption column for CO_2-H_2S	50–95% RE	15 g/m^2/day	57–79% TOC	94% CH_4	Complete removal of H_2S, alkalinity highly influenced CO_2 removal	Posadas et al. (2017a)
				80–87% TN			
	Raw untreated centrate			100% $N\text{-}NH_4^+$			
	Simulated biogas $CH_4/CO_2/H_2S$ = 70:29.5:0.5			84–92% TP			
Scenedesmus sp.	0.5 L glass bottle PBR, modified Chu13 medium, continuous illumination at 5.5 Klux, initial biomass 10^7 cells/ml	99% RE, 2.59 g-CO_2/day/L CO_2 fixation rate	2.8 g/L	–	>90% CH_4	Lipid-rich biomass with a lipid productivity of 96 mg/L/day, with desirable fuel properties	Srinuanpan et al. (2017)
	Simulated biogas CH_4/CO_2 = 60:40, gas flow rate of 0.3 L h^{-1}						

(continued)

Table 15.3 (continued)

Microalgal species	Cultivation conditions	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Chlorella minutissima	Indoor 180 L HRAP interconnected to a bubble column for absorption of CO_2-H_2S, agitated at 20 cm s^{-1}, co-current flow, 1500 µmol/m^2/s, 14 h:10 h light/dark cycle	98.8% RE	15 g/m^2/day	83–89% COD	98.5% CH_4		Toledo-Cervantes et al. (2017b)
				98% TN	0.8% CO_2		
					0.7% N_2		
					0.01% O_2		
	Untreated rendering digestate						
	Simulated biogas CH_4/CO_2/H_2S = 70:29.5:0.5						
Chlorella vulgaris FAHCB 31 with fungus *Ganoderma lucidum*	Cylindrical 16.8 L PBR, 25 °C, 200 µmol/m^2/s, mixed LED light red:blue as 5:5, 12 h:12 h light/dark cycle	75.6% RE	0.183 g/L/day	78.09% TOC	–		Cao et al. (2017)
				86.24% TN			
				86.74% TP			
	10% diluted biogas slurry						
	Synthetic biogas, 62–67% CH_4, 37–41% CO_2						
Picochlorum sp. and *Halospirillum* sp.	Indoor 180 L HRAP interconnected to a bubble column for absorption of CO_2-H_2S, modified MSM medium, pH 9.5, high alkalinity, agitated at 20 cm s^{-1}, 500 µmol/m^2/s	91.5% RE	–	–	–	99.5% RE for H_2S	Franco-Morgado et al. (2017a)
	Simulated biogas CH_4/CO_2/H_2S = 70:29.5:0.5 at a flow rate of 22 l/day						

Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 153 mg/L, 25 °C, stepwise increase of light intensity from 400–1000 µmol/m^2/s, red LED light, light dark cycle as 12 h:12 h, mixing by shaking the bag thrice a day Filtered and UV sterilized biogas slurry Synthetic biogas 63–68% CH_4, 31–35% CO_2	–	446.98 mg/L	92.67% TOC 80.87% TN 79.33% TP	92.87% CH_4		Yan et al. (2016a)
Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 180 mg/L, 25 °C, stepwise increase of light intensity and photoperiod, mixing by shaking the bag thrice a day Filtered and UV sterilized biogas slurry Synthetic biogas 60–67% CH_4, 38–43% CO_2	85.46% RE	582.4 mg/L	85.23% TOC 87.1% TN 92.4% TP			Yan et al. (2016b)
Microalgal consortium	Indoor 180 L HRAP interconnected to a bubble column for absorption of CO_2-H_2S, agitated at 20 cm s^{-1}, continuous illumination75 µmol/m^2/s Raw centrate diluted to 1:70 Simulated biogas N_2/CO_2 = 70:30 at a flow rate of 38.7 l/day	99% RE	–	100% TN 82% TP	–	>95% harvesting efficiency because of the algal-bacterial flocs, lipid content 2.9–11.2%	Posadas et al. (2016)

(continued)

Table 15.3 (continued)

Microalgal species	Cultivation conditions	CO_2 removal and utilization	Biomass production	Nutrient removal efficiencies from slurry	Upgraded biogas	Remarks	References
Chlorella vulgaris and nitrifying-denitrifying aerobic activated sludge	Indoor 180 L HRAP interconnected to a bubble column for absorption of CO_2-H_2S, agitated at 20 cm s^{-1}, continuous illumination 104 µmol/m^2/s, 26 °C	80% RE	12 g/m^2/day	51% TOC	–	100% H_2S RE, biomass rich in carbohydrate: 60–76%	Serejo et al. (2015a)
				35% TN			
				86% TP			
	Anaerobically digested vinasse						
	Simulated biogas						
	BM1:N_2/CO_2 = 70:30, BM2: $CH_4/CO_2/H_2S$ = 70:29.5:0.5 at a flow rate of 1.2 m^3 m^{-2} h^{-1}						
Chlorella sp.	Transparent polyethylene bag as PBR, initial biomass of 285 mg DW, 25 °C, 300 µmol/m^2/s, red:blue LED light at ratio 5:5, light dark cycle as 12 h:12 h, mixing by shaking the bag thrice a day	85.29% RE	614 mg/L	85.73% TOC	92.6% CH_4		Yan et al. (2014)
				73.21% TN			
				73.89% TP			
	Filtered and UV sterilized biogas slurry						
	Synthetic biogas 45–65% CH_4						

Chlorella sp. TISTR 8263	1 L glass PBR, modified Chu13 medium, pH 7.8, initial biomass of $10^{7.5}$ cells/ml, 4500 lux	89.3% RE		–	94.7% CH_4	94.7 mg/L/day lipid productivity	Tongprawhan et al. (2014a)
	Synthetic biogas: 50% CH_4, 50% CO_2. At a gas flow rate of 0.03 L/min						
Spirulina platensis and an alkaliphilic H_2S oxidizing bacterial culture	Indoor 180 L HRAP interconnected to a bubble column for absorption of CO_2-H_2S, agitated at 20 cm s^{-1}, continuous illumination 80 μE/m^2/s, 26 °C, 10 pH	40% RE	1.2 g/L	–	–	100% H_2S RE, algal-bacterial biomass with a biomethane yield of 0.21–0.27 L/g volatile solids	Bahr et al. (2014)
	Anaerobically digested sludge from WWTP						
	Simulated biogas N_2/CO_2 = 70:30, H_2S =500/1000/5000 ppm						

PBR photobioreactor, *RE* CO_2 removal efficiency, *WWTP* wastewater treatment plant, *DW* dry weight, *Chl.a* Chlorophyll a, *COD* Chemical oxygen demand, *TN* total nitrogen, *TP* total phosphorus, *HRAP* high rate algal pond

is questionable and the carryover of microorganisms from the AD slurry or even in biogas is possible, such biological consumption of methane could be attributed to microorganisms other than microalgae. A marine microalga *Nannochloropsis gaditana* CCMP 567 (wild type) was grown in methane concentrations of 0%, 50%, and 100%, and it was found that the biomass concentrations and specific growth rate (1 g/L and 0.1 day^{-1}, respectively) were not affected by the increasing concentrations of methane (Meier et al. 2015). Three microalgal strains, *C. protothecoides* TISTR 8243, *Chlorella* sp. TISTR 8263, and marine *Chlorella* sp., capable of high growth potential in 50% CO_2, were evaluated for their ability to grow in the presence of 50% CH_4 and 50% CO_2 simulating the biogas composition (Tongprawhan et al. 2014a). Of these the marine *Chlorella* sp. fared well, with no significant differences in biomass and lipid production in the presence of 50% CH_4. The CO_2 removal efficiency from 50% CO_2 in air and 50% CO_2 in methane were 70.4% and 68.9%, respectively (Tongprawhan et al. 2014a). Another related study for screening microalgae for tolerance to high levels of CH_4 in biogas led to the isolation of a *Scenedesmus* sp. with high biomass and lipid productivity. *Scenedesmus* sp. showed 99.3% CO_2 removal efficiency in simulated biogas (CH_4:CO_2 = 60:40), with a CO_2 fixation rate of 2.59 g-CO_2 day/L (Srinuanpan et al. 2017). The biomass concentration and lipid productivity were estimated to be 2.83 g/L and 96.18 mg/L/day, respectively, with lipids that could produce biodiesel with high stability and ignition quality (Srinuanpan et al. 2017). Other than the wild types, mutant strains were developed by random mutagenesis for tolerance to CH_4. A mutant *Chlorella* sp. MM-2 was developed by random mutagenesis which was resistant to up to 80% CH_4 retaining 70% of the growth potential compared to growth in the absence of CH_4, with a biomass productivity of 0.116 g/L/day (Kao et al. 2012a). Another mutant, *Chlorella* sp. MB-9, also could grow in the presence of 80% CH_4 and 20% CO_2 retaining 82% of growth potential and biomass productivity of 0.243 g/L/day (Kao et al. 2012b). It has been shown that even in biogas-tolerant strains, presence of moderate levels of CH_4 in the range of 45–55% can enhance biogas upgrading (Yan et al. 2014).

The second important component in the biogas that could severely influence the outcome of biogas upgrading is H_2S. The concentrations of H_2S in biogas vary from 0 to 10,000 ppm (Table 15.1), and dissolution of H_2S in the culture medium could reduce the pH of the medium drastically inhibiting microalgal growth. The tolerance of microalgae to H_2S could be attributed to the presence of certain sulfur oxidizing bacteria carried over from the AD slurry. A *Scenedesmus* sp. was reported to be tolerant to H_2S up to 3000 ppm with complete removal of CO_2 and H_2S. However, it must be noted that the microalga was grown in raw unsterilized AD digestate with the fermentation microbes from the AD process (Prandini et al. 2016). A high rate algal pond (HRAP) at pH 10 with *Spirulina platensis* and an alkaliphilic H_2S oxidizing bacterial consortium could remove up to 5000 ppm H2S effectively, and it was unaffected by the presence of other components in the AD slurry used as nutrient source (Bahr et al. 2014). Another HRAP harboring *Chlorella vulgaris* and nitrifying-denitrifying activated sludge showed 100% removal efficiency for 0.5%

v/v H_2S (Serejo et al. 2015a). Also, mutant microalgal strains resistant to biogas level H_2S has not been isolated yet. Since desulfurization of biogas is a routine procedure of biogas purification for feeding into the CHP stations, most studies use desulfurized biogas where the H_2S concentrations are reduced to 50–100 ppm to which most microalgae are generally tolerant (Table 15.2).

The CO_2 removal efficiencies of microalgae from biogas are in the range of 50–99% based on the culture conditions and the microalgae used (Tables 15.1 and 15.2). All experiments based on simulated biogas used CO_2 at 30%, and it was efficiently removed by microalgae. Algal bacterial bioreactors or microalgae with fungi or bacterial co-culture performed better than mono-algal culture, due to the synergistic effect of bacteria on algal growth and their pollutant removal efficiency to an extent. A wild-type strain *Nannochloropsis gaditana* which can tolerate up to 100% methane was inhibited by CO_2 concentrations of 9% (Meier et al. 2015). *Chlorella vulgaris*, *Chlorella protothecoides*, *Chlorococcum* sp., *Chlorella* sp., and *Scenedesmus armatus* were evaluated for their biogas upgrading potential by growing in 50% CO_2 in air under phototrophic conditions. Of these, the marine *Chlorella* sp. TISTR 8263 showed better tolerance to 50% CO_2 with a specific growth rate, biomass content, lipid content, and lipid productivity of 0.457 day^{-1}, 601 mg/L, 28.2% DW, and 21.3 mg/L/day, respectively (Tongprawhan et al. 2014b). A similar screening was performed with another set of strains comprising freshwater *Chlorella* sp., marine *Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Botyrococcus* sp. (Srinuanpan et al. 2017). The culture was phototrophic with 40% CO_2, and among the strains *Scenedesmus* sp. showed better performance based on biomass, lipid content, and lipid productivity. Even though the lipid content of *Botyrococcus* sp. (42%) was higher than *Scenedesmus* sp. (27%), the specific growth of *Botyrococcus* sp. was the lowest at 0.21 day^{-1} thereby reducing the lipid productivity (Srinuanpan et al. 2017). In the presence of 60% CH_4, *Scenedesmus* sp. showed 98% CO_2 removal efficiency, escalating the methane content in the simulated biogas to 99.39% (Srinuanpan et al. 2017). Three microalgae, *Chlorella vulgaris* FACHB 31, *Scenedesmus obliquus* FACHB 416, and *Neochloris oleoabundans* UTEX 1185, were co-cultured with activated sludge on biogas slurry and evaluated for CO_2 and H_2S removal from biogas. The CO_2 removal efficiency was over 95% for all the strains 45–55% CO_2 and 55–75% CH_4. The H_2S present in the simulated biogas were also removed from the biogas at an efficiency ranging from 70% to 80% (Sun et al. 2016). Hence CO_2 tolerance and carbon fixation efficiency are highly strain dependent. As it can be seen form Tables 15.1 and 15.2, *Chlorella* sp. dominate the scene for biogas upgrading, closely followed by *Scenedesmus* sp. *Chlorella* sp. are known to be robust, easily adaptable to any environment with higher growth rates. *Chlorella vulgaris* is known to be rich in proteins, lipids, carbohydrates, pigments, antioxidants, and other vitamins and minerals (Safi et al. 2014). They are a perfect feedstock for any valuable product generation and the methodology has been perfected over the years. *Scenedesmus* sp. are also appreciated as potential bio-mitigation candidates and can be applied for biogas upgrading (Ho et al. 2010).

6 Factors Affecting Nutrient Removal from the Effluents of AD and DF

Microalgal biomass can be obtained by cultivation in open systems or closed photobioreactors (PBR). Open pond systems are the most preferred method for microalgal cultivation, because of its inexpensive nature and easier methods. However, the stringent requirements for pharmaceutical compounds insist the use of closed PBRs for axenic cultivation of specific microalgae, which will yield the desired product of interest (Chang et al. 2017). PBRs also offer the advantage of proper control of the process parameters like temperature, pH, light intensity, and mixing. It must be noted that the optimal process parameters for the cultivation is chosen based on the microalgal strain used. Biogas upgrading by microalgae works on the same principle, and optimization of the process occurs based on the microalgal strains used. Here we discuss some important external factors affecting microalgae-based biogas upgrading.

6.1 Light Intensity

Light intensity is essential for microalgal cultivation, and the supply of optimal light intensity is one of the major challenges in microalgal cultivation. Light is the source of energy for photosynthesis, the primary metabolism in microalgae. Increase in light intensity may result in light limitation and subsequent inhibition of growth, while a decrease in light intensity cannot sustain biomass growth. It has also been shown that light intensity is a key factor regulating lipid accumulation in microalgae. Under high light intensities, lipid accumulation serves as an electron sink for the over-reduced photosynthetic apparatus (Liu et al. 2012). *Scenedesmus* sp. 11-1 accumulated 40% by weight as lipids under a light intensity of 400 μmol m^{-2} s^{-1}, while only 26% was obtained at 40 μmol m^{-2} s^{-1} light intensity (Liu et al. 2012). Lipid synthesis requires uninterrupted supply of ATP and NADP(H), which is provided by photosynthesis under high light intensities, simultaneously protecting the cells from photo oxidative damage (He et al. 2015). The neutral lipid content of both *Chlorella* sp. L1 and *Monoraphidium dybowskii* Y2 were higher at high light intensities, which was 71% and 60% of the total lipids at 100 μmol m^{-2} s^{-1} (He et al. 2015). Also, high light intensities (600 μmol m^{-2} s^{-1}) resulting in moderate photoinhibition could promote neural lipid accumulation in *Pseudochlorococcum* sp. LARB-1 (Li et al. 2011a). However, high light intensities could inhibit the uptake of organic carbon in microalgae (Perez-Garcia et al. 2011). *Chlorella sorokiniana* UTEX 1230 could rapidly import and metabolize glucose in the absence of light under heterotrophic growth conditions with a 9 h doubling time, accumulation of 39% total lipids and TAG productivity of 28.9 mg L/day. In the presence of light under mixotrophic conditions, TAG productivity was reduced to 18.2 mg L/day (Rosenberg et al. 2014). Light was also known to inhibit glucose uptake in *Chlorella*

vulgaris, even in a non-photosynthetic mutant (Kamiya and Kowallik 1987). Low light intensities under mixotrophic conditions might help overcome light inhibition of both photosynthesis and organic carbon uptake, making it an effective strategy for microalgal cultivation in the presence of VFAs (Chen et al. 2018). Also, choosing the strains without light inhibition is of importance in mixotrophic cultivation (Perez-Garcia et al. 2011). Moderate light intensities were preferred for efficient biogas upgrading in microalgae. *Scenedesmus* sp. obtained high nutrient removal rates from biogas slurry at moderate light intensities of 150–170 μmol m^{-2} s^{-1} (Ouyang et al. 2015). *Chlorella* sp. showed higher biogas CO_2 removal and better biogas upgrading at 350 μmol m^{-2} s^{-1} compared to 400 μmol m^{-2} s^{-1} (Yan and Zheng 2013). Illumination of the microalgal culture with lights at different wavelengths revealed that red light was found optimal for *Chlorella* sp.. Some studies indicate an optimal light intensity of 400–1000 μmol m^{-2} s^{-1} (Yan et al. 2016a), while another related study reported an optimal light intensity if 1200–1600 μmol m^{-2} s^{-1} (Zhao et al. 2013). A mixture of red and blue lights at a ratio of 5:5 was shown to be optimal for many studies (Yan et al. 2014, 2016b; Zhang et al. 2017b; Yan and Zheng 2014). Also, moderate photoperiod of 14 h light/10 h dark was preferred for biogas upgrading by *Scenedesmus obliquus* FACHB-31 (Wang et al. 2016) and *Chlorella* sp. (Yan and Zheng 2013). The introduction of photoperiod for the culture of *Chlorella sorokiniana* enhanced biogas CO_2 removal even in the dark conditions, and the authors speculated that a decrease in the culture temperature in the dark can increase CO_2 solubility with biogas CO_2 removal even in dark periods (Meier et al. 2017). The photoperiod or difference in light/dark periods did not influence biogas upgrading by an alkali-tolerant microalgal culture of *Picochlorum* sp. and *Halospirulina* sp. in a high rate algal pond (Franco-Morgado et al. 2017b).

6.2 Culture pH

The pH of the culture medium is another important factor governing microalgal growth, metabolism and other cellular functions. The optimal pH for each microalgal strain might vary depending on the natural habitat and subsequent laboratory conditions for which they were primarily adapted. Variations in the medium pH might interfere with nutrient uptake, as pH of the medium determines the available form of inorganic carbon as CO_2 or bicarbonates (Juneja et al. 2013). Alkaline conditions are best suited for biogas upgrading by microalgae, as alkaline conditions can enhance the solubility of CO_2 from biogas. Under alkaline AD conditions, the CO_2 generated in the fermentation process remains as dissolved carbonate in the fermentation medium generating highly pure biogas (Nolla-Ardevol et al. 2015). Also alkaline conditions could promote the absorption of other impurities present in biogas via chemical reactions (Franco-Morgado et al. 2017a). Maintenance of the medium pH at slightly alkaline conditions of pH 7.8 enhanced CO_2 removal from biogas by *Chlorella* sp. TISTR 8263 (Tongprawhan et al. 2014b). The pH of AD effluents ranges from acidic to alkaline depending on the process conditions and

microbial inoculum, so the microalgal cultivation medium pH should be maintained at the optimal pH of the microalga cultivated. Also, VFAs uptake by microalgae together with CO_2 solubilization from biogas might reduce the medium pH drastically which could be highly inhibitory for microalgal growth (Chen et al. 2018). Microalgal photosynthesis makes the medium alkaline, and hence maintenance of the optimal pH via acidification of the medium could be required. Maintaining the optimal pH of the cultivation medium at 7 greatly enhanced the biomass productivity and nutrient removal efficiency of *C. vulgaris* when cultivated in undiluted AD effluent of activated sludge. In pH controlled cultures (maintained at pH 7), a biomass productivity of 433 mg/L/day was achieved, whereas in pH uncontrolled cultures biomass productivity was reduced to around 296 mg/L/day (Cho et al. 2015). High ammonia concentrations and the high pH (pH = 9) in piggery wastewaters could also affect microalgal growth and nutrient removal efficiency (Tan et al. 2016). It was also observed that the high pH levels could protect the microalgal culture from extraneous contaminants, and alkaline pH could be considered as a stress factor for triggering lipid accumulation in microalgae (Bartley et al. 2014). It has been shown that the CO_2 removal efficiency of an algal-bacterial co-culture comprising of *Chlorella* sp. and activated sludge increased from 23% at pH 7 to 62% at pH 8.1 (Lebrero et al. 2016). However, for microalgae that have an optimal around 6–7, pH over 9 is severely inhibitory. The culture pH of a microalgal consortium grown in undiluted piggery wastewater was maintained under 8 with CO_2 acidification for effective nutrient removal and biogas upgrading (Ayre et al. 2017).

6.3 Temperature

Temperature is another important factor governing microalgal growth and beneficial product accumulation in microalgae. In biogas upgrading by microalgae, temperature of the process is mainly chosen based on the optimal growth temperature of the microalgal strain. Biogas is fermentation off-gas of AD process, and it is at ambient temperature. Hence, cooling of the biogas to reduce the temperature or selection of thermotolerant microalgal strains is not needed. As it can be seen form the table on biogas upgrading, most of the processes occur at ambient temperature or the temperature being controlled at the optimum level of the microalgal strain. An attempt to determine the optimal temperature for biogas upgrading by *Leptolyngbya* sp. indicated that temperature influences the biomass growth, but not biogas upgrading by carbon capture (Choix et al. 2017). A central composite design for the determination of optimal temperature and light intensity revealed that light intensity significantly influences carbon capture and carbon assimilation, while the effect of temperature is statistically insignificant for the same. *Leptolyngbya* sp. is known to grow in a temperature range of 20–45 °C, and an optimal temperature of 27.1 °C was best suited for biogas upgrading and biomass accumulation (Choix et al. 2017). However, temperature is also known to influence the solubility of biogas CO_2 in the culture medium. The growth rate of *Chlorella sorokiniana* increased during

the light period of light/dark cycle (12 h:12 h), when grown on M8a medium using biogas (65% CH_4, 32% CO_2) from a laboratory scale brewery wastewater AD process (Meier et al. 2017). The authors also stated that CO_2 solubility is inversely related to the culture temperature; as temperature decreases, solubility increases and vice versa. Hence, CO_2 solubility, desorption, and accumulation performs well under dark conditions and that biogas feeding can be continued in the dark period to enhance biogas upgrading (Meier et al. 2017). The effect of temperature on organic acid accumulation by microalgae has not been studied in detail; however it has been stated that suboptimal temperatures are preferred for growth on inhibitory VFAs like butyrate, since optimal or close to optimal temperatures can exacerbate the inhibitory effect (Turon et al. 2016).

7 Bottlenecks in Microalgae-Based Biogas Upgrading and Future Perspectives

Microalgae are currently being touted as the ultimate solution for most pressing problems like global warming, climate change, and the search for alternative energy. Some researchers feel that microalgae could not fit the bill as a potential carbon mitigation candidate or as an effective carbon sink for emission reduction due to the difficulties in longtime carbon storage (Acien Fernandez et al. 2012). Still, they are the best known sustainable alternative for biofuels, pigments, and fatty acids. Also, microalgae can be effectively used for the treatment of various wastewaters before release into the environment. Competent design of microalgal cultivation in wastewaters with minimal requirement of valuable resources like water, nutrients, or CO_2 can greatly enhance the energy balances of wastewater treatment and turn them into potential power houses (Menger-Krug et al. 2012). In this book chapter, we discussed extensively about the integration of microalgal cultivation with anaerobic digestion as a solution for treating nutrient-rich AD slurry with concomitant improvement in biogas quality. The microalgal biomass composition can be manipulated (high carbohydrate/lipid) by carefully controlling the process parameters and the microalgal strain chosen for cultivation (Srinuanpan et al. 2018a; Serejo et al. 2015b). Also, they make an excellent feed for aquaculture or animal husbandry. The major problem associated with any microalgae-based bioremediation/energy generation system is the constraints in commercialization of the proof-of-concept level studies carried out under controlled laboratory conditions with skilled personnel. Technology carryover to commercialization at this stage should also consider the economics of the process, cost competitiveness with available alternatives in the market, and the availability of skilled individuals for operation. Biogas upgrading by microalgae also face considerable challenges at the cultivation level, and some of the major bottlenecks in biogas upgrading by microalgae and the potential solutions are listed in Table 15.4.

Table 15.4 Bottlenecks faced in biogas upgrading by microalgae and possible solutions

Challenges faced	Potential solution
High concentrations of CO_2 in the biogas (30–50%)	Selection of a high CO_2 tolerant microalgal strain
	Genetic engineering of available strains for CO_2 tolerance
	Regulate the inflow of biogas in the culture to allow optimal biomass production
Presence of very high concentrations of methane (>60%) in the biogas	Select methane-tolerant microalgae
	Genetically engineered methane tolerance in microalgae
	Adapt various biogas feeding strategies to control influent methane concentrations
Toxicity to microalgae due to the presence of H_2S in biogas	Selection of tolerant strains
	Cultivation under alkaline conditions for chemical conversion of sulfide to sulfates
	Maintaining a nontoxic level of H_2S in the influent biogas (<5 mg/L)
	Desulfurizing prior to injection into microalgal culture
High COD level of AD slurry impairs light penetration in microalgal cultures	Preliminary pretreatment to remove suspended solids and particulate matter, improve slurry quality
	Mixotrophic/heterotrophic cultivation without light energy
	Use diluted slurry instead of undiluted or highly concentrated slurry
Presence of inhibitory or non-utilizable VFAs in the slurry	Increase the food to microorganism ratio for effective uptake of inhibitory VFA
	Adapt efficient lighting strategy as light could sometimes inhibit VFAs uptake in microalgae
Energy and cost-intensive sterilization of the slurry	Chose microalgal strain with robust growth characteristics to overcome competitive bacteria
	Cultivation under alkaline conditions to keep off common contaminants
Expensive microalgal culture methods	Effective outdoor culturing
	Resource efficiency
	Manipulating biomass composition to achieve valuable coproducts
	Obtained biomass as a feedstock for AD in a biorefinery concept
	Complete energy recovery from the biomass by thermochemical conversion methods
	Combustion of microalgal biomass for residual energy generation

Integration of microalgal cultivation with AD is mainly challenging due to the difficulties in choosing an appropriate algal strain that is capable of mixotrophic growth in the presence of organic and inorganic carbon. In our previous review, we

have pointed out the essential qualities required in a microalgal strain to be used for biogas upgrading and nutrient removal from slurry: (1) the strain should be capable of mixotrophic growth, utilizing both inorganic and organic carbon under low light intensities; (2) the strain must possess robust growth properties, with high tolerance to extreme conditions like high CO_2, high CH_4, variations in pH, and certain toxic compounds present in biogas; and (3) the strain should be capable of accumulating higher levels of either carbohydrates or lipids for subsequent energy generation in the form of biofuels. Apart from these characteristics of the microalgal strain, the cultivation process itself needs to be optimized based on the chosen strain minimizing energy input and lowering the carbon footprint of the total system (Chen et al. 2018). Microalgae are very diverse with very flexible metabolic potentials, and hence it has always been the way to prospect for microalgal strains in natural habitats that could be used in a particular process. A *Scenedesmus* sp. was isolated from an open pond in a wastewater treatment plant for effective nutrient removal from swine water digestate. The microalga could grow well in raw unsterilized digestate, with a maximum CO_2 assimilation from biogas at 219 mg/L/day (Prandini et al. 2016). A *Chlorella vulgaris* strain was isolated form an open pond used for the storage of vinasse in a sugar industry. The strain was slowly acclimated for growth in vinasse AD digestate for 21 days under optimal light intensity of 61 μmol m^{-2} s^{-1} before being introduced in an HRAP for biogas upgrading (Serejo et al. 2015b). On the other hand, acclimation of the microalgal cultures for growth in biogas slurry has been carried out. A co-culture of *C. vulgaris* FAHCB31 with fungi *Ganoderma lucidum* and *Pleurotus ostreatus* were acclimated or slowly adapted to diluted biogas slurry until the cells were tolerant to slurry conditions along with higher growth rates, which were then used for slurry nutrient removal (Cao et al. 2017). Specific genetic engineering of microalgae for tolerance to biogas components or flue gas components have not been performed yet due to the unclear nature of the mechanisms involved. However, random mutagenesis has been performed to improve tolerance to high CO_2 or high methane concentrations (Kao et al. 2012a, b). Thus, choice of the microalgal strain is crucial for the success of the integrated AD-microalgal system. Another efficient strategy would be to control the inflow rate of biogas for low tolerance strains, which could improve the biomass production. Biogas might contain up to 70% methane based on the feedstock used, and hence control of biogas loading in the culture is a practical way to overcome methane inhibition. Nutrient removal from slurry and CO_2 removal from biogas by *Chlorella* were shown to be higher when the influent methane concentration ranged from 45% to 55% (Yan et al. 2014). The other important component present in biogas that could inhibit microalgal growth is hydrogen sulfide (H_2S). Desulfurization of biogas is a common biogas cleaning step, and hence most of the studies applied desulfurized biogas for microalgal cultivation. The presence of 5 mg S/L reduced the photosynthetic oxygen production rate of a microalgal consortium by 43%, and inhibitory effects were observed above a sulfide concentration of 20 mg S/L (Gonzalez-Camejo et al. 2017). The sulfides present in the biogas were known to be converted to sulfates under alkaline conditions and illumination, and sulfates were shown to be assimilated by *Chlorella* sp. resulting in enhanced growth rates (González-Sánchez and Posten 2017).

Liquid digestates are very high in COD, in the range of 210–6900 mg/L (Xia and Murphy 2016b). Such high COD levels results in increased turbidity of the liquid, which will severely affect light penetration in microalgal cultures. Hence, cultivation of microalgae under low light intensities in mixotrophic mode is a viable option for overcoming this hindrance. Also, mixotrophic mode enables microalgae to utilize both organic and inorganic carbon sources in the presence of light. The initial growth utilized the organic carbon via respiration, and when the organic carbon level decreases, photosynthesis is initiated, resulting in higher biomass productivities compared to autotrophic and heterotrophic modes of cultivation (Zhan et al. 2017). *C. vulgaris* was shown to grow with glucose as carbon source under respiratory mode, and in the presence of light, it is funneled to lipid synthesis, increasing lipid productivities under mixotrophic mode. The lipid productivity of *C. vulgaris* UTEX 259 under photoautotrophic mode and mixotrophic mode with glucose were shown to be 4 mg/L/day and 54 mg/L/day, respectively, under similar light intensities (Liang et al. 2009). Liquid digestates are also rich in total nitrogen (TN – 139–3456 mg/L) and total phosphorus (TP – 7–381 mg/L), with over 90% of both available as ammonia and phosphates (Xia and Murphy 2016b). Such high concentration of N and P combined with the high COD could be inhibitory to microalgal growth, and hence dilution of the digestate to obtain optimal level of these essential nutrients in the culture medium can improve light penetration as well. *S. obliquus* (FACHB-31) was grown in piggery wastewater AD digestate with a COD of 3200 mg/L, and the COD, N, and P removal efficiencies were 65%, 63% and 71%, respectively, with a biomass productivity of 241 mg/L/day. When the liquid digestate was diluted to COD 1600 mg/L, the COD, N, and P removal efficiencies increased to 75%, 74% and 81%, respectively, with a simultaneous increase in biomass productivity of 311 mg/L/day (Xu et al. 2015). Similarly, *C. vulgaris* (FACHB-31) performed well in biogas upgrading and nutrient removal from sewage treatment plants when the COD and total nitrogen levels were maintained at relatively lower concentrations (Xu et al. 2017). The total COD, N, and P removal efficiencies were 72%, 71%, and 69% with a medium influent COD of 200 mg/L, and the biogas methane was increased to 92% from 67%. When the COD level raised to 400 mg/L, the removal efficiencies were considerably lower. And when the total nitrogen was maintained at a medium level of 40 mg/L, the total COD, N, and P removal efficiencies were 77%, 77%, and 73% respectively, with an increase in biogas methane to 93%, and it was considerably higher compared to the high TN levels of 80 mg/L (Xu et al. 2017). Thus, maintaining the COD and TN concentrations in optimal levels enhances both light penetration and nutrient removal efficiencies. Another interesting option is to isolate microalgal strains from local environments that could be resistant to the extreme conditions of the digestate to be treated. A *Chlorella* strain was isolated from centrate (highly concentrated municipal wastewater), and the strain was able to grow in raw centrate with COD, TN, TP, and ammonia removal efficiencies of 90.8%, 89.1%, 80.9%, and 93.9%, respectively. The lipid-rich algal biomass obtained could be used for biodiesel production with a yield of 0.12 g-biodiesel/ for every liter of algae culture (Li et al. 2011b). The microalgal culture was scaled up to 25 L in a coil reactor, and a net

biomass productivity of 0.92 g algae/L/day was achieved (Li et al. 2011b). Isolation and screening of microalgae specifically for remediation of wastewater might increase the chances of success for economic biofuel production (Li et al. 2011c; Zhou et al. 2011). Enhanced COD levels of liquid digestate are mainly due to the presence of increased concentration of VFAs as well. Acetate is the primary VFA present in most digestates, in addition to butyrate, isobutyrate, propionate, and valerate, along with certain alcohols. As discussed in detail in Sect. 4, butyrate and lactate are known to be inhibitory to microalgal growth at concentrations above 0.1 g/L and 0.5 g/L, respectively. An increase in the food-to-microorganism ratio or an increase in the acetate/butyrate ration can aid in overcoming the inhibition, and the details are discussed in Sect. 4 as well. Light intensity is also known to affect the uptake and utilization of organic acids by microalgae, and hence optimization of light intensity should be carried out while determining the process parameters for microalgal cultivation in AD slurry.

Another major issue in the use of AD digestate for microalgal cultivation is the carryover of pathogenic or harmful anaerobic bacteria from the AD process. The microbial community in AD process is very diverse, ranging from facultative anaerobes to obligate sporulating anaerobes that could survive extreme environmental conditions, and it could be present in the slurry after processing. Hence, autoclaving is essential to destroy the pathogenic bacteria (Zhu et al. 2016), but it is both expensive and energy intensive. If the anaerobic fermentation is carried out with a single nonpathogenic bacterium like dark fermentation for hydrogen production, pretreatment of effluent for pathogen removal could be deemed unnecessary. *C. sorokiniana* was cultivated in undiluted raw dark fermentation effluent containing acetate and butyrate under heterotrophic mode. *C. sorokiniana* grew efficiently in the raw effluent consuming acetate, and the presence of any contaminating bacteria present in the raw effluent did not affect the biomass productivity when compared to the use of sterile effluent (Turon et al. 2015a). Raw unsterilized centrate has also been used for biogas upgrading and slurry nutrient removal by *Scenedesmus* sp. (Prandini et al. 2016) and *Chlorella* sp. (Posadas et al. 2017b).

The inability to produce microalgae-based bioproducts and biofuels in a cost-competitive manner compared to the available products in the market is a major barrier in commercialization of the same, mainly owing to the high cost associated with microalgal cultivation, harvesting, and processing. This applies to integration of microalgal cultivation to biogas upgrading, and effective measures needs to be taken for economic cultivation of microalgae. Outdoor cultivation of microalgae is known to be economic and has been adapted by various commercial organizations for mass production. Culture contamination could be prevented by growing the microalgae in alkaline conditions, which is inhibitory to many of the common contaminants. Biogas upgrading has been performed in pilot scale in HRAPs under alkaline conditions with the use of a microalgal consortium comprising of *Leptolyngbya lagerheimii*, *Chlorella vulgaris*, *Parachlorella kessleri*, *Tetradesmus obliquus*, and *Chlorella minutissima* (Marín et al. 2018). The alkaline conditions helped increase the solubility of CO_2, and summer months proved to be best for both nutrient removal and biogas upgrading. Maximum biogas removal occurred in May with

the resultant biogas with 0.1% CO_2, while the biogas with highest methane concentrations (99.6%) was achieved in August (Marín et al. 2018). *Chlorella pyrenoidosa* FACHB-9 was cultivated in outdoor rectangular photobioreactors on anaerobically digested activated sludge, and effective nutrient removal was achieved in summer months. The authors also proposed an innovative method to control contamination: by cutting off CO_2 supply intermittently the medium pH tends to rise to 8.5–9.8 before resuming CO_2 supply, which would inhibit contaminants (Tan et al. 2015). Other simulated and outdoor pilot scale studies have been reported indicating the feasibility of outdoor cultures for AD waste treatment (Tan et al. 2016; Posadas et al. 2017b; Sheets et al. 2014). Water is an essential resource required for microalgal cultivation and the water footprint of microalgae-based biodiesel production ranges from 1600 to 3360 L water/L biodiesel without recycling (Farooq et al. 2015). Recycling is an effective way to reduce the water footprint of microalgal cultivation, and care should be taken about the carryover of growth-inhibiting substances present in the recycled water resulting in the crash of the cultivation. Microalgal allelopathy is a well-reported phenomenon, and the secondary metabolites released by certain harmful algae can totally inhibit other related algae (Bacellar Mendes and Vermelho 2013). Even in the absence of harmful bacteria and allelopathy, harvest water can be recycled only once or twice based on the buildup of growth inhibitory substances in harvest water (Zhu et al. 2016). Extracellular polysaccharides and certain nitrogen-rich small organic molecules were observed to accumulate in the recycled culture media of *C. vulgaris*, and the water was recycled for over 60 days without any significant inhibition of growth (Hadj-Romdhane et al. 2013). The liquid digestate could provide other essential nutrients like N, P, and carbon in the place of expensive fertilizers, making the cultivation more economic.

Proper utilization of the biomass obtained can greatly improve the economics of microalgal cultivation, and proficient harvesting and downstream processing techniques are pivotal for cost-cutting measures. It has been reported that algal cultivation, harvest, and dewatering might contribute to up to 70% of the production costs of any algae-based product. So effective harvesting and dewatering strategies with lower energy input might help decrease the associated production costs (Chen et al. 2011). The composition of the biomass obtained (lipid/carbohydrate/protein rich) might vary depending on the microalgal strain chosen, and proper control of process parameters can result in high accumulation of beneficial component. Carbohydrate-rich biomass can be used for production of biofuels like bioethanol, biobutanol, and biohydrogen by fermentation of the sugars released after simple pretreatment (Serejo et al. 2015b; Nwoba et al. 2016). Lipid-rich algae can be used for the production of biodiesel (Srinuanpan et al. 2018b). Microalgal biomass rich in protein can be used as animal feed components (Singh et al. 2011). The residual biomass after product extraction can be processed by a number of thermochemical ways like pyrolysis, hydrothermal liquefaction, gasification, or torrefaction for complete energy recovery from the biomass (Chen et al. 2015). Pyrolysis resulting in the production of algal biochar has been proposed as the most effective option for the treatment of residual biomass in integrated AD-microalgal cultivation systems, since soil amendment of

biochar can result in closing of the carbon cycle (Chen et al. 2018). Also, biochar can be used as a sustainable adsorbent for the removal of various harmful pollutants (Ho et al. 2017). Another similar study integrates microalgal cultivation with biogas production, and the microalgal biomass obtained was returned as AD feedstock in a loop. Life cycle analysis indicated that the use of obtained algal biomass as AD feedstock has a net energy ratio of 1.54, with reduced land use changes compared to other terrestrial crops. This strategy could help increase the annual biomethane production of the proposed Sweden biogas plant by 9.4% (Wang et al. 2013). An integrated AD of distillery stillage with microalgal cultivation providing biogas upgrade and nutrient removal was proposed. The microalga *Chlorella* sp. consumed CO_2 from simulated flue gas and raw biogas in the range of 2–50%, simultaneously removing ammonia from biogas slurry with higher growth rates (Doušková et al. 2010). The obtained biogas was designed to be used at the plant for heat and electricity, while the microalgal biomass obtained was to be processed as food or feed supplement making this a closed technology (Doušková et al. 2010). Integration of microalgal cultivation with AD of cattle manure resulted in an annual production of 160–190 ton of microalgal biomass, with valuable components like lipids, proteins, and carbohydrates (Ledda et al. 2016). Hence, integration of microalgal cultivation with biogas production is technically feasible and economically viable. A possible integration scenario of microalgal cultivation with AD is illustrated in Fig. 15.3. All the specifics had been discussed previously, and the integration will be sustainable and beneficial giving high precedence to the following: choice of microalgal strain (preferably indigenous and robust), effective upstream and downstream process design, and complete energy recovery from the resultant biomass.

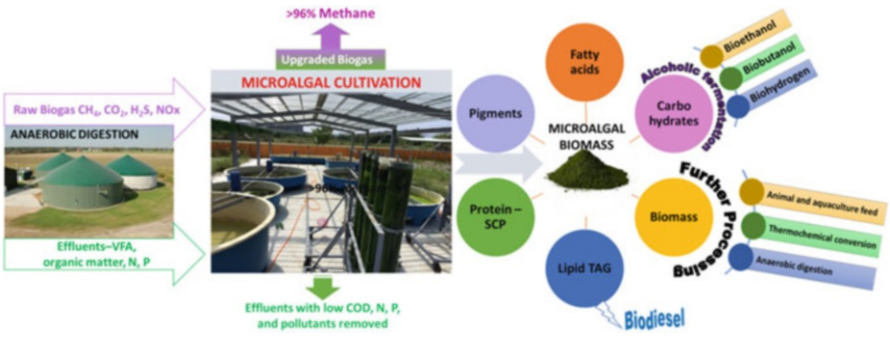

Fig. 15.3 Schematic illustration of the integration of anaerobic digestion with microalgal cultivation

8 Conclusions

Microalgae-based carbon capture is a superior method for biogas upgrading in terms of environmental impacts and process operating conditions, when compared to the available chemical-based methods. The major constraint in realizing the potential of the technology is the development of cost-competitive methods for commercialization. With a boom in biogas production plants particularly in Europe and the USA, simultaneous development of microalgal technology to suit the needs of the biogas industry is essential. Isolation of an indigenous strain capable of tolerating the extreme condition of biogas slurry and biogas is pivotal for biogas upgrading. The existing information gap between microalgal genetic engineering and biogas upgrading needs much research attention, developing genetically engineered strains for excellent carbon capture from biogas- and slurry-based nutrient removal. With the ideal strain, the desired metabolic potential and optimal process parameters, outstanding performances in biogas upgrading, and bioremediation of biogas slurry can be accomplished. The biomass composition of the obtained microalgal biomass can help reduce the economic burden of the process, and the production of value-based chemicals in a biorefinery-based concept could be pragmatic. The return of the obtained biomass to soil in the form of biochar or fertilizer can help decrease carbon footprint of the system with long-term carbon sequestration. It has been shown that many closed loop sustainable technologies for microalgal cultivation and biogas upgrading have been carried out at the pilot scale. Commercial-scale carbon capture from flue gas has already been established at the St. Mary's cement factory in Canada, and similar attempts are needed in biogas plants for the realization of microalgae-based biogas upgrading.

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Chapter 16
Anaerobic Digestion of Microalgae Biomass for Methane Production

Hossain M. Zabed, Xianghui Qi, Junhua Yun, and Huanhuan Zhang

Abstract Biomethane is one of the most promising biofuels that is produced from a wide variety of biomass using anaerobic digestion (AD) process. Microalgae, among these biomass sources, have received significant attention since the past years due to their rapid growth rate, capability of accumulating different biomolecules, effective CO_2 sequestration, and requirement of relatively small land area. However, despite these advantages of microalgae and potential of AD, conversion of microalgae into methane is bottlenecked by the low biomass loading and recalcitrance of digestible components, low C/N ratio, and interferences of various factors. Eventually, it is necessary to make effective efforts for addressing the shortcomings of the overall process to achieve a state-of-the-art technology for commercial scale methane production. This chapter will discuss the major aspects of biomethane production from microalgae focusing on the potential of these biomass sources for methane production, technical aspects in the conversion of microalgae into methane, and factors affecting methane yield in AD of microalgae.

Keywords Biofuels · Microalgae · Biomethane · Pretreatment · Microalgae cultivation · Anaerobic digestion · Biogas · Methanogenesis

1 Introduction and Overview

The critical dependence of human societies on fossil fuels for meeting energy demands has triggered much interests in alternative fuels to mitigate the growing concerns with natural gas and petroleum oils, pertaining to energy insecurity, greenhouse gas emissions, and climate change (Zabed et al. 2016a, b, 2017a). Biogas or more specifically biomethane, in this aspect, is one of the most promising alternative fuels having potential to bring about the movement of current fossil fuels-dependent energy to a sustainable energy future. Compared to other

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hydrocarbon fuels, burning of methane generates a lower CO_2, while the ratio of the molecular mass (16.0 g/mole) to the heat of combustion (891 kJ/mole) shows that methane generates more heat per unit mass than other complex hydrocarbons (Shuba and Kifle 2018).

Methane can be produced by the anaerobic digestion (AD) of various biomass sources including food wastes, agricultural residues, animal manure, forestry residues, energy crops, microalgae, organic-rich wastewaters, organic fraction of municipal solid waste, and industrial organic waste (Cucchiella and D'Adamo 2016; Jankowska et al. 2017). Among these sources, microalgae can be more attractive feedstock due to their faster growth rate (five to ten times), high biomass yield, and suitability to cultivate in the nonarable lands and nutrient-rich wastewaters, in addition to having potential to utilize CO_2 and thereby reducing its accumulation in the atmosphere (Stephens et al. 2013; Kröger and Müller-Langer 2012; Ward et al. 2014). The promising of microalgae as biomethane feedstocks is further extended due to containing good quantity of biodegradable compounds, such as carbohydrates (4–57%), lipids (2–40%), and proteins (8–71%) of the total solids (Prajapati et al. 2013), which can produce biomethane with the theoretical yield of 0.42, 1.01, and 0.5 L_{STP} CH4/g, respectively (Guiot and Frigon 2012).

The AD is one of the most widely used biological approaches for converting biomass into methane (CH_4) (Ho et al. 2018). It is a naturally occurring process where organic matters are decomposed by the synergistic activities of various methanogenic bacteria under anaerobic conditions (Frigon and Guiot 2010). The final products of AD are biogas composed primarily of CH_4 (55–75%) and CO_2 (25–45%) and a solid organic residue rich in nitrogen (Li et al. 2011; Harun et al. 2010). Biogas production by the AD is an old and established technique that offers significant benefits including low sludge production, low costs, and low energy consumption (Adarme et al. 2017). Methane generated during AD can be used in various purposes in a clean way, by generating heat or electricity for alternative energy, liquefying into methanol, compressing into a source of car fuel similar to that of compressed natural gas (CNG), and purifying into fed gas in the distribution grids (Roubaud and Favrat 2005; Ghosh et al. 2000).

Methane production from microalgae or other biomass can be done by using two types of AD, which are liquid AD (L-AD) and solid-state AD (SS-AD). Both techniques are similar in the basic principles but differ in physical conditions of the system, particularly moisture content of the substrate load (Li et al. 2011). Moreover, methane yield is almost similar under both techniques, but volumetric productivity is much higher in SS-AD than that of A-AD as estimated to twofold higher in the former (Brown et al. 2012). In addition, compared to L-AD, SS-AD is characterized by a higher solid load, which is typically greater than 15% (Li et al. 2011).

Methane yield from the AD of microalgae can be accomplished by several steps including cultivation, harvesting, pretreatment, and finally AD of the pretreated microalgae (Fig. 16.1). However, due to the wide variation in the composition of various microalgal species, the biomethane potential also varies significantly among the species, which is required to be considered prior to selecting any strain as methane producer. In addition, several other factors, particularly process parameters,

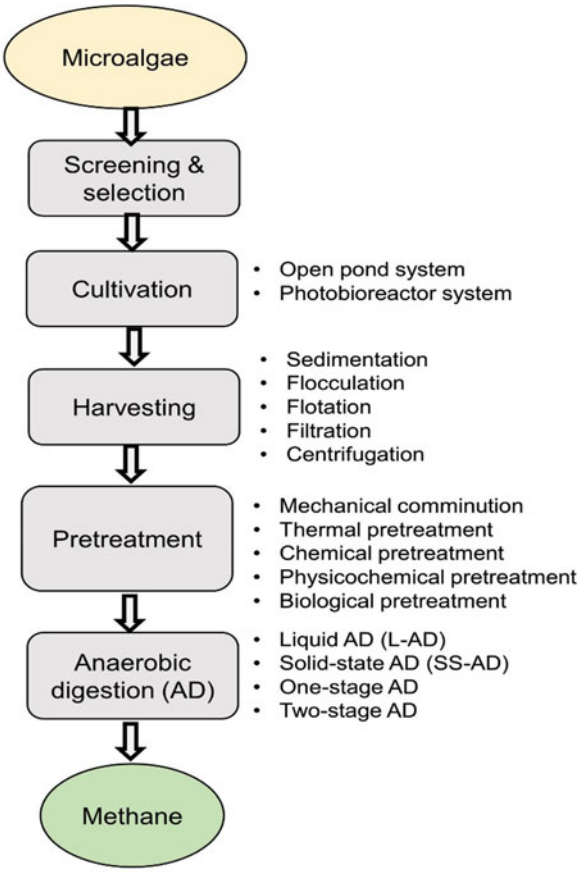

Fig. 16.1 Overview of methane production from microalgae through anaerobic digestion. (Source: Drawn by the authors)

significantly affect the yield and efficiency of the overall process. Therefore, this chapter is designed to discuss these aspects in-depth aiming to present an overview on the generation of methane from microalgae using AD process.

2 Selection of Microalgae for Methane Production

Microalgae species differ in their biochemical composition (Table 16.1), which further vary depending on the different biotic and abiotic factors. For instance, lipid accumulation is generally increased in the cells under the nitrogen starvation conditions. The productivity as well as yield of methane during AD is the result of the conversion of biochemical components of microalgal biomass (Fig. 16.2). Consequently, microalgae are versatile in nature, and it is one of the major challenges in the sustainable microalgae-based methane production. In this aspect, a microalgal species with more stable biomass composition could be a better choice for methane

Table 16.1 Chemical composition of biofuel source microalgae (% of dry matter)

Microalgae species	Lipid (%)	Protein (%)	Carbohydrate (%)
Chlorella protothecoides	55	10–52	10–15
Chlamydomonas reinhardtii	21	48	17
Chlorella vulgaris	14–22	51–58	12–17
Dunaliella salina	6	57	32
Dunaliella bioculata	8	49	4
Scenedesmus dimorphus	16–40	8–18	21–52
Scenedesmus obliquus	35–55	50–56	10–17
Spirogyra sp.	11–21	6–20	33–64
Anabaena cylindrical	4–7	43–56	25–30
Spirulina maxima	6–7	60–71	13–16
Spirulina platensis	4–9	46–63	8–14
Synechococcus sp.	11	63	15
Chaetoceros calcitrans	39	58	10
Chaetoceros muellerii	33	44–65	11–19
Porphyridium cruentum	9–14	28–39	40–57
Euglena gracilis	4–20	39–61	14–18
Isochrysis galbana Parke	21–38	30–45	7–25
Prymnesium parvum	22–38	28–45	25–33

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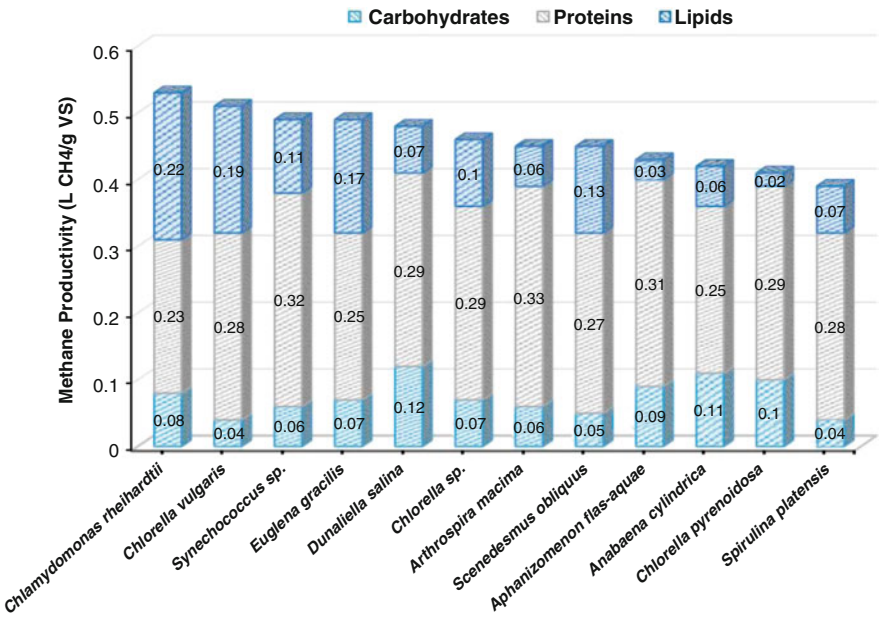

Fig. 16.2 Latent methane productivity in relation to the biochemical components (carbohydrates, proteins, and lipids) of various microalgal species. (Reproduced from Tijani et al. 2015 with the permission from Elsevier, copyright 2015)

production. In addition to the composition, productivity of biomass during cultivation could be another important parameter for selecting an ideal microalgal species as the biomethane feedstock. In fact, efficient multiplication of microalgal cells is the most complex and challenging issue required for mass production of microalgal.

Obtaining the stable microalgal strains capable of producing large volumes of methane is a fundamental issue need to take into consideration during screening and selection process. It should be noted that methane production during AD is highly strain-specific due to varying features of cell walls in microalgae. A good methane producing microalgal species is therefore desired to have thin or no cell wall, large cytoplasmic contents, maximum growth rate in non-sterile media, high tolerance to the contaminants, and carbohydrate-based cell wall (Tijani et al. 2015). Among these criteria, cell wall structure and composition are of paramount important as microalgae with no cell wall or a protein-based cell wall containing no polysaccharides are reported to be more promising for biodegradability during AD (Tijani et al. 2015). Moreover, the efficiency of cell wall biodegradability of microalgae has been considered as the rate-limiting aspect in the kinetics biofuels generation (Sialve et al. 2009; Chen and Oswald 1998).

In addition to the specific selection criteria mentioned above, some general aspects also need to be considered during selection of microalgal strains for methane production. Firstly, solar energy yield should be 6–12 times higher in microalgae than that of terrestrial plants due to their inherently more efficient solar energy conversion as estimated to 3–8% higher than the terrestrial plants. Secondly, absence or presence in the polysaccharides can minimize the necessity or importance of pretreatment step for degrading the biopolymers into smaller compounds prior to subjecting to AD. Thirdly, metabolic and environmental diversity should allow the selected strains to grow in the local aquifers. Fourthly, the morphological traits of the microalgae strains should offer a cost-effective harvesting process. Finally, the strains should offer feasible genetic manipulation for controlling the end-products synthesis and improving the tolerance to nutrient and ecological stresses (Shuba and Kifle 2018).

Selection of the efficient methane producing microalgal strains can also be done by considering the theoretical methane yield, which is calculated from the stoichiometric relationship between methane production and the elemental composition of biomass (C, H, O, and N contents) using Eqs. 16.1 and 16.2 (Ward et al. 2014; Buswell and Boruff 1932)

$$\begin{aligned}(C_aH_bO_cN_d) + \left(\frac{4a - b - 2c + 3d}{4}\right)H_2O \\ \rightarrow \left(\frac{4a + b - 2c - 3d}{8}\right)CH_4 + \left(\frac{4a - b + 2c + 3d}{8}\right)CO_2 + dNH_3 \end{aligned} \quad (16.1)$$

where a, b, c, and d are the C content, H content, O content, and N molar composition, respectively

$$Y_{CH4} \rightarrow \left(\frac{4a + b - 2c - 3d}{12a + b + 16c + 14d}\right) * V_m \tag{16.2}$$

where Y_{CH4} is the methane yield (L/g/VS) and V_m is the molar volume of CH_4, i.e., 22.14 L at 0 °C and 1 atm (Sialve et al. 2009).

3 Cultivation of Microalgae

Majority of microalgae strains are strictly phototrophic where light is an essential parameter required for proliferation of microalgal cells, while some heterotrophic strains are light independent and can grow using organic substrates for carbon source. Even though autotrophic cultivation is widely used, heterotrophic cultivation is recognized for relatively greater biomass productivity (Tijani et al. 2015). Cultivation of microalgae can be done using different strategies such as batch, fed-batch, repeated fed-batch, semicontinuous, and continuous systems (Tan et al. 2018).

Microalgal cultivation is usually done by two major systems, namely, open raceway ponds and closed photobioreactor systems, where the former is relatively more convenient compared to the latter in the techno-economic perspectives. However, raceway pond cultivation system is bottlenecked by the risk of unwanted microbial contamination (Balat 2011) and low biomass productivity (Rawat et al. 2011). The raceway pond system is generally designed with a paddle wheel for mixing and circulation as well as maintaining the consistent integrity (Schenk et al. 2008). A typical raceway pond contains three basic parts, (1) endless loop for continuous circulation of the cells by the paddle wheels, (2) rotating arm to maintain agitation, and (3) inclined system that combines pumping and gravity flow (Tan et al. 2018).

Photobioreactor offers controlled conditions for phototrophic cultivation of single microalgae species. It provides necessary light intensity to carry out the photobiological reactions by the cells needed for their proliferation (Mata et al. 2010). Photobioreactor has been used extensively for producing large quantity of microalgae bulk biomass. A comparison between open raceway pond and photobioreactor systems used for microalgae cultivation is presented in Table 16.2.

4 Harvesting and Processing of Microalgae

Harvesting of microalgae basically includes concentration and separation of mature biomass from the culture medium. Usually, the cultures of microalgae contain a very dilute biomass as dry matter that may vary from 0.5 to 0.75 g/L in open pond systems to 3–4 g/L in closed systems (Fasaei et al. 2018). The dilute biomass solution is then concentrated to around 10–25% or more dry solids (Barros et al. 2015). Concentrating of the microalgae cultures from the cultivation medium can be done by three

Table 16.2 Comparison between photobioreactor and raceway pond systems used for microalgae cultivation

Parameters	Photobioreactor facility	Raceway ponds
Biomass productivity (kg/year)	100,000	100,000
Volumetric productivity (kg/m^3/d)	1.535	0.117
Areal productivity (kg/m^2/d)	0.048	0.035
Biomass concentration (kg/m^3)	4.00	0.14
Dilution rate (/d)	0.384	0.250
Area needed (m^2)	5681	7828
CO_2 consumption (kg/year)	183,333	183,333
System geometry	132 parallel tubes/unit; 80-m-long tubes; 0.06 m tube diameter	978 m^2/pond; 12 m wide, 82 m long, 0.30 m deep
Number of units	6	8

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Table 16.3 Overview of the microalgal harvesting techniques

Harvesting method	Stage of concentration	Total suspended solids (%)	Developmental stage[a]
Sedimentation	1st	0.5–3	Pilot
Auto/co-flocculation/ biofilm	1st	1.4–5	Pilot
Inorganic flocculation	1st	1.2–7	Pilot
Organic flocculation	1st	0.6–15	Lab
Electrolytic flocculation	1st	3–5	Bench
Magnetic flocculation	1st	4.4	Lab
Hydro-cyclone	1st	0.4	Bench
Dissolved air flotation	1st	1–8	Pilot
Electrolytic flotation	1st	3–5	Bench
Suspended air flotation	1st	1.4–5	Pilot
Micro-strainer filtering	1st	1.5–3	Pilot
Acoustic aggregation	1st	7.6	Lab
Decanter	2nd	12–30	Bench
Disk stack centrifugation	2nd	10–22	Bench
Spiral plate rotor	2nd	31.5	Pilot
Membrane filtration	2nd	2–27	Bench
Belt filtering	2nd	12–50	Pilot
Chamber filtering	2nd	5–27	No data
Vacuum belt filtering	2nd	9.5–18	Pilot
Vibrating screen filtering	2nd	1–10	Pilot

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[a]Lab, volumes <10 gal; bench, volumes 10–1000 gal; pilot, volumes >1000 gal (Christenson and Sims 2011)

strategies, which are (i) a single-step harvesting and dewatering, (ii) one-step harvesting with a separate dewatering step, and (ii) one-step harvesting with two steps of subsequent dewatering (Fasaei et al. 2018). However, the most common and general harvesting technique is achieved by the second strategy, in which microalgal biomass is thickened in the first step to a slurry usually containing 1–5% of biomass for facilitating the separation of biomass from culture medium. The secondary step mainly includes dewatering of the thickened biomass that produces microalgal sludge with an average concentration of 20% biomass (Deconinck et al. 2018). Till to date, a variety of unit operations have been used either individually or in combinations for concentration as well as harvesting of the microalgae (Table 16.3). However, most of these harvesting techniques are still confined to the laboratory or pilot scales and remains in the infancy in consideration of their commercial applications (Table 16.3).

Concentration of microalgal cultures can be done by physical, chemical, and biological ways. Physical methods include mainly mechanical or electrical forces to concentrate the biomass. For example, ultrasonic sounds and electrolysis are the

most promising physical methods used to disrupt the microalgal cells (Hincapié Gómez and Marchese 2015; Bosma et al. 2003). In the chemical concentration of microalgae, cultures are coagulated using some chemical substances including inorganic and organic or neutralization of the microalgal negative charge using nanoparticles for improved coagulation (Deconinck et al. 2018; Lee et al. 2015). Biological concentration of microalgae is usually based on the bio-flocculation that can be achieved by introducing natural flocculation (Alam et al. 2016).

The concentrated microalgae biomasses are subsequently subjected to drying for enhanced shelf life and ease of handling in the further bioprocessing steps, which can be done by basically three techniques including drum drying, spray drying, and solar drying. Among the drying techniques, drum and spray drying are mature techniques but energy intensive. Although solar drying is a low-cost technology, it has some disadvantages in the techno-economic perspective, such as time-consuming and risk for contamination (Fasaei et al. 2018).

5 Pretreatment of Microalgae

The bioconversion efficiency of microalgae biomass into methane depends on the digestibility of the cell wall polymers, particularly holocellulose contents. However, the cell wall polysaccharides are recalcitrant in their natural stage to both the direct microbial conversion and hydrolytic enzymes. Eventually, an additional pretreatment step is required for enhanced digestibility of the microalgal biomass prior to subjecting them to the digestion step. To date, many pretreatment methods have been studied for lignocellulosic biomass under the major categories of physical (mechanical and thermal), chemical, physicochemical, and biological approaches (Zabed et al. 2017b) (Fig. 16.3), which are also applied for microalgae pretreatment under the same conditions (Cardeña et al. 2017; Córdova et al. 2018; Jankowska et al. 2017). However, the feasibility, suitability, and outcome of each pretreatment methods depend on its techno-economic conditions.

Physical pretreatment methods act on the microalgae biomass by enlarging the surface area and pore volumes. This category of pretreatment mostly includes size reduction of the biomass using mechanical approaches, which are energy intensive and eventually not sustainable for commercial facilities (Zabed et al. 2016c). Moreover, only size reduction is not enough for efficient conversion, and hence it requires combination with other techniques. Another approach of physical pretreatment is the heating of microalgae biomass that has been reported to enhance methane yield significantly as estimated to 123% when compared with the yield with the untreated biomass (González-Fernández et al. 2012).

In the chemical pretreatment method, acids like H_2SO_4 and alkali (e.g., NaOH) are widely used (Zabed et al. 2017c). However, chemical pretreatment requires high temperatures, in addition to requiring acid or alkali. Although thermochemical pretreatment has been reported to be effective for improving methane yield from microbial biomass (Penaud et al. 1999), some techno-economic issues like corrosion

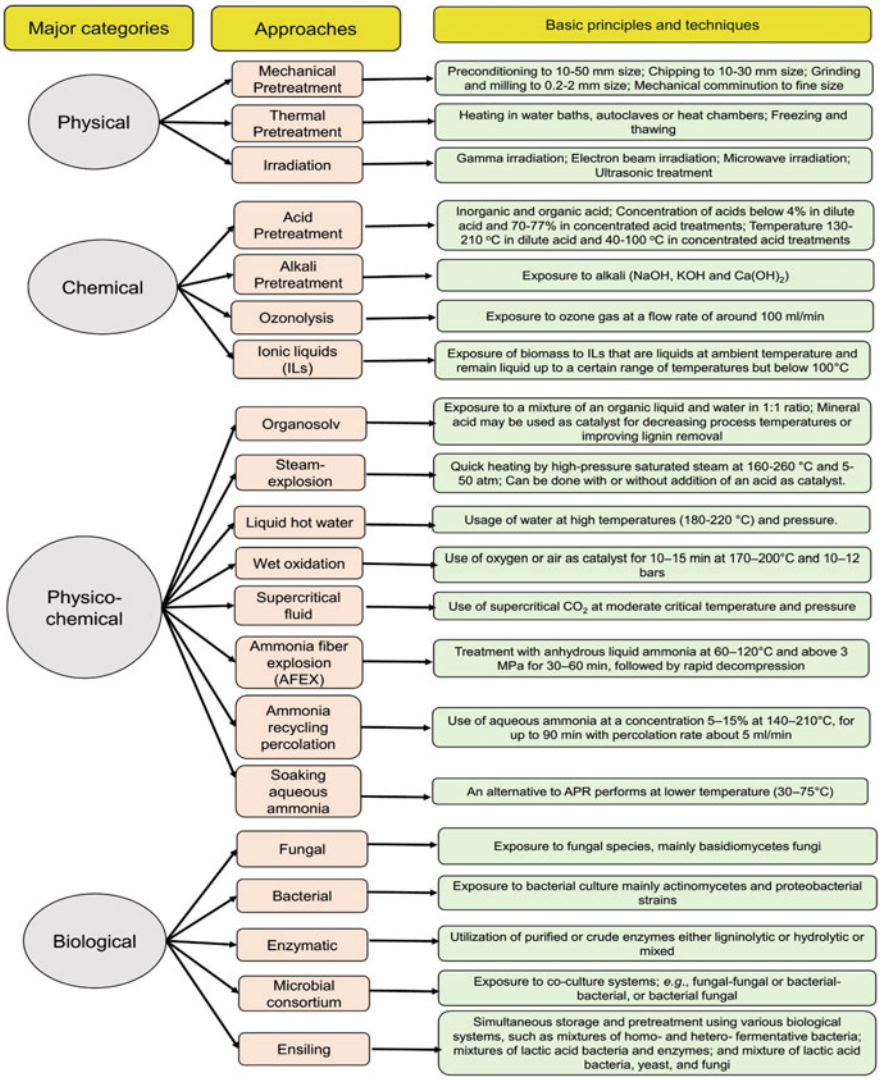

Fig. 16.3 Approaches and techniques of various pretreatment methods. (Source: drawn by the authors)

of reactor, requirement of removing or neutralizing the chemicals, and energy consumption may make it challenging for large-scale implementation. On the other hand, the progress in pretreatment is more apparent in physicochemical techniques as substantial research efforts have been made in this major category using various unit operations (Fig. 16.3).

Biological pretreatment is another approach of pretreatment, which is considered more attractive compared to the conventional pretreatment methods, as it uses natural microorganisms or their enzymes. The major advantages of biological pretreatment over other pretreatment methods are the requirement of low energy, simple operation methods and equipment, no or less inhibitors formation, low downstream processing costs, and no requirement for recycling of chemicals after pretreatment (Sindhu et al. 2016; Millati et al. 2011). Despite these promising advantages and extensive studies, commercial implementation of biological pretreatment is, however, still in its infancy and bottlenecked by long incubation time and loss of carbohydrates during pretreatment (Wan and Li 2012).

6 Anaerobic Digestion of Microalgae

6.1 Basic Principles and Steps

The AD of microalgae biomass is the decomposition of the organic fractions of biomass through a sequential internal redox reaction brought about by the synergistic action of various microorganisms. There are four basic phases in AD for methane production, which are hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 16.4) (Yang et al. 2011). Each of these steps has its own microflora that facilitates the overall process (Yu and Schanbacher 2010).

In the hydrolysis step, the polymeric components in microalgal biomass including carbohydrates, proteins, and lipids are biodegraded by the action of the respective microflora in the digester that results in the obtainment of simple substrates like glucose, fructose, amino acids, and long-chain fatty acids. The hydrolysis step is then followed by the acidogenic phase of AD when simple components are converted into a mixture of short-chain volatile fatty acids (VFAs) and other minor metabolites (CO_2, H_2, acetic acid). In the third phase, VFAs are converted into acetic acid and H_2 by the action of acetogenic bacteria, which are finally metabolized into methane in the methanogenesis step by a variety of methanogenic bacteria (Li et al. 2011). Despite the common steps in AD of all microalgae, the final methane yield after AD varied significantly among the microalgal species even if the loading rate is sometimes similar (Table 16.4).

As discussed above, the final product of AD is a mixture of CH_4 and CO_2 called biogas, where the amounts of these gases are related to the biochemical composition and biodegradability of the biomass. In case of carbohydrates (glucose, starch, and cellulose), the proportion of CH_4 and CO_2 is 50:50 (Eq. 16.3), while a higher amount of CH_4 is produced if the substrates are protein or lipid estimating the CH_4 to CO_2 ratios are 55:45 (Eq. 16.4) and 70:30 (Eq. 16.5), respectively (Annonymous 2018). However, the actual methane yield can be varied due to the incompleteness of the reactions, generation of by-products and consumption of substrates by the bacteria for their multiplication (Annonymous 2018).

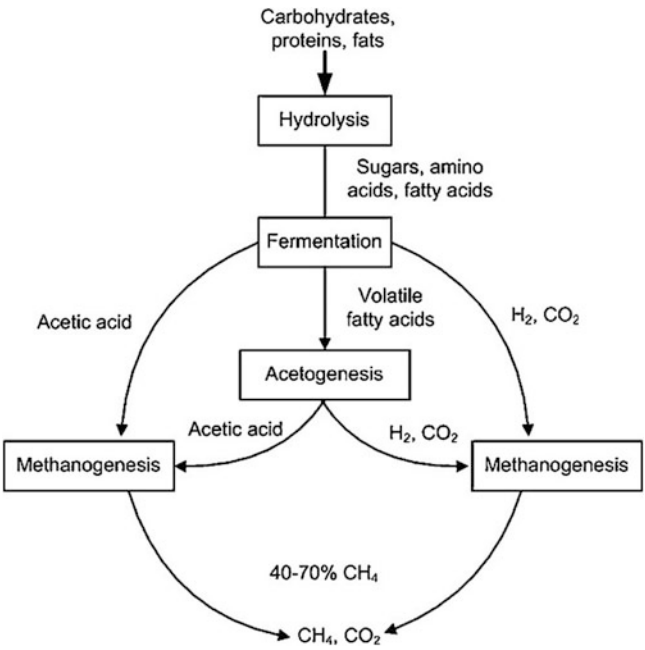

Fig. 16.4 Process flow of the degradation of organic material through anaerobic digestion. (Reproduced from Li et al. 2011 with the permission from Elsevier, copyright 2011)

$$C_nH_{2n-2}O_{n-1}(\text{Strach or cellulose}) + nH_2O \rightarrow 0.5\ CH_4 + 0.5\ CO_2 \tag{16.3}$$

$$C_{10}H_{20}O_6N_2(\text{Proteins}) + 3H_2O \rightarrow 5.5\ CH_4 + 4.5\ CO_2 + 2NH_3 \tag{16.4}$$

$$C_{54}H_{106}O_6(\text{Lipids}) + 28H_2O \rightarrow 40\ CH_4 + 17\ CO_2 \tag{16.5}$$

6.2 *Microbial Community*

The AD of microalgae or other biomass is the result of synergistic actions of a wide variety of microorganisms in different steps, except the pretreatment process that is usually conducted before subjecting the biomass into the digester using various physical, chemical, and biological methods. Hydrolysis and acidogenesis of the pretreated biomass then occurred in the digester by the action of various bacterial community including roughly 50 kinds of bacteria, such as *Clostridium* sp., *Bacteroides* sp., *Bifidobacterium* sp., *Butyrivibrio* sp., *Proteobacteria* sp., *Pseudomonas* sp., *Bacillus* sp., S*treptococcus* sp., *Eubacterium* sp., and others (Xu et al. 2018).

Table 16.4 Generation of methane from various microalgae species through anaerobic digestion

Microalgae species	Loading rate (g/TS/L* or g/VS/L**)	Methane yield (mL g^{-1}VS)	References
Chlamydomonas reinhardtii	2*	587	Mussgnug et al. (2010)
Arthrospira platensis	2*	481	Mussgnug et al. (2010)
Scenedesmus obliquus	2*	287	Mussgnug et al. (2010)
Dunaliella salina	2*	505	Mussgnug et al. (2010)
Chlorella kessleri	2*	335	Mussgnug et al. (2010)
Euglena gracilis	2*	485	Mussgnug et al. (2010)
Dunaliella tertiolecta	5**	24	Lakaniemi et al. (2011)
Chlorella vulgaris	5**	286	Lakaniemi et al. (2011)
Spirulina maxima	22.5**	330	Varel et al. (1988)
Scenedesmus obliquus	2**	240	Zamalloa et al. (2012)
Chlorella sorokiniana	–	212	Polakovičová et al. (2012)
Chlorella vulgaris	2**	403	Lü et al. (2013)
Chlorella vulgaris	1**	240	Ras et al. (2011)

The initial stage of AD is mainly influenced by the species of *Bacteroidetes* and *Firmicutes*, while the acidogenesis is carried out predominantly by the species of *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria* (Wang et al. 2017). In the final stage or methanogenesis step, mostly three kinds of methanogens, namely, acetoclastic, hydrogenotrophic, and methylotrophic, are involved, where the first two types are the predominant (Shin et al. 2010). The acetoclastic methanogens include mainly *Methanosaeta* and *Methanosarcina* (Xu et al. 2018), while the common hydrogenotrophic bacteria which are *Methanobacterium*, *Methanothermobacter*, *Methanospirillum*, *Methanobrevibacter*, and *Methanoculleus* are the most commonly identified hydrogenotrophic methanogens (Collins et al. 2003; Ziganshin et al. 2013).

6.3 Liquid Versus Solid-State AD

The common technique of generating methane is the L-AD, where biomass is mixed with water prior to loading in the digester. However, the major drawback of this technique is the limited solid load that is usually confined to 15% or less. To

overcome the technical difficulties of L-AD, recently SS-AD has drawn much attention in the conversion of biomass into methane using more than 15% of total solid (Yang et al. 2015). In a typical SS-AD system, pretreated or untreated biomass is thoroughly mixed with inoculum until achieving the target total solid content and then loaded in the reactor, followed by the incubation at the suitable temperature for the prescribed time period varied from 30 to 45 days.

Compared to the L-AD, SS-AD has been reported to be more advantageous due to requiring smaller reactor volume, consuming lower energy for heating, easy processing of the materials, and reducing the loss of energy (Guendouz et al. 2008). In addition, the residues of SS-AD can be used as fertilizer or pelletized fuel for its low moisture content, which is more convenient to handle than the effluent of liquid AD (Li et al. 2011). The reactions in a SS-AD are much faster than those that occur in a landfill, while the reactions are slower in SS-AD compared to the reactions in L-AD (Martin et al. 2003). However, the retention time required in SS-AD is around threefold longer than that of L-AD because of the lower mass transfer in the former (Li et al. 2011).

Over the last 20 years, there have been substantial progress and achievements in the SS-AD with the implementation of this technology in commercial facility, where both batch and continuous reactors have been introduced using thermophilic or mesophilic digestion of organic wastes (Li et al. 2011). The solid contents in these reactors varied between 20% and 40% with the yield of biogas in the range from 0.3 to 0.5 m^3/kg of volatile solids (Li et al. 2011; Rapport et al. 2008).

6.4 One-Stage Versus Two-Stage AD

Traditionally, methane is produced by one-stage AD, which is completed through the basic four steps of AD yielding CH_4 and CO_2 as the final products. One-stage AD is particularly of interest when separate hydrogen is not expected. In this type of AD system, hydrogen is not detected as it is consumed during the final stage of AD (methanogenesis) (Pakarinen et al. 2009). Most of the earlier studies on the methane production from microalgae were done using one-stage AD (Yang et al. 2011).

In contrast to one-stage AD, separate acidogenesis and methanogenesis steps are maintained in two-stage AD (Pakarinen et al. 2009). In this technique, organic components in the biomass are first converted into VFAs and H_2 in the acidogenesis phase, while VFAs are converted in the subsequent methanogenesis step producing CH_4 and CO_2 (Ueno et al. 2007). The main objective of the two-stage AD is the combined production of H_2 and CH_4 which offers some advantages over one-stage AD, such as feasibility to optimize acidogenesis and methanogenesis steps separately as both stages are influenced by the respective bacterial community, increased biogas yield, enhanced conversion efficiency, and high stability of the process (Yang et al. 2011).

In a typical two-stage AD, two bioreactors are used in series as the digesters. The first reactor is operated for a very shorter hydraulic retention time (HRT), roughly

one-tenth or less of the HRT used for a typical single-stage reactor. The second reactor is operated at typical anaerobic digestion HRT, generally over 15 days. Thus, the first reactor is much smaller than the second reactor, in which nearly all conversion to methane occurs (Annonymous 2018). In a two-stage AD, the ideal stoichiometry, for the simple case of carbohydrate (glucose) breakdown, can theoretically be expressed as (Annonymous 2018):

$$\text{1st stage}: C_6H_{12}O_6(\text{Glucose}) + 2H_2O \rightarrow 4H_2 + 2C_2H_4O_2\ (\text{acetic acid}) + 2\ CO_2 \quad (16.6)$$

$$\text{2nd stage}: 2C_2H_4O_2\ (\text{acetic acid}) \rightarrow 2CH_4 + 2\ CO_2 \quad (16.7)$$

7 Factors Affecting Cultivation and AD of Microalgae

7.1 Influencing Factors in Cultivation and Biomass Yield of Microalgae

7.1.1 Nutrients

The cultivation of microalgae as well as biomass yield is affected by various factors categorized as nutritional, physical, chemical, and biological factors. The quality and concentration of essential nutrients are the major concerns for microalgal growth. The primary growth nutrient for microalgal cultivation is carbon (C) that is mostly used in the form of inorganic C derived from the atmospheric CO_2, even though some microalgae can utilize organic C from the wastewaters (Pittman et al. 2011). Although CO_2 is essential for microalgal growth, the effective concentration of CO_2 varies depending on the types and species of microalgae, and even high concentration of CO_2 acts as the toxic component of microalgae (Jankowska et al. 2017). For example, the concentration of CO_2 above 5% has been reported to affect the growth rate of freshwater microalgae like *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, and *Scenedesmus obliquus* because of the changes in the photosynthesis by decreasing the affinity to CO_2 and activity of carbonic anhydrase and increasing the photosynthetic sensitivity to O_2 (Lee and Lee 2003; Yang and Gao 2003). Unlike the freshwater microalgae, some species of green microalgae, such as *Scenedesmus* sp. and *Chlorella* sp., have been reported to be the more tolerant to the CO_2 level with high growth rate that was up to 50% (Lee and Lee 2003).

Among the other nutrients, inorganic nitrogen (N) as the form of ammonium or nitrate and phosphorous (P) in the form of phosphate are second and third important nutrients, respectively (Jankowska et al. 2017). The optimum concentration of N is required for fast growth of microalgae, and under the stress condition of this nutrient, growth rate and productivity drop significantly even though lipid accumulation is increased under such conditions (Lardon et al. 2009). The optimum N/P ratio

adopted in most of the microalgal cultivation systems is 16 (Angelidaki and Ahring 2000). In addition, trace metals including chelated salts of iron, nickel, manganese, selenium, cobalt, and zinc have also been reported as micronutrient supplements [29]. In addition to the macronutrients, some microelements and vitamins are also necessary as they influence the growth and biomass yield of microalgae, which include Fe, Se, Ni, Co, Mn, Zn, Cu, and Mo (Jankowska et al. 2017).

7.1.2 Light

The intensity and uniform distribution of light as well as the ratio of light to dark are important factors for the growth and biomass production of microalgae. Bioreactors should be designed in such a way that they could have sufficient surface area to volume ratio with a short light path for circumventing the light attenuation during growth and improving the light absorbance by the culture medium (Richmond et al. 2003). Despite the photosynthetic sensitivity of microalgal cells increases as the intensity of light increases, light intensity above the saturation point may cause photo-inhibition that results in an irreversible damage in the light receptors of the chloroplasts and thereby decrease the photosynthesis rate in the cells (Dismukes et al. 2008). The negative influence of light intensity can be offset by applying short light to dark cycles (Pulz 2001).

The optimum light wave length for the growth of microalgae varies depending on the species, which necessitates the optimization of light intensity for the target microalgal species prior to considering it for cultivation. Several studies were conducted to investigate the effects of LED light on the cultivation of microalgae, and it was observed that growth rates of *Chlorella* sp. and *Nannochloropsis* sp. significantly increased with the blue and/or red LED (Yan et al. 2014; Das et al. 2011).

Maintaining the optimum and uniform light intensity is a challenge in the dense cultures where light accessibility to the cells at the interior fluid layers is affected by the mutual shading effects, which is a common growth-restraining issue as it decreases the rate of cell proliferation and eventually the high biomass production (Tijani et al. 2015). In order to overcome this problem and uniform distribution of light, some control parameters including fluid depth, viscosity, and mixing frequency were partially modified (Williams and Laurens 2010).

7.2 Influencing Factors in AD and Methane Yield

7.2.1 Composition of Microalgal Biomass

The efficiency of AD as well as methane yield is affected by the composition and biodegradability of microalgae. The recalcitrance of microalgal cell wall components (lignin, cellulose, and hemicellulose) is one of the reasons for inefficient

biodegradability. On the other hand, biodegradability and methane yield from the major components also varied depending on the chemical nature of these macromolecules. For example, lipid is the most attractive macromolecule in the microalgal biomass that theoretically yields higher amounts methane than carbohydrates and proteins (Zamalloa et al. 2011).

The cultivation conditions, harvesting time, and storage conditions of microalgae indirectly influence the AD process and methane yield by affecting the composition of the biomass. Microalgae grown under the conditions of nitrogen starvation results in the accumulation of a higher lipids in the biomass (Richardson et al. 1969). However, the nitrogen starvation conditions may reduce the productivity and digestibility of the microalgae biomass by, firstly, changing the cell morphology due to accumulating higher biochemical components (lipid, proteins, and carbohydrates) that increase the cell volumes and wall thickening (Donk et al. 1997; Tillberg and Rowley 1989) and, secondly, secreting the exudates that accumulate external to the cell wall and resist the accessibility to the hydrolytic enzymes (Malej and Harris 1993).

The accumulation of biochemical components fluctuates over the cultivation time. As a result, harvesting of the biomass in an appropriate time will ensure the desired concentrations of the major biochemical components as well as efficiency of the AD process. However, unlike the time, harvesting methods do not have any significant influence on the AD (Harith et al. 2010).

The storage condition of microalgae is also important for maintaining the favorable composition of the biomass. During storage under the freezing conditions, it has been reported that carbohydrates and protein in the biomass were significantly reduced (Babarro et al. 2001). Likewise, the composition of microalgal biomass has also been reported to be changed during storage at the temperature ranged between 40 and 60 °C. The reason of changing the biomass composition under these storage conditions could be due to the bacterial contamination as well as degradation of the macromolecules for their proliferation, chemical oxidation, and accumulation of protease enzymes (Cordero Esquivel et al. 1993; Grabner et al. 1981).

7.2.2 Biomass Loading

Biomass loading in the digester significantly influences the growth of microflora as well as conversion efficiency and methane yield. In general, methane yield increases with the increase in the initial biomass loading up to a certain level. For example, significant drop in COD removal and methane yield was noticed in an SS-AD study, which recorded a drop in COD removal from 80.69% to 69.05% upon an increase in the biomass load from 20% to 30%, while 17% lower methane yield was found at 30% solid load compared to the yield obtained at 20% biomass concentration (Fernández et al. 2008). The optimum solid load in AD process may further be varied depending on the types of AD, such as whether it is L-AD or SS-AD.

7.2.3 Hydraulic Retention Time (HRT)

Methane production has been reported to be increased over the HRT, but too long period of digestion will not bring about any significant changes in the overall conversion efficiency due to the exhaustion of available substrates and nutrients, reaching the microbial growth to the stationary phase, and accumulation of toxic metabolites that leads to death of cells. Therefore, establishment of the optimum retention time is a preliminary and necessary technical issue in the experimental design of the microalgae-based AD system. In a typical pilot or large-scale AD plant, optimum retention time may vary between 30 and 50 days (Sreekrishnan et al. 2004), while it varies from 15 to 30 days in the lab-scale experiments (Jankowska et al. 2017).

7.2.4 Carbon/Nitrogen (C/N) Ratio

The C/N ratio is one of the most important parameters in AD process, and any significant deviation from the optimum C/N ratio reduces the efficiency of methanogenesis as well as methane yield by releasing high total ammonia nitrogen and/or volatile fatty acids during digestion (Parkin and Owen 1986; Yen and Chiu 2007). In general, high C/N ratio is desired for effective methanogenesis. However, depending on the feedstocks, the effective C/N ratio varies between 20 and 30 with an optimum ratio of 25 (Li et al. 2011; Pang et al. 2008; Parkin and Owen 1986). In fact, ammonia (NH_4) is accumulated in the digester during AD as a result of dissociation of protein that acts as potential inhibitor for target microbial growth and eventually decreases the rate of methanogenesis.

When C/N ratio reached to less than 20, the microbial community in the digester faces an imbalance situation between C and N, which leads to the release of N in the form of NH_3. The release of this form of N is inhibitory to the methanogenic bacteria causing the accumulation of volatile fatty acids (VFA) in the digester (Sialve et al. 2009), particularly if the C/N ratio reaches to 15 or below (Ehimen et al. 2009). Although N in the form of NH_3 and VFA are important intermediates in AD, it can also act as a potential inhibitor upon its accumulation in the digester (Parkin and Owen 1986). The minimum concentration of N in the form of NH_3 essential for the growth of microflora in AD has been reported to be ranged between 50 and 200 mg/L (Parkin and Owen 1986; Chen et al. 2008).

To overcome this situation as well as increase the net C/N ratio, C-rich materials can be co-digested with the microalgae biomass (Jankowska et al. 2017). In addition to increasing the C/N ratio, co-digestion approach may also improve VFA to alkalinity ratios and decrease the unfavorable ratios of single substrates (Ward et al. 2014). In several earlier studies, microalgae were successfully co-digested with waste streams and other biomass that resulted significantly high C/N ratio and enhanced methane yield. The co-digested substrates included pig manure (González-Fernández

et al. 2011), water hyacinth (Saxena et al. 1984), cow manure (Ramamoorthy and Sulochana 1989), corn stalk (Peng et al. 2012), municipal solid wastes (Yuan et al. 2012), glycerol (Ehimen et al. 2009), paper waste (Yen and Brune 2007), and lipid-rich fats, oil, and grease (Park and Li 2012).

7.2.5 Temperature and pH

The optimum temperature in AD for mesophilic and thermophilic fermentation ranged from 30 to 38 °C and 50 to 55 °C, respectively (Jankowska et al. 2017). However, AD process is not efficient and effective under the mesophilic conditions, while thermophilic conditions of AD were found to be well-established, reliable, and acceptable (Hartmann and Ahring 2006). In addition, thermophilic operation of AD minimizes the risk for unwanted and pathogenic bacterial contaminations. The requirement of high energy for thermophilic operations seems to be a challenge for sustainability of the process, but it can be counterbalanced by the enhanced methane yield and productivity (De Bere 2000). In an earlier study, methane yield was found to be significantly higher under the thermophilic conditions compared to the yield obtained during mesophilic AD (El-Mashad et al. 2004).

The pH level in the digester also has the significant effects on the efficiency of AD and methane yield. The microbial activities in AD are affected under too high or too low pH levels (Franke-Whittle et al. 2014; Gomez-Romero et al. 2014). The optimal pH for overall AD was reported to be in the range between 6 and 8 (Shi et al. 2014).

8 Conclusion

Biomethane is one of the most promising biofuels and microalgae is an attractive feedstock for generating this biofuel. The extensive research efforts made on the microalgae for various purposes provide much knowledge and understanding of the overall structure and complexity of many microalgal species as the feedstocks for AD. Methane production from microalgae could be more promising than utilizing other biomass sources for this purpose as the former can integrate waste management and biofuels production by being grown in the waste effluents. Till date, a good number of microalgal species have been studied for methane production using the AD process, which produced satisfactory amounts of methane, even though there is still a need to improve some technical issues for better yield and implantation in the industrial arena. The major technical issues need to be improved for industrial aspects include low biomass loading during AD and recalcitrance of the biomass to biodegradability, which can be overcome by using SS-AD and pretreatment technology, respectively.

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Chapter 17
Advancement of Bio-hydrogen Production from Microalgae

Mamudul Hasan Razu, Farzana Hossain, and Mala Khan

Abstract In the twenty-first century, ensuring energy security is a key challenge to economic and political stability of the globe. Biological hydrogen production from microalgae is the promising alternative source for potential renewable energy which only releases water vapor as by-product without polluting environment as it does by fossil fuel, emitting CO_2 when burnt. Microalgae can generate hydrogen by bio-photolysis or photo-fermentation. Two enzymes, viz., hydrogenase and nitrogenase, are responsible for biological hydrogen production process in metabolic pathway of microalgae. Though successful research has been conducted at laboratory scale producing hydrogen from microalgae, low yield has been recognized as challenge due to light capturing efficiency, oxygen sensitivity of enzyme, CO_2 fixation efficiency, etc. during its bulk production for commercialization. In biological H_2 production, cost reduction in algae culture and downstream process is required to make it economically feasible. Therefore present research emphasizes overcoming key challenges for scaling up biomass and H_2 production through genetic and low-cost designed photo-bioreactors. This chapter depicted the principles of photobiological hydrogen production in microalgae along with various recent approaches and emerging strategies to mitigate the present limitations for hydrogen production.

Keywords Microalgae · Renewable energy · Bio-photolysis · Hydrogenase · Biological hydrogen · Photo-bioreactors

1 Introduction

At present century, most of the countries in the world realize that energy security is an important weapon for modern economic stability and national safety. Instantly our globe is fronting the challenges of huge energy loads as well as depletion and rising of price in fossil fuel, reliance on foreign sources of energy, geopolitics,

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economic efficiency versus prompt growth of population, and industrialization. So, we have to manage such dare and for this, scientists are now giving much significant attention by endorsing bearable and cost-effective methods in energy production. Another most important issue is worldwide climate fluctuations caused by anthropogenic rise of the atmospheric greenhouse gasses as CO_2 concentration has been estimated that around 41 million metric tons CO_2 is emitted globally in 2017 due to fossil fuel depletion which create catastrophic impact on our environment (Welch 2017). For this reason, it makes urge to researchers to find renewable sources of energy being environmental friendly. Biomass is one of the most promising renewable resources that can provide different types of biofuels like biodiesel, bioethanol, biogas, and bio-hydrogen. The Intergovernmental Panel on Climate Change (IPCC) noted in 2008 that renewable energy contributed to a huge 12.9% of global primary energy supply and the immense majority of renewables is biomass (Intergovernmental panel on climate change (IPCC) 2011). Molecular hydrogen from biomass is a hygienic energy, an ecologically safe, renewable fuel resource, and an admirable auxiliary of fossil fuels which is a prospective contender with highest energy density, many of the technical, socioeconomic, and ecological profits to its acceptance among all other known energy, and is the only recognized energy that does not create CO_2 as a by-product when used in fuel cells for electricity generation (Azwar et al. 2014; Chang and Lin 2004). It has been estimated that 141.65 MJ/kg heating value can produce from a unit weight of hydrogen gas (Perry 1963).

By the time mentioned, first-generation biofuels are produced directly from most widely used food crops such as wheat, sugar, and oil seed by extracting the oils for use in bioethanol or producing biodiesel through fermentation (Report 2007). However, first-generation biofuels have a number of associated problems like it produces negative net energy gains, releasing more carbon in their production than their feedstock's capture in their growth and scarcity of food. Second generation biofuels produced from non-food crops such as wood, organic waste, food crop waste and specific biomass crops, as well as lignocellulosic, cellulose and hemicellulose material. In bio-hydrogen production as biofuel, it needs to convert complex composition and polymeric structure of lignocellulosic or cellulose to simple sugar like glucose, sucrose, fructose, etc. However, great cost in the hydrolysis of lignocellulosic or cellulose ingredients are a matter of concern (Azwar et al. 2014; Report 2007; Behera et al. 2015). In recent years, among the other microorganisms, green microalgae have concerned attention not only for producing valuable molecules but also for third- and fourth-generation biofuels through bio-hydrogen production because it is competent at transforming sunlight into the chemical energy and requires a smaller footprint, less water for cultivation, and storing capacity of CO_2 (Table 17.1).

The journey started from 70 years ago; the pioneer German plant physiologist Hans Gaffron and co-researchers (Gaffron 1939; Gaffron and Rubin 1942) discovered the capacity of unicellular green algae to harvest hydrogen gas upon lighting has been an interesting field for sustainable energy. Prior to the finding of Gaffron and co-researchers, Jackson and Ellms (1896) reported that a natural bloom of the cyanobacterium *Anabaena*, when placed into a glass jar, promptly started to produce

Table 17.1 Various bio-hydrogen production mode by several microorganisms with their benefits and limitations

Microorganisms	Strains	Mode of operation	Benefits and limitations
Green algae	*Chlamydomonas reinhardii* *C. moewusii* *Chlorella vulgaris*	Direct biophotolysis $2H_2O + \text{light} + Fd_{(ox)} \rightarrow 4H + Fd_{(red)}\ (4e^-) + O_2$ $4H + \ + Fd_{(red)}\ (4e^-) \rightarrow Fd_{(ox)} + 2H_2$	Benefits: H_2 is produced from water and sunlight Tenfolds more solar conversion energy is produced then to trees, crops CO_2 is decreased in environment
		Photo-fermentation $6CO_2 + 12H_2O \rightarrow 6O_2 + C_6H_{12}O_6$ $6H_2 + C_6H_{12}O_6 \rightarrow 12H_2 + 6CO_2$	Limitations: H_2 production is inhibited of by oxygen
Cyanobacteria	*Anabaena variabilis* *Cyanothece sp.* *Synechocystis* PCC 6803	Direct biophotolysis $\text{light} + 2H_2O + Fd_{(ox)} \rightarrow 4H + Fd_{(red)}\ (4e^-) + O_2$ $4H + \ + Fd_{(red)}\ (4e^-) \rightarrow Fd_{(ox)} + 2H_2$	Benefits: H2 is produced from water and sunlight CO_2 is decreased in environment
	Anabaena sp. PCC 7120	Indirect biophotolysis $N_2 + 8H + Fd_{(red)}\ (8e^-) \rightarrow \ + 16ATP$ $2NH_3 + H_2 + Fd_{(ox)} + 16ADP + 16Pi$ $8H + \ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi$ $12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2$ $C_6H_{12}O_6 + 6H_2 \rightarrow 6CO_2 + 12H_2$	Limitations: H_2 is consumed by hydrogenase enzyme H_2 production is inhibited of by oxygen
Photosynthetic bacteria	*R. capsulatus* *R. sulidophilus* *Thiocapsa roseopersicina*	Photo-fermentation $CH_3COOH + 2H_2O + \text{light} \rightarrow 4H_2 + 2CO_2$ $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$	Benefits: Different types of waste resources like, whey, distillery, etc. can be utilized Wide spectrum of light can be used
			Limitations: Light is required for the H_2 production Fermentation causes water pollution

(continued)

Table 17.1 (continued)

Microorganisms	Strains	Mode of operation	Benefits and limitations
Fermentative bacteria	*Enterobacter aerogenes* *Clostridium butyricum* *Magashaera elsdenii*	Dark fermentation $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$ Pyruvate + CoA → acetyl − CoA + formate Pyruvate + CoA + $2Fd_{(ox)}$ → acetyl − CoA + CO_2 + $2Fd_{(red)}$	Benefits: H_2 can be produced without presence of light in anaerobic condition Different carbon sources like, starch, cellobiose, sucrose, etc. can be used Valuable metabolites such as butyric acid, lactic acid can be made
			Limitations: Carbon and nitrogen sources is needed for growth Fermentation causes water pollution Gas contained CO_2

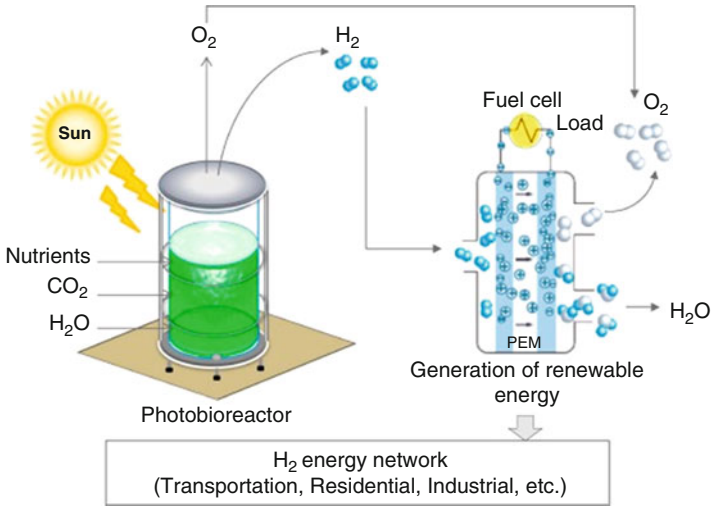

Fig. 17.1 Photobiological H_2 production from microalgae that may be used as H_2 energy cells that can be applied in transportation, resident, and industry. (Khetkorn et al. 2017)

hydrogen gas (Jackson and Ellms 1896). Those days renewable hydrogen production was made less imperative by the excess of fossil fuel but is now again of growing significance due to global warming and reduction of petroleum resource.

Various kinds of process have been applied for hydrogen manufacture, like steam reforming, electrolysis, photolysis, bio-hydrogen production, etc. (Holladay et al. 2009; Yilmaz et al. 2016). In steam reforming technique high-temperature steam is used to yield hydrogen gas from fossil fuel or natural gas. In this case, a number of catalysts have been discovered for improving the productivity process (Obradovic et al. 2013). In electrolysis, water molecule converted into O_2 and H_2 through electrolytic techniques. But, these two methods are required high energy and also create CO/CO_2 and other pollutants as by-products, which is not eco-friendly. Besides several methods of H_2 production, photobiological may be one of the effective, eco-friendly, and less energy demanding techniques (Debabrata and Veziroglu 2001; Khetkorn et al. 2013; Nyberg et al. 2015).

By using as fuel or fuel cell, hydrogen gas is the most encouraging and a model future energy to produce electricity for resident, industry, and transportation (Ramachandran and Menon 1998) (Fig. 17.1). It is also used as an important raw material for production of fertilizer and petroleum. However, high manufacture cost, difficulties in preservation and transportation, and an emergent hydrogen setup all create problems in economic viability as fuel energy (Khetkorn et al. 2013; Chu and Majumdar 2012; Rashid et al. 2013; Oey et al. 2016). The prospective of H_2 manufacture in green microalgae and cyanobacteria depends on strain capability to produce different enzymes liable for H_2 metabolism and environmental factors like light, pH, temperature, substrate concentration, etc. Additionally, various genetic engineering techniques such as targeted engineering of certain enzymes (e.g.,

hydrogenases and nitrogenases) and metabolic pathways may considerably increase the H_2 production (Khetkorn et al. 2013). Some strains of microalgae have been developed using a genetic engineering approach to enhance their capability of hydrogen production (Oey et al. 2016; Baebprasert et al. 2011; Maneeruttanarungroj et al. 2012; Khetkorn et al. 2012a, b; Nyberg et al. 2015).

The design and operation of photo-bioreactors are being considered for efficient hydrogen productions from algal biomass is a matter of concern. Many promising bioreactors have been designed like fixed-bed, membrane bioreactor, continuous stirred-tank reactor, etc. which can generate H_2 at satisfactory level. In all kinds of bioreactors, the basic requirement is energy supply, either light, carbohydrates, or carbon dioxide, for H_2 production. This chapter highlights the recent improvement and technical advance en route for a sustainable bio-hydrogen production from microalgae.

2 Biological Mechanisms of Hydrogen Production

In biological H_2 production, H_2 gas is produced by the utilization of microbes. Various kinds of photosynthetic and non-photosynthetic bugs like green algae, cyanobacteria, and photosynthetic and dark fermentative bacteria have the ability to yield H_2 gas.

2.1 Bio-photolysis

Microalgae are one of the clusters of photosynthetic microbes appropriate for the photobiological manufacture of H_2 (Hansel and Lindblad 1998; Nagarajan et al. 2017). A number of microalgae like *Botryococcus*, *Chlamydomonas*, *Chlorococcum*, *Chlorella*, *Scenedesmus*, *Synechocystis*, *Tetraspora*, *Anabaena*, *Nostoc*, etc. are capable with potent photosynthetic machinery for hydrogen production (Eroglu and Melis 2011; Eroglu and Melis 2016; Alam et al. 2017) in presence of enzymes (hydrogenase and nitrogenase) encoded in the nucleus (Adams 1990; Meyer and Gagnon 1991; Happe et al. 1994), whereas electrons derived by expending of water and energy derived from sunlight (Esper et al. 2006). Simple nutrient is given to culture the algae, with the capability to capture CO_2 from atmosphere as carbon source, and some strains are accomplished to decrease atmospheric nitrogen to ammonia by using of sunlight for energy to yield H_2.

In this procedure photosystem II (PSII) arrested light energy from sun to break water, produce oxygen, and create a high-energy, low-potential reductant which is able for reducing protons (H+) to hydrogen (H_2) in presence of hydrogenase or nitrogenase enzyme. The produced electron from water molecule splitting moves through the membrane-bound electron transport chain which consists two photosystems (I and II), later can yield ATP, and then reduces ferredoxin that take part in a

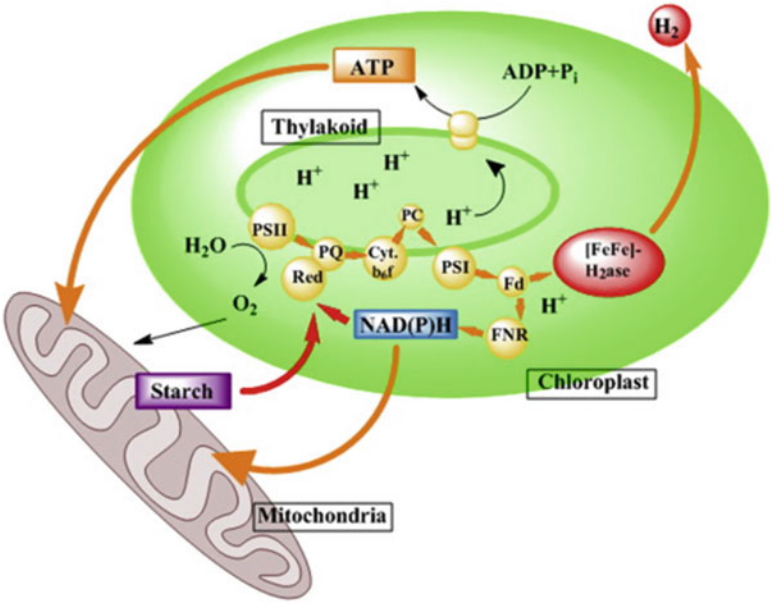

Fig. 17.2 A general pathway for hydrogen production from microalgae. (Mathews and Yiwangb 2009)

number of metabolic reactions (Fig. 17.2). Bio-photolysis is categorized into two major groups: direct and indirect bio-photolysis.

2.1.1 Direct Bio-photolysis

In direct bio-photolysis, photosynthetic apparatus chlorophyll a and other pigments of eukaryotic green microalgae have the ability to arrest light and energy from the sun and are improved by using water splitting to yield a low-potential reductant or ferredoxin, which can moderate a hydrogenase or nitrogenase enzyme directly without transitional CO_2 fixation. The generated hydrogen ions are transformed into hydrogen gas in the medium with donated electrons by reduced ferredoxin in the presence of hydrogenase enzyme. The following incidents occurred during hydrogen production (Fig. 17.3):

1. Using light energy, photosystem II generated electrons are moved into the photosynthetic electron transport chain through a series of transport molecules like plastoquinone (PQ), cytochrome bF (Cyt bf), and plastocyanin (PC) and transfer through photosystem I (PSI) to reduce ferredoxin (Fdx).
2. Ferredoxin (Fdx) energies on to reduce $NADP^+$ to NADPH with the help of enzyme ferredoxin or $NADP^+$ reductase (FNR).

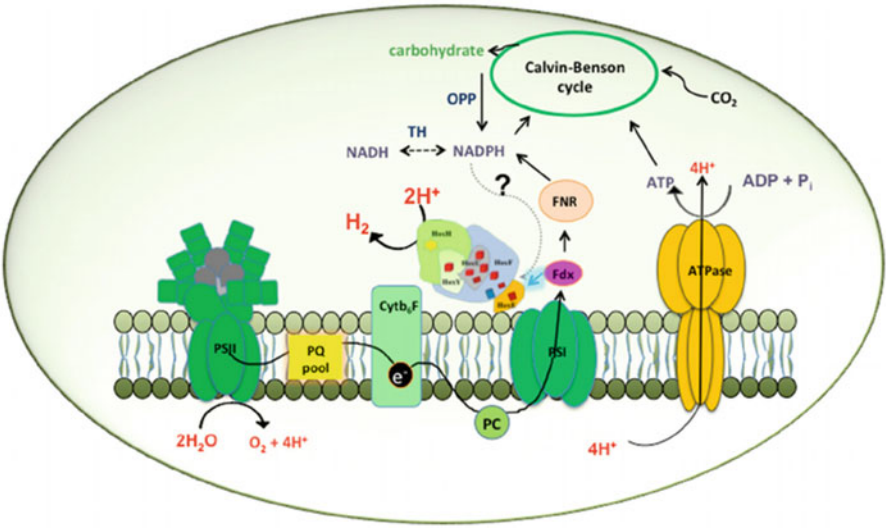

Fig. 17.3 Pathway of direct bio-photolysis for hydrogen production in microalgae. (Khanna and Lindblad 2015)

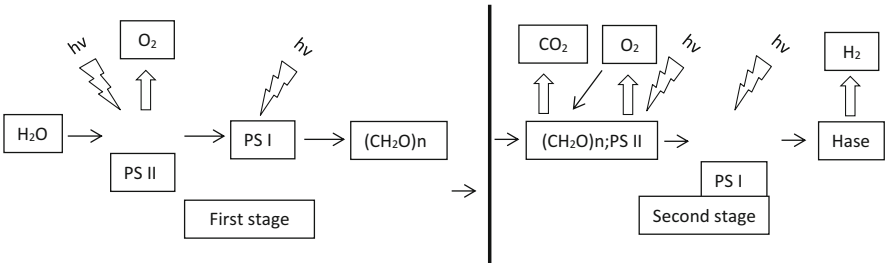

Fig. 17.4 Direct bio-photolysis reaction by removing O_2

3. Simultaneously the reduced ferredoxin also has the capability to donate the electrons to the hydrogenase directly.
4. Then at the present conditions hydrogenase enzyme can compete with the Calvin cycle for hydrogen production.

Naturally, direct bio-photolysis is a very attractive method because of using solar energy to alter a readily obtainable substrate, water, to oxygen and hydrogen, but in practical, it is restricted by other issues. The enzyme hydrogenase activity affected by O_2 because it is related during direct bio-photolysis process and therefore inhibits the H_2 yield. A method has been explored to succeed this limitation by rising respiration process using endogenous or exogenous substrates (Fig. 17.4). Some green algae naturally hold an inducible alterable hydrogenase, and it could be used in a direct bio-photolysis system. *C. reinhardtii* the familiar H_2 generating green algae, under anaerobic environments, can either produce H_2 or practice H_2 as an electron

donor. There are some microalgae such as *Scenedesmus obliquus*, *Chlorococcum littorale*, *Platymonas subcordiformis*, and *Chlorella fusca* where hydrogenase activity has also been observed (Winkler et al. 2002; Nandi and Sengupta 1998).

In this aspect to succeed the obstruct outcome of O_2 on hydrogenase, numerous approaches have been studied with partial success:

1. In in vivo to progress the efficacy, response conditions, stabilizing mechanisms, and demonstration of direct bio-photolysis, it has been carried by spending inert gas sparing, which declines gas and liquid phase of oxygen and hydrogen concentrations >0.1%. It has been verified that the microalgae *C. reinhardtii* concurrently creates H_2 and O_2 with four photons/H_2, though inert gas sparing is not applied (Kosourov et al. 2002; Ghirardi et al. 2000). On the other hand, to cover the pond with a simple floating transparent reduce the gas remove; this system would cut the cost of sparing inert gas mixing as well. However the major issue would be separation of the hydrogen and oxygen gas which is costly. Besides this the greatest complication of direct bio-photolysis method is the safety issue, production of oxygen-hydrogen mixture can be fiery and unsteady under even normal atmospheric circumstances. These boundaries bring to the researchers to manage the separation of O_2 from H_2 for three causes: firstly, to decrease the effect of oxygen on hydrogenase; secondly, lower the cost of treating to isolate H_2 from O_2; and thirdly, overcome the safety issue.
2. In some case O_2 can be removed by protest of glucose and glucose oxidase (Benemann et al. 1973). It has been showed that myoglobin and hemoglobin could diminish O_2 concentration efficiently (Rosenkrans and Krasna 1984). However, such kind of restoration would not be applied in full-scale system.
3. Many scientists (Pow and Krasna 1979; Mahro and Grimme 1982, 1986; Randt and Senger 1985) used a tough reducing agent dithionite that quantitatively reacts with O_2 and leading to anaerobic environment, permitting a continuous direct bio-photolysis reaction. Acetate is a good carbon substrate for *C. reinhardtii* cells to retain a high respiration rate (Kosourov et al. 2002) on exogenous acetate and/or endogenous carbohydrates (Ghirardi et al. 2000) help to lesser the photosynthetic oxygen production rate than the rate of O_2 uptake by respiration and the cells culture slowly becomes anaerobic, which switches the action of reversible hydrogenase (Kosourov et al. 2005).
4. It was studied that sulfur-deprived culture medium of *C. reinhardtii* can decline the photosystem II activity and oxygen production rates become lower in respiration and provide anaerobic condition for hydrogenase enzyme (Kosourov et al. 2005).
5. The basic problem in two stage of direct bio-photolysis process is that the carbohydrate stored in the first phase of the reaction, holds about the same quantity of energy as the hydrogen produced in the second phase Fig. 17.4, and during high rate of respiration this energy is fully misused in the process of oxygen feeding, thus the hydrogen production become due to low-energy transformation efficiency from light (Yoon et al. 2006).

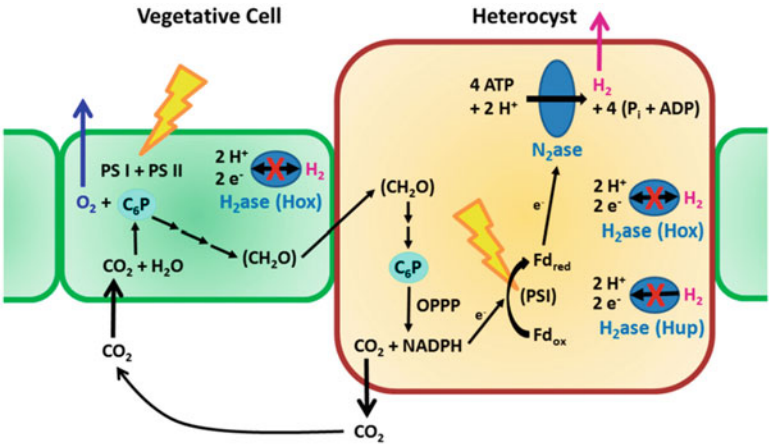

Fig. 17.5 Metabolic route of heterocystous cyanobacteria for indirect bio-photolysis H_2. (Masukawa et al. 2012)

2.1.2 Indirect Bio-photolysis

In indirect bio-photolysis, presence of light energy PSII is responsible to produce oxygen by splitting water and produce reduced ferredoxin that is used to capture carbon dioxide and the subsequent reduced carbon compound involved to initiative hydrogen evolution in a distinct reaction.

2.1.2.1 Single Stage of Indirect Bio-photolysis

One of the most common options in indirect bio-photolysis is heterocystous cyanobacteria also called blue-green algae. Heterocystous cyanobacteria are filamentous in which vegetative cells are differentiated with specified heterocyst cell where nitrogen is fixed with the help of enzyme nitrogenase and in deficiency of nitrogen; enzyme produces hydrogen (Fig. 17.5).

1. In this process, there are two compartments in cyanobacteria where heterocyst contains only PSI responsible to generate ATP for nitrogenase activity and PSII is completely absent.
2. Heterocysts are joined with vegetative cells through a pore and permit the dispersion of carbohydrates from vegetative cells produced by regular photosynthesis with additional development of O_2 and uptake of CO_2.
3. Then heterocysts accept the carbohydrate which goes to produce NADPH through hexose phosphate (C_6P) and oxidative pentose phosphate pathway (OPPP) and use them as the sources of e− for N_2ase action.
4. In presence of light energy, PSI transport the electron to reduced ferredoxin (Fd_{red}) to activate N_2ase enzyme for hydrogen production.

5. Beside that net production of H_2 can be efficient by inactivation of H_2ase (s) through uptake H_2ase Hup and bidirectional H_2ase Hox.
6. *Anabaena* sp., heterocystous nitrogen-capturing cyanobacteria, serves an oxygen-free environment to nitrogenase that reduces molecular nitrogen into NH_3 as well as protons into hydrogen (Smith et al. 1992), and in absence of molecular N_2 in atmosphere, cyanobacteria use carbohydrate for H_2 production.

For the first time, *Anabaena* sp. was used to demonstrate the indirect bio-photolysis mechanism that could yield hydrogen and oxygen concurrently using nitrogenase. Even though the heterocyst system is very attractive for H_2 production from biological point of view, it has three major disadvantages:

1. Simultaneous production of oxygen and hydrogen which is fiery as well as costly to separate.
2. Large closed photo-bioreactor is required.
3. In comparison of hydrogenase, nitrogenase is a very incompetent enzyme.

2.1.2.2 Two Stage of Indirect Bio-photolysis

To improve the inadequacy of single-stage bio-photolysis is to convey out the two reactions in individual stages by using light energy: the first stage is oxygen manufacture and carbon dioxide capturing and second stage is hydrogen production with CO_2 discharge. This procedure is functionally similar to single-stage heterocyst system, except that oxygen production with carbon dioxide capturing and hydrogen making with CO_2 production are divided with period of time (Fig. 17.6). Two-stage indirect bio-photolysis was first time demonstrated in non-heterocystous cyanobacteria *Plectonema boryanum* with frequent substitute cycle of aerobic light-driven carbon dioxide fixation and oxygen manufacture by Weare and Benemann (1974). The nitrogen-fixing cyanobacterium *Plectonema boryanum* was used as an ideal organism to study the hydrogen production in nitrogen-limited batch cultures where argon/CO_2 were maintained for anaerobic condition constantly (Huesemann et al. 2010).

This procedure can be passed out either in two single reactors, open pond for CO_2 fixation and oxygen manufacture and closed photo-bioreactor for hydrogen

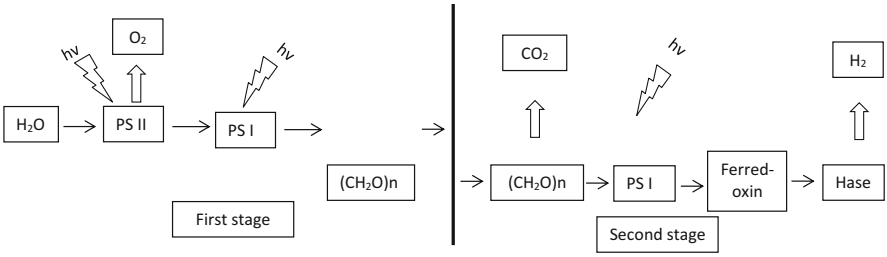

Fig. 17.6 Indirect two-stage bio-photolysis with the presence of light

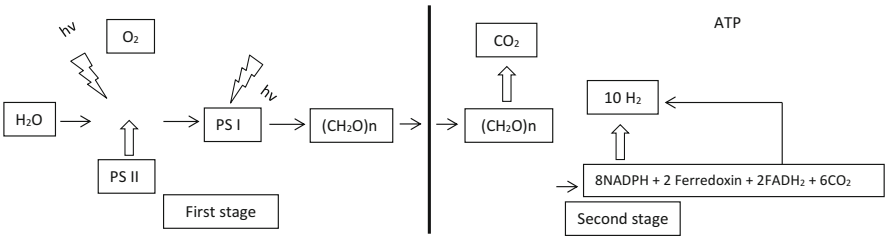

Fig. 17.7 Indirect two-stage bio-photolysis with dark fermentation second stage

manufacture or single closed photo-bioreactor for CO_2 fixation with oxygen manufacture and hydrogen yield in alternate cycle. The two-stage bio-photolysis is altered by substituting light-driven second phase of H_2 production with dark fermentation of second phase (Fig. 17.7).

The alteration of two-stage bio-photolysis indirect method might be achieved into the day-night cycle, where the dark period permits development of anaerobic fermentation circumstances (Redwood et al. 2009) with hydrogen yield and CO_2 fixation in day. The main benefit in two-stage dark fermentation procedure is that smaller sealed photo-bioreactors are used in the second stage and do not need light exposure. The noticeable candidate microalgae like *Chlorella* and blue-green algae *Spirulina* responsible for this process in high accumulation of carbohydrates and nitrogen limitation condition (Redwood et al. 2009). However, in dark anaerobic situation, water replacement is not essential for green algae *C. reinhardtii*, well-known in hydrogen production capability with sulfur-lacking medium (Melis et al. 2000).

2.2 Dark Fermentation

In dark fermentation hydrogen is manufactured without the presence of sunlight, water, and oxygen. Fermentative microorganisms hydrolyze the complex organic polymers to monomers which are further converted to a mixture of organic acids and alcohols along as well as necessary hydrogen production (Das and Veziroglu 2008; Lin and Jo 2003; Schara et al. 2008). Through this process, significant by-products like acetic acid, butyric acid, lactic acid, etc. can be produced. Advantage of this process is there is no need for presence of light energy and continuous use of carbohydrate. Disadvantages are relatively low hydrogen production, and during production, it contains carbon dioxide which needs to separate (Lin and Lay 2004a). In order to increase yield more hydrogen in the dark fermentation process, it is necessary to control several parameters, namely, organic food, nutrition feed rate, temperature, solids retention time (SRT), and pH. One of the most important parameters on hydrogen production is pH, because it influences the activities of the enzyme hydrogenase.

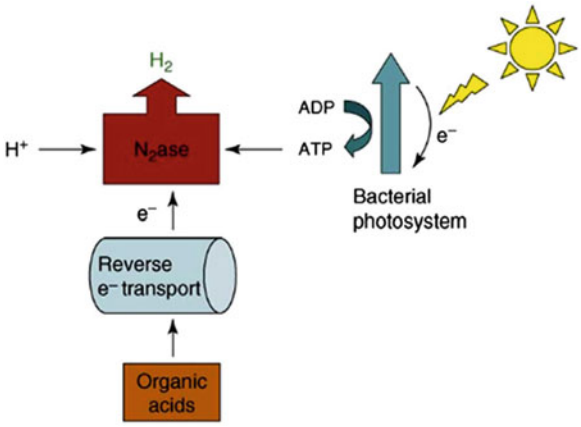

Fig. 17.8 Photo-fermentation processes by photosynthetic bacteria. (Hallen beck and Ghosh 2009)

2.3 Photo-fermentation

It is a fermentative conversion of organic substrates into hydrogen and carbon dioxide by use of sunlight as an energy source.

$$CH_3COOH + 2H_2O + \text{light} \rightarrow 4H_2 + 2CO_2$$

In presence of light as the energy source, the organic acid substrates are oxidized through tricarboxylic acid cycle (TCA), producing electrons, protons, and carbon dioxide. The advantage of this process is removal of environmental pollutants, use of industrial waste and organic acids can be used which is produced in dark fermentation. On the other hand, disadvantages of the process is need to supply partial nitrogen condition and pretreatment of industrial effluent as it may be toxic (Lin and Lay 2004b) (Fig. 17.8).

3 Hydrogen Production from Microalgae

3.1 Enzyme-Dependent Hydrogen Production

In microalgae hydrogenases (H_2ase) and nitrogenases (N_2ase) are believed to be the crucial cellular apparatus liable for biological H_2 yield (Winkler et al. 2009). The enzyme H_2ase is mostly originated in green microalgae and cyanobacteria, but their enzymatic action, enzyme development, and mechanical multiplicity may diverge in different species. On the other hand, nitrogenase can yield major amounts of H_2 under anaerobic, and nitrogen-lacking situations are current only in cyanobacteria (Carrieri et al. 2011; Kim and Kim 2011). Both enzymes act a vital part in the manufacture of hydrogen by reduction of proton (H+).

3.1.1 Hydrogenase

Green microalgae and cyanobacteria have various kinds of hydrogenase enzymes. There are three diverse kinds of hydrogenase, i.e., [NiFe] hydrogenase, [FeFe] hydrogenase, and [Fe] hydrogenase. The hydrogenase enzyme is a homodimer originate only in certain methanogenic archaea (Vogt et al. 2008). It has been thought that both the green microalgae and cyanobacteria produce hydrogenases individually, but enzymatic mode of action via their particular metallo-clusters is same. The properties and mode of action of hydrogenase enzyme are given below:

1. In microalgae, H_2 yields due to connection of the photosynthetic electron transport chain and the plastidial [FeFe] hydrogenase enzyme.
2. The [FeFe] hydrogenase has the 100-fold higher ability to produce hydrogen than other enzymes because of having exceptional active center (H cluster) (Vogt et al. 2008).
3. In nucleus, hydA gene responsible for [FeFe] hydrogenase enzyme encoding and after enzyme maturation it contained in the chloroplast. The enzyme has a monomeric arrangement whose molecular weight is about 45–50 kDa. The catalytic H-cluster site is composed by [FeFe] bonds with sulfur bridges and 4Fe-4S residue (Meyer 2007), nonproteinous ligands CN and CO which linked with both Fe atoms. Under oxygenic conditions it is tough to produce H_2 naturally because electrons are shifted from ferredoxin to the active site of the enzyme, which is highly sensitive to oxygen.
4. It has been proposed that both ferredoxins and flavodoxins could play as electron giver to the cyanobacterial hydrogenase (Gutekunst et al. 2014; Khanna and Lindblad 2015). The basic structure of the enzyme consists of two parts, one is larger active subunit site which is around 60 kDa molecular weight and has [NiFe] bonds and the other is the small subunit that has around 30 kDa molecular weight along with a Fe-S (4Fe-4S or 3Fe-4S) cluster. Four cysteine residues are linked with metallogenic compartments through sulfur bonds. The small subunit shifts electrons to the active sites that reduce hydrogen production.
5. Hox-hydrogenase is a bidirectional heteropentameric which encoded by hoxEFUYH gene and produce NAD+-reducing enzyme. It contains two protein complexes: hydrogenase complex (HoxY and HoxH) and a diaphorase complex (HoxE, HoxF, and HoxU). The bidirectional Hox-hydrogenase is usually found in all cyanobacteria (Tamagnini et al. 2007; Aubert-Jousset et al. 2011) which catalyzes both ingesting and producing of molecular hydrogen.
6. The heterodimeric Hup-hydrogenase enzyme found in almost all nitrogen-capturing cyanobacteria consists of at least two subunits, HupS small subunit which is encoded by hupS gene and large subunit HupL encoded by hupL that catalyzes ingesting of the hydrogen (Tamagnini et al. 2007).

The main enzymatic differences between [FeFe] and [NiFe] are:

1. The [FeFe] hydrogenases are significantly less oxygen tolerant than [NiFe] hydrogenases.

2. The O_2-inhibition of [NiFe] hydrogenases can be altered, whereas [FeFe] hydrogenases alteration inhibited by O_2 (English et al. 2009).
3. It has been revealed that [NiFe] hydrogenases are constitutively expressed, while anaerobic induction is required for the expression of [FeFe] hydrogenases (Ghirardi et al. 2014).

3.1.2 Nitrogenase

Numerous cyanobacteria are capable to capture atmospheric nitrogen into ammonia (NH_3) and discharge H_2 as a by-product. Nitrogenase enzyme is a multiprotein complex composed of two proteins: MoFe protein or protein I and Fe protein or protein II which is also called dinitrogenase and dinitrogenase reductase, respectively. The nitrogenase enzyme properties and mode of action are given below:

1. The dinitrogenase or protein I is a heterotetramer $\alpha_2\beta_2$ consisting a and b subunits encoded by nifD and nifK gene, respectively. The molecular weight is around 220–240 kDa. It is responsible for reduction of N_2 bonds that lead the formation of ammonia (NH_3).
2. The dinitrogenase reductase or protein II is a homodimer which encoded by nifH gene and the molecular weight is around 60–70 kDa. It has vital role for shifting electrons from the external electron donor to the e protein I (Bothe et al. 2010).
3. Nitrogenase may exclusively catalyze the production of H_2 and high-potential electrons when N_2 substrate is absent. However, nitrogenase needs significant amount of electrons, reductants, and a minimum of 16 ATP molecules which came from photosynthesis or carbohydrate degradation in the cell for considerable H_2 production (Srirangan et al. 2011).

3.2 Factors Related to Hydrogen Production

3.2.1 Environmental Factors

Various environmental factors have impact on bio-hydrogen production from microalgae are described below:

3.2.1.1 pH

pH is one of the most vital environmental features that influence metabolic pathways and H_2 production. It has been revealed that the perfect pH range should be around 5.2 and 6.0 (Park et al. 2006; Oh et al. 2006). The influence of pH in a continuous stirred-tank reactor (CSTR) must be in the range of 4.0–7.0, optimum at pH of 5.5 (Zhang et al. 2008a). The maximum hydrogen production was achieved when microbial reactions engaged in ethanol fermentation at a pH 4.5 (Zhang et al. 2008b).

3.2.1.2 Hydraulic Retention Time (HRT)

HRT is necessary for algal growth rates and mechanical dilution set up by continuous volumetric flow. It was observed that limitation of HRT from 8 to 6 h would decrease microbial diversity, responsible to escalate H_2 production (Fang and Liu 2002). Moreover, the methane concentration ranged between 0.0011 and 0.0058 mol l^{-1} at low dilution rates ($D = 0.002–0.0167\ h^{-1}$) was hardly countable at higher dilutions ($D > 0.075\ h-1$), representing negligible methanogenic activity at high dilution rates which means HRT is capable of preventing methanogenesis in hydrogen production through anaerobic fermentation (Wang et al. 2009).

3.2.1.3 Hydrogen Partial Pressure

Hydrogen partial pressure is the key factors that affecting algal pathways by the addition of dissolved hydrogen concentration. Hydrogen productions become low when the hydrogen partial pressure rises. So it is required to remove excess hydrogen from the system for conserving hydrogen production (Fang and Liu 2002; Wang et al. 2009; Zhang et al. 2006a).

3.2.1.4 Nutrients

For maintaining optimal cell growth and hydrogen production nitrogen, phosphate and other inorganic trace minerals are vital balances in carbohydrate-based feedstocks. Organic nitrogen is more favorable than inorganic one. Phosphate is required for optimal hydrogen production. The elements like Mg, Na, Zn, and Fe are essential supplements for optimum nutrient preparation. Iron have crucial significance in the enzymatic activity of [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenase for hydrogen production (Tiwari et al. 2006; Mizuno et al. 2000; Yokoi et al. 2001; Lin and Lay 2004a; Lin and Lay 2004b; Shima et al. 2008; Bleijlevens et al. 2004; Frey 2002).

3.2.1.5 Temperature

The optimum temperature range of 15–85 °C is suitable for microalgae's growth and in mixed cultures; it may vary 15–34 °C (Chang and Lin 2004; Kanai et al. 2005). It has been observed that the hydrogen production volume in a mixed culture can be increased at 30–34 °C and 28–32 °C temperature, which produce 359 mmol l^{-1} day^{-1} and 1.42 mol H_2 mol^{-1} glucose, respectively (Chang and Lin 2004).

3.2.1.6 Substrate Concentration

Though this factor has controversy, current research have found that H_2 production can be scaled up with increasing glucose concentration (Adams and Hall 1979). It was studied that when glucose concentration is increased from 10 to 35 g l^{-1} at a hydraulic retention time of 12 h, then H_2 yield can be scaled up (Ginkel and Logan 2005), whereas yield reduced from 1.7 ± 0.2 mol H_2 mol^{-1} by using of hexose at 10 g l^{-1} sucrose to 0.8 ± 0.1 mol H_2 mol l^{-1} hexose (Ginkel and Logan 2005).

3.2.1.7 Seed Culture

Many investigations for fermentative H_2 production using pure cultures of microalgae have revealed that in batch mode glucose was used as substrate (Kyazze et al. 2006; Wang and Wan 2009) in pilot program. Mixed algal cultures used in anaerobic sludge, compost, and soil are inoculums for fermentative H_2 yields.

3.2.1.8 Feedstock

Simple sugars such as glucose, sucrose, and lactose being biodegradable are favored as model substrates for H_2 yields. But, the costs for pure carbohydrate sources are high (Zhang et al. 2006b, 2008b; Lo et al. 2008, 2009). In many investigations of hydrogen fermentative processes, complex carbohydrates, wastes, and biomass rich in sugars are the essential source for hydrogen generation (Luo et al. 2011).

3.2.2 Bioreactor

Traditional industrial methods for H_2 Yields are quite expensive. Design of bio-reactors is categorically a pre-mandatory factor for large-scale hydrogen production by microalgae (Akkerman et al. 2002; Younesi et al. 2008).

3.2.2.1 Photo-bioreactors

Design of photo-bioreactors depends on microbiological microalgae or cyanobacteria species. To design photo-bioreactor, some factors like photochemical efficiency, absorption coefficient and size, and the light regime including light and dark cycles need to be considered (Laurinavichene et al. 2008). Therefore, competence of a photo-bioreactor associated with ratio of culture volume and reactor space as well as access on economical, rapid multiplication and high density of the microalgae culture (Luo et al. 2011; Evens et al. 2000; Doenitz et al. 1988). Several

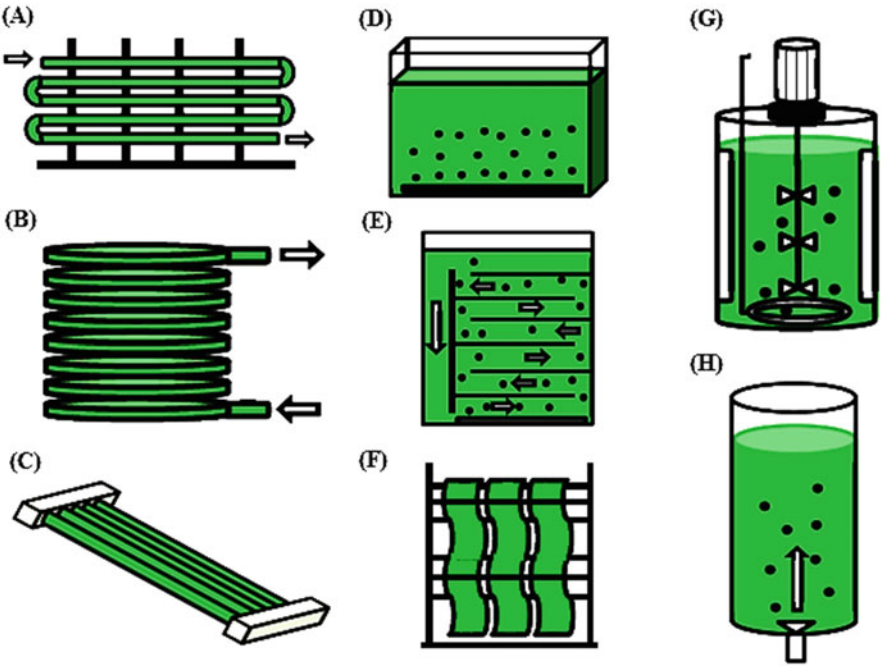

Fig. 17.9 Diagram signifies the different PBRs like fence tubular (**a**), helical tubular (**b**), horizontal tubular (**c**), vertical flat panel (**d**), airlift type (**e**), accordion type (**f**), stirred tank (**g**), bubble column (**h**). (Oncel 2015)

types of photo-bioreactors have been used for hydrogen production. These can be mainly divided into three types of photo-bioreactors (PBRs) (Fig. 17.9):

(i) Vertical PBR comprises a transparent column usually made up of high-quality glass and surrounded by a water jacket for maintaining the temperature with circulating water allows adequate entry of light (Miron et al. 1999). A limitation of these systems is that the power consumption of aeration is high.

(ii) Tubular type photo-bioreactor contains of long transparent tubes where the algal culture is pumped through these tubes by mechanical or airlift pumps (Slegers et al. 2013; Oncel and Kose 2014). The advantage of using this photo-bioreactor is flexibility in volume to surface area ratio and flexibility in shifting the place for light capturing (Molina et al. 2001).

(iii) Flat-panel photo-bioreactor consists of two compartments which is stainless steel frame and three polycarbonate panels, placed side by side. A specially made control system is used in flat-plate photo-bioreactor that can monitor and maintain pH, temperature, optical density, and amount of produced H_2 and dissolved O_2 concentration (Skjånes et al. 2013). Moreover, flat-plate photo-bioreactors found more cost-effective than other bioreactors when it is skillful with high photosynthetic efficiencies and effective gas pressure controller.

Table 17.2 Assessment of microalgal hydrogen production by culturing in different photo-bioreactors

Cultivation	Photo-bioreactor types	H_2 production rate	H_2 production assay condition	References
Continuous photoautotrophic	Helical tubular (4.35 mL total)	14.9 mL H_2 L^{-1} (373 L)	BG11 medium with nitrogen-free, argon atmosphere, 7 days	Lindblad et al. (2002)
Batch photoautotrophic	Vertical flat panel (450 mL)	4.1 mL H_2 g dcw^{-1} h^{-1}	BG11 medium, anaerobic conditions, 120–140 lEm^{-2} s^{-1}, 40 h	Yoon et al. (2006)
Batch photomixotrophic, using immobilized cells	Panel (160 mL)	45 mL day^{-1}	Sulfur-limiting TAP medium, anaerobic environments, 23 days	Laurinavichene et al. (2006)
Batch photomixotrophic	Fence tubular (110 L)	0.6 mL H_2 L^{-1} h^{-1}	Sulfur-free TAP medium with silica nanoparticle to boost scattering, 48 h	Giannelli and Torzillo (2012)
Semicontinuous photomixotrophic	Stirred tank (2.5 L)	1108 mL	Sulfur-free TAP medium, anaerobic environments, 127 days	Oncel and Vardar-Sukan (2009)

Based on a relative investigation of photo-bioreactors, it was concluded that the flat-panel photo-bioreactor is more appropriate for H_2 production, because accumulated back pressure of H_2 could be escaped in flat photo-bioreactors (Oncel and Kose 2014; Nyberg et al. 2015). Moreover, various photo-bioreactors and their efficiency (Oncel and Kose 2014) for biomass and hydrogen production from microalgae as presented in Table 17.2. It is still challenging in establishment of a suitable photo-bioreactor for microalgal H_2 production (Sevda et al. 2017; Kroumov et al. 2017). Some severe factors that should be measured for a specific reactor are:

(i) Photo-bioreactor should be an enclosed system so that the produced H_2 may be collected without any loss.
(ii) The reactor plan must allow disinfection with suitability and simplicity.
(iii) Microalgal growth and hydrogen production can be high and should provide high surface to volume ratio if area of light incident is maximized.

3.2.2.2 Continuous Stirred-Tank Reactor (CSTR)

Continuous stirred-tank reactors (Fig. 17.10) are regularly being used for hydrogen production because microalgae are mixed and suspended well in the reactor liquid through a pattern. This bioreactor provide good contact between microalgae and substrate and due to continuous stirred of tank reactor follow the rapid mixing

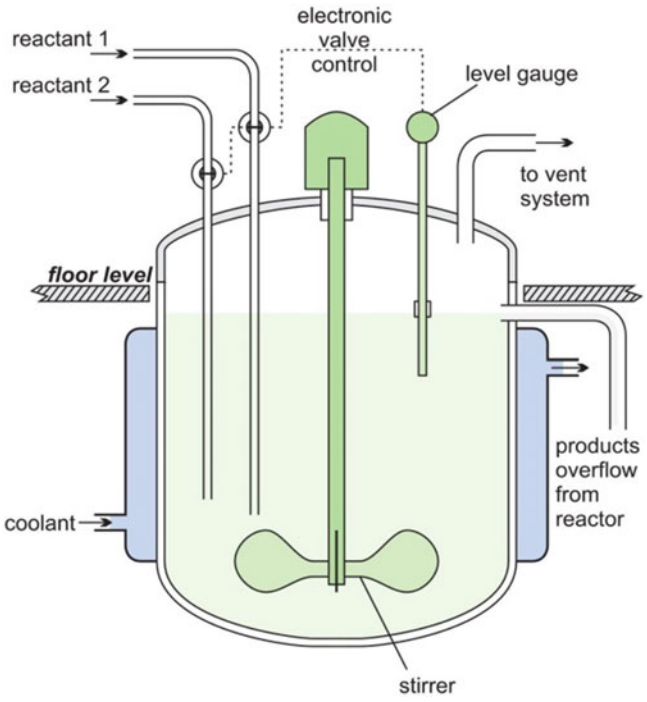

Fig. 17.10 Schematic diagram of a CSTR bioreactor. (http://www.essentialchemicalindustry.org/processes/chemical-reactors.html)

operating pattern which helps to maintain little care during fermentation of biomass. Through this reactor hydrogen production rates are markedly controlled because washout of biomass is possible at short hydraulic retention times (HRTs) (Luo et al. 2011; Kosourov and Seibert 2009; Younesi et al. 2008; Ding et al. 2010).

3.2.2.3 Fixed-Bed Bioreactor

Packed-bed or fixed-bed bioreactors commonly used with attached microalgae act as carriers packed within the tank (Fig. 17.11). The fixed-bed reactors are widely used with immobilized cells. The bed compaction which generally occurs during fermentation results in high. Hydrogen yield become decreased in a fixed-bed reactor due to pH gradient distribution along the reactor column which responsible for heterogeneous distribution of algal activity. So, recirculation flow was recommended to solve this limitation (http://www.essentialchemicalindustry.org/processes/chemical-reactors.html; Mizuno et al. 2000).

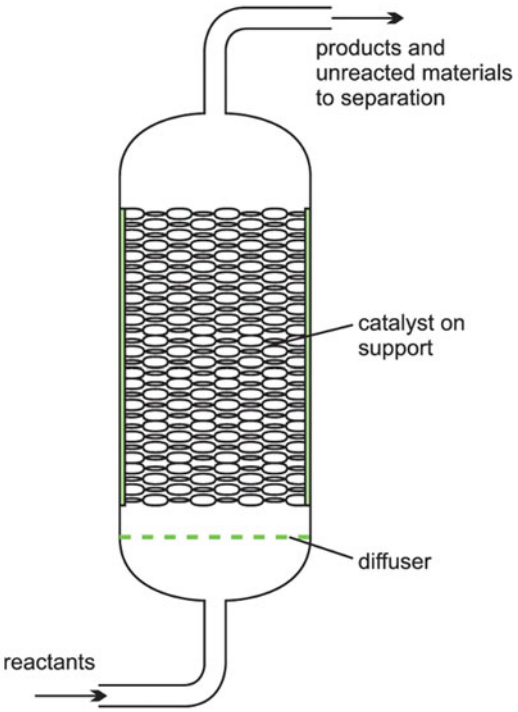

Fig. 17.11 A schematic diagram of a fixed-bed reactor. (http://www.essentialchemicalindustry.org/processes/chemical-reactors.html)

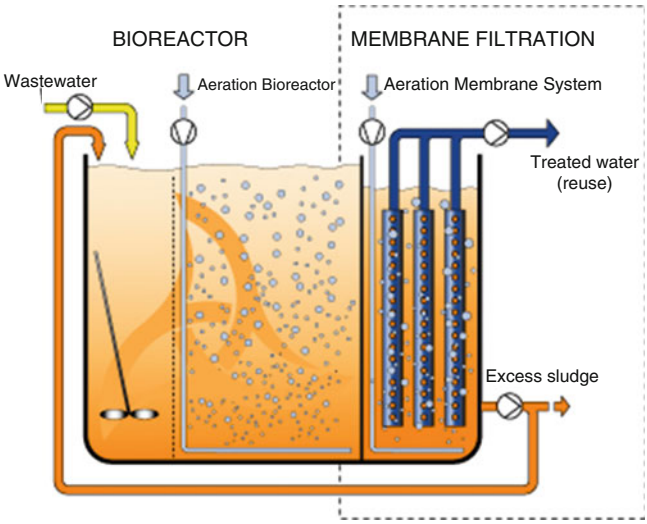

Fig. 17.12 A schematic diagram of membrane bioreactor. (Ntaikou et al. 2010)

3.2.3 Membrane Bioreactor

Membrane bioreactor (MBR) is mainly used to regulate biomass concentration (Fig. 17.12). MBR did not exhibit dominance on other high-rate hydrogen production systems. In bio-hydrogen, fermentation membrane fouling and high operating cost also limit the use of membrane bioreactor process (Wang et al. 2011). The gas separation membrane allows one component in a gas stream to pass through faster than the other components. Solvent-assisted membranes are being developed to combine the best features of membranes and solvent scrubbing. Different types of gas separation membranes include porous inorganic membranes, palladium membranes, polymeric membranes, and zeolites are used in the bioreactor. In the membrane bioreactor, different characteristics may be required to separate pure hydrogen from CO_2 and mixture of gasses. Membranes cannot usually complete high degrees of separation. Therefore, multiple-stage recycling is essential.

3.2.4 Multistage Bioreactors

Multistage bioreactors comprising of three or four stages engaged to maximize the production from the substrate (Luo et al. 2011; Kosourov et al. 2012). Sunlight is mandatory in direct photolysis reactor because visible light will be broken by blue-green algae and the unfiltered infrared light is used by photosynthetic microbes in the

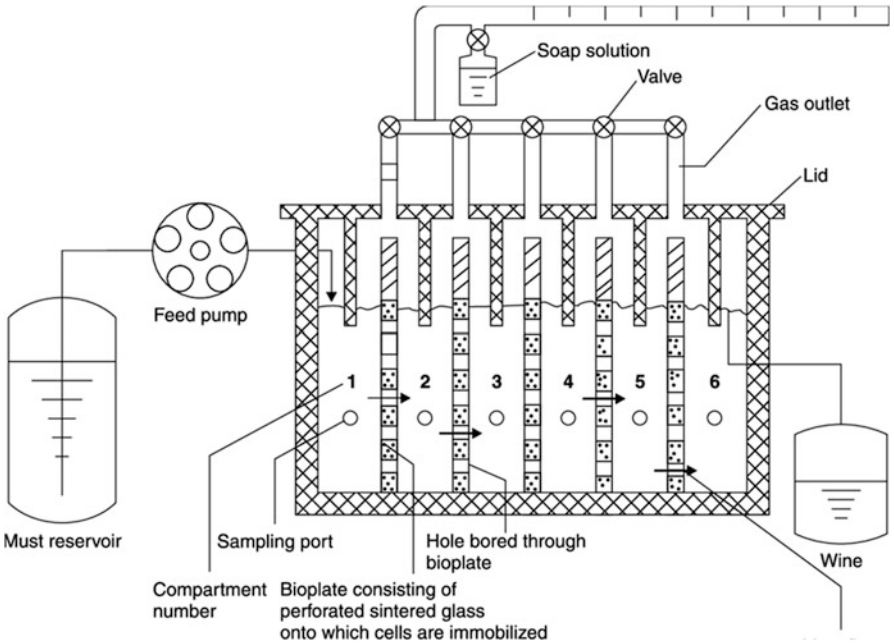

Fig. 17.13 A schematic design of a multistage bioreactor. (Ogbonna et al. 1989)

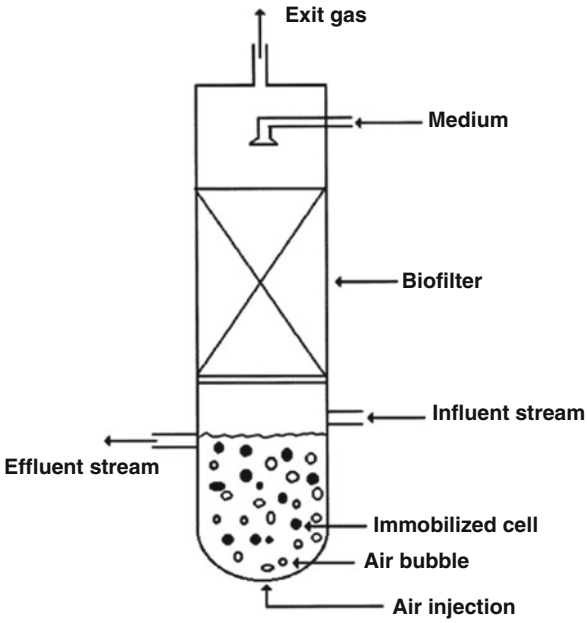

Fig. 17.14 A schematic diagram of a hybrid bioreactor. (Yeom and Yoo 1999)

second stage called photo-fermentative reactor. In third stage, called dark fermentation effluent along with feedstock is satisfied into reactor where the bacteria convert the substrate to hydrogen and organic acids. The fourth stage is the use of a MEC by which organic acids product produced in the dark fermentation under light-independent process. So it can be controlled during night or in low light situation (Moreira et al. 2006) (Fig. 17.13).

3.2.5 Hybrid Bioreactors

Principle of the hybrid two-stage bioreactors is to produce hydrogen and organic acids through bio-fermentation of substrate and other gaseous energy like methane is being removed from the second stage. For enhancing gas production, second stage is being operated under different conditions like higher pH and longer hydraulic retention times. Hydrogen yield can be improved in a hybrid two-stage system in combination of dark and photo-fermentation. In the first stage, under thermophilic dark fermentation, biomass is fermented to produce acetate, carbon dioxide, and hydrogen, and in the second stage, acetate is converted into hydrogen and carbon dioxide (Show et al. 2011; Markov 2012) (Fig. 17.14).

4 Challenges in Microalgal Hydrogen Production

Many algal species, like *Chlamydomonas*, *Chlorella* (Rashid et al. 2013), *Scenedesmus* (Schulz et al. 1998), and *Tetraselmis* (D'Adamo et al. 2014), have been stated to produce hydrogen at lower levels. During hydrogen production using of *C. reinhardtii* has the advantage because genome sequencing of nucleus, chloroplast, and mitochondria gives complete information on the organization of photosynthetic complexes (Hemschemeier and Happe 2011; Tolleter et al. 2011). Gene transfer techniques like particle bombardment (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990), electroporation (Shimogawara et al. 1998), and *Agrobacterium tumefaciens*-mediated are well established, and using of these techniques on model organisms *Chlamydomonas* may enhance the H_2 production.

4.1 Light Capture Efficiency

The light-harvesting antenna proteins (LHC) of photosystem have a double function, to arrest photons and disperse additional light energy to deliver photoprotection (Niyogi 1999; Pascal et al. 2005; Takahashi et al. 2006). Following incident may take to increase light capture efficiency of microalgae:

1. Biomass production productivity at laboratory scale can be enhanced through LHC antenna reduction that improves light distribution through the bioreactors, responsible to increase cell concentrations and to improve overall photosynthetic efficiencies (Beckmann et al. 2009; Mussgnug et al. 2007; Oey et al. 2013; Polle et al. 2003).
2. Moreover, under sulfur deprivation, *C. reinhardtii* antenna mutants have been stated to develop three factors for enhancing H_2 production (Oey et al. 2013): (i) developed light circulation leading to higher photon transformation proficiencies of the overall culture; (ii) the capacity to lower the dissolved oxygen concentration through the use of higher cell compactness, which equilibrium O_2 yields with metabolic load; and (iii) altered photo-inhibition and steadying of PSII required for subsequent hydrogen production (Volgusheva et al. 2015).
3. In larger-scale production systems, in-depth understanding of the structural complexity and dynamic light response of algae LHC antenna is required for precise engineering (de Mooij et al. 2015). Introduction of foreign DNA into the nuclear genome of antenna can be done through chemical or random insertion mutagenesis (Polle et al. 2002, 2003), the operation of antenna regulation proteins (e.g., NAB1 (Beckmann et al. 2009) or RNAi knockdown approaches (Mussgnug et al. 2007; Oey et al. 2013) and random insertion (Zhang et al. 2014).
4. A more targeted approach to engineer antenna cell lines is via the indirect route of manipulating antenna regulation proteins. For example, the overexpression of the translational repressor NAB1 results in the downregulation of exact LHC proteins (Beckmann et al. 2009). A challenge for exact antenna engineering is that the

coding regions of the LHC genes are highly homologous (Natali and Croce 2015), which complicates the specific downregulation of target LHC genes.

5. The most well-designed technologies expedite accurate and stable genome editing enabling the fine modification of antenna genes and corrections to the pigment content, intensifying the available solar spectrum (Blankenship and Chen 2013; Blankenship et al. 2011). Several genome editing techniques have been established in recent years, including zinc finger nucleases (ZFN) (Sizova et al. 2013), transcription activator-like effector nucleases (TALENs) (Gao et al. 2014), and the CRISPR/Cas systems (Cho et al. 2013; Mali et al. 2013), all of which depend on nuclease-induced DNA strand breaks and endogenous cell repair mechanisms to gain mutants. Another magnetism of these methods is to grow mutants which are nongenetically modified organisms through the transient introduction of required nucleases.
6. ZFN (Sizova et al. 2013) and TALENs (Gao et al. 2014) have been used for genome editing in *Chlamydomonas*, which is labor-intensive cloning steps. On the other hand, the CRISPR/Cas (Cas 9) system is a (Cho et al. 2013; Mali et al. 2013) potential simple technique only looking for expression of RNA-guided nuclease. Although effective CRISPR/Cas usage in *Chlamydomonas* is yet to be published, it is predicted that this technique will be possible in *Chlamydomonas* in near future (Jiang et al. 2014).

4.2 Availability of Electron and PETF

For viable hydrogen production, another prospective logjam is inadequate electron flow to the hydrogenase (Hallenbeck and Benemann 2002). This can be occurred due to the limited availability of reduced ferredoxin (PETF) as a result of other competing pathways (Winkler et al. 2011), e.g., ferredoxin-NADP+ reductase (FNR), sulfite reductase, nitrate reductase, glutamate synthase, and fatty acid desaturases (Hemschemeier and Happe 2011). In this phenomenon:

1. The hydrogenase (HYDA) can accept e^- through two routes: (i) direct route which is PSII-dependent provides two photons per electron to HYDA, or (ii) indirect route which is PSII-independent from starch provides three photons per electron to HYDA (Chochois et al. 2009; Fouchard et al. 2005).
2. To progress electron flow to PETF, FNR and the hydrogenase have all been engineered (Long et al. 2009; Lubner et al. 2011; Rumpel et al. 2014; Sun et al. 2013; Wittenberg et al. 2013; Yacoby et al. 2011), with a specific attention on the enhancement of (i) attraction of the hydrogenase to PETF, (ii) decline of the affinity of PETF for FNR, and (iii) fusion of PETF and PSI with the hydrogenase.
3. However, most engineering efforts have concentrated on the chloroplast-localized nuclear-encoded genes toward the in situ overexpression of the hydrogenase in the plastid to separate it from its natural regulator system (Reifschneider-Wegner et al. 2014).

4. Engineering has also focused on indirect targets to increase hydrogen production level, including electron competitors such as RuBisCo (Pinto et al. 2013), cyclic electron flow (Kruse et al. 2005; Tolleter et al. 2011), starch degradation (Chochois et al. 2009), and respiration (Ruehle et al. 2008).
5. Hydrogen production also be increased through added additional components like electron transfer pathway, including a plastid-expressed NAD(P)H dehydrogenase (Baltz et al. 2014), a hexose transporter (Doebbe et al. 2007), and exogenous hydrogenases (Chien et al. 2012).

4.3 Alteration of the Thylakoid Proton Gradient

Another potential break through is alteration of the thylakoid proton gradient in which proton supply to the hydrogenase.

1. In this process transport of e^- from water to PETF via the photosynthetic electron transport chain is responsible in founding a proton gradient across the thylakoid membrane which drives ATP synthesis. ATP is essential during CO_2 fixation, and hydrogen production becomes low due to the lack of ATP (Volgusheva et al. 2015) responsible to reduce electron transport at the point of Cytb6f (Antal et al. 2009; Burgess et al. 2011), leading to an impaired dissipation of the proton gradient and therefore decreased proton availability for the hydrogenase. To improve H_2 production via artificially introduce of the proton gradient in the presence of chemical uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP), which causes an efflux of H^+ from the thylakoid lumen into the stroma (Kruse et al. 2005; Lee 2013; Lee and Greenbaum 2003).
2. A similar strategy involves the development of a leaky ATPase to increase proton flow and reduce ATP production (Das et al. 2014; Robertson et al. 1990). Reduced ATP production triggered by the introduction of a proton channel or mutated ATPase may additionally decrease reactions competing for reducing equivalents and therefore increase electron supply to the hydrogenase (Kumar and Das 2013).

4.4 Oxygen Sensitivity

Sustainable H_2 production under standard growth condition is a major challenge, due to O_2 sensitivity to the hydrogenase. Oxygen inhibits not just hydrogenase enzyme function but also transcription and protein maturation (Cohen et al. 2005). Remarkably, two algae strains were recently reported that hydrogenase enzyme can be expressed in the presence of more than 21% O_2 and to produce low levels of H_2 at

15% atmospheric O_2 (Hwang et al. 2014). Several genetic engineering approaches have also been applied to reduce the O_2 sensitivity on the hydrogenase:

1. Due to random and targeted mutagenesis of catalytic site (Flynn et al. 2002) and targeted mutagenesis of the catalytic site limit O_2 access (Stiebritz and Reiher 2012). This approaches have been effective for a bacterial [NiFe] hydrogenase (Dementin et al. 2009), not been the case with the microalgal [FeFe] hydrogenase. For this reason, the identification of hydrogenase with reduced O_2 sensitivities will increase the scope for genetic engineering.
2. A hydrogenase with a reduced O_2 sensitivity would open up a direct path in H_2 production from water splitting but would also lead to the co-production of O_2, which then requires subsequent gas separation. However, this approach would overcome through two-phase aerobic/anaerobic process which is engaged in a continuous H_2 production and also eliminating the ATP and NADPH, are required to produce starch- and oil-based feedstocks for alternative fuels.
3. Alteration of O_2 sensitivity and extension of H_2 production can be controlled through increasing the cell density and respiratory load at anaerobic condition (Oey et al. 2013; Sch€onfeld et al. 2004).
4. Moreover, downregulation of PSII (Surzycki et al. 2007) and engineering of O_2 evolution activity (Makarova et al. 2007; Scoma et al. 2012; Torzillo et al. 2009) have been exploited to reduce sensitivity of O_2 on hydrogenase.
5. Leghemoglobins, capable to sequester O_2, have also been expressed in *C. reinhardtii* (Wu et al. 2010, 2011), and a sulfate permease mutant was developed, which allowed greater control of sulfur deprivation (Chen et al. 2005).

The development of H_2 screening methods can also accelerate the engineering of improved H_2-producing algal strains. H_2 mutants can be detected through a chemochromic sensing assay, which utilizes a palladium/tungsten oxide film (Seibert et al. 1998). A current H_2-detection screen method developed (Wecker and Ghirardi 2014), which uses *Rhodobacter capsulatus* expressing an emerald green fluorescent protein (emGFP) driven by the hupR H_2-sensing promoter, deals a simple, inexpensive, and semiquantitative screening method. The development of a chlorophyll fluorescence assay (Godaux et al. 2013) and an inorganic hydrogenase active site mimic will also permit for quicker screening of hydrogenase mutants (Berggren et al. 2013; Esselborn et al. 2013). Indirect process as genetic engineering and biotechnology simplify cultivation of microalgae, analysis, and H_2. The ability to easily report lumen pH (Ben_cina 2013; Demaurex 2002), hydrogenase expression, and key metabolic pathways such as sulfur, starch, and lipid metabolism, combined with external control of gene expression levels and physiological mechanisms via the supply of stimuli such as hormones or light, would enable the rapid dissection of the changes in cell state occurring during H_2 production.

5 Microalgae H_2 Systems Within a Global Perspective

It is also important to understand which technology is fit for hydrogen production at global context, as hydrogen linked several global challenges. In global perspective following scenarios is related with hydrogen production from microalgae:

1. In 2010, the G20 nations accounted demand of global energy and level of CO_2 emissions is similarly 83% (BP 2013). The maximum emission level of CO_2 was reported in China (8205.9 Mt CO_2), the USA (5074.1 Mt CO_2), and India (1954.0 Mt CO_2) (International Energy Agency (IEA) 2014). In 2013, among these G20 nations' per capita, highest CO_2 emission range is found in Saudi Arabia ~17.2 tCO_2/year/person and lowest in India (1.8 tCO_2/year/person) (Global Carbon Project (GCP) 2013; Population Reference Bureau (PRB) 2013). This has resulted in a debate between high and low CO_2 emitters, all of whom are looking for to limit their emission liabilities. On this burning issue hydrogen offers the forthcoming per capita energy demand without the CO_2 emissions which is associated with carbon-based fuels.
2. Instead of fossil fuel significant challenges, in hydrogen production some technical difficulty remains such as capturing, storing, purifying and compressing of H_2, and marketing (Bimbo et al. 2013; Hruzewicz-Kołodziejczyk et al. 2012).
3. Significant educations can be learnt from demonstration scale systems on volatile fuels market because the photosynthetic element of hydrogen yields is a vital structure, for example, ethanol, which are apparently close to commercial production using cyanobacteria for less than $2 per gallon (Perkins 2014). In storage and distribution systems of H_2, there is yet no global infrastructure enabling the use of H_2 as a "drop-in" fuel.
4. It has been forecasted that 8–10% to the energy market would be contributed by hydrogen within 2025 (Gupta et al. 2013), which will provide a development driver for H_2 production, storage, circulation, and transformation technologies, and these are actively being developed and deployed internationally. Policies such as tax incentives are also gradually being established (Fuel Cell and Hydrogen Energy Association (FCHEA) 2014).

Therefore, an international movement is starting to bond H_2-producing technologies with advancements in storage, distribution, and fuel cell technologies. Analysis of the global H_2 patent scenario delivers valuable visions for developing microalgal H_2 biotechnology industry, along with challenges and opportunities (Fig. 17.15). National consumption levels on H_2 technologies by the G20 nations are shown in Fig. 17.15a, where Japan, the USA, and EU assisted the largest number of developments. Current patent submissions of the G20 countries in the areas of H_2 energy (Fig. 17.15b) and H_2 production (Fig. 17.15c), as well as renewable H_2 production (Fig. 17.15c), H_2 storage (Fig. 17.15d), H_2 fuel cells (Fig. 17.15e), and H_2-driven transport (Fig. 17.15f), focus the advancement of future H_2 economy. The majority number of patents 87 and 85 related to H_2 as a renewable energy source were filed in the USA and China (Fig. 17.15c), respectively, though among the other

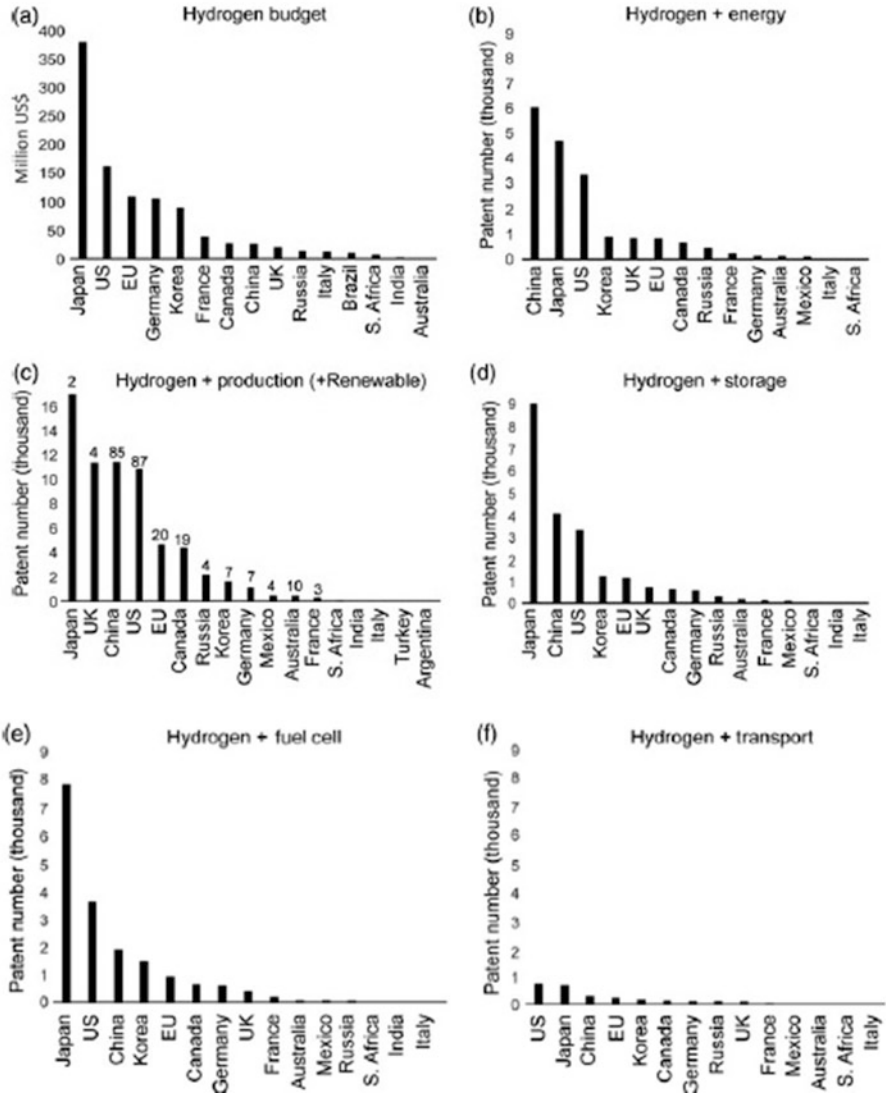

Fig. 17.15 Hydrogen budget and potential market in G20 countries. (**a**) Allocated budget per country for the development of hydrogen technology and (**b**–**f**) patents filed in the respective countries

countries, they are highest national CO_2 emitters. Even Japan stated the highest H_2 fuel cell patent submissions (Fig. 17.15e) (Government of Japan (GoJ) 2014). These international patenting patterns may be a sign of early technology adopter hotspots and focus the potential of developing renewable H_2 production platforms to supply emerging global H_2 markets and distribution networks.

6 Future Perspectives

At present, in total global energy, 80% is utilized and as fuel 20% is utilized as electricity. Hydrogen energy has been proposed as the future energy ruler because of clean and alternative energy. Solar-driven microalgal hydrogen production is a promising, which play a vital role in the global drive to reduce greenhouse gas (GHG) releases. In hydrogen economy the major obstacles are its production rate through effective strain and inefficient cost in purification, storage, and transport systems, which are essential to be fixed.

(i) Recent investigations are intensive on strain improvement by system metabolic engineering such as various genome-wide tools in systems biology, including high-throughput analytical techniques, computational analyses, and integrated analyses that cover genomic, transcriptomic, proteomic, and metabolomics to find proper environments to increase the levels of hydrogen yields.
(ii) In the near future, it may be promising to execute knockouts and insertions techniques on the basis of data available by modeling research. The start of synthetic biology demands such models, since it aims for systematizing biology to give projected responses. The commercial sustainability of hydrogen production may depend on proficient production strategies with projecting yield, well-organized storage systems and transport ensuring the secured supply of hydrogen.

In near future many industries will receive a renewable hydrogen energy when hydrogen production will economically feasible. Last but not least, the combined strength of both scientists and engineers is required to fully implement hydrogen energy as the energy for the future green world.

7 Conclusion

Day by day the human society faces an unappeasable desire for energy to meet up with global prosperity. But paradoxically dependence on fossil fuels as the primary energy source becomes major cause to global warming, environmental degradation, and health problems which threaten the survival of mankind. Hydrogen from microalgae resources is a sustainable source for energy not limited and with different applications. Microalgae can be used for hydrogen production because of their genetic capacity to split water into H_2 and O_2 using light energy. Photobiological H_2 yield is reflected as a more efficient and less energy-intensive process. Due to its high production cost, hydrogen energy from microalgae became economically less viable. However, recent metabolic and genetic engineering approaches can be augmented bio-hydrogen yields from microalgae.

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Part III
Microalgae in CO_2 Sequestration and Wastewater Treatment

Chapter 18
Metabolomics Analysis of Microalgae for the Cellular Physiology Adjustment to High CO_2

Ying Guo and Zhenhong Yuan

Abstract Carbon dioxide capture by microalgae may be a potential approach to reduce carbon emission from industrial plants. The high CO_2 concentrations can constrain the growth of most microalgae. For the tolerant species, the metabolites were found to enhance the cellular physiology mechanisms. These cellular physiology adjustments of the high CO_2 were related to signal transduction, nutrition availability, intracellular pH adjustment, and other pathways.

Keywords Carbon dioxide capture · Cellular physiology mechanisms · Metabolomics · Microalgae

1 Introduction

Microalgae CO_2 capture may be a potential approach to reduce carbon release of industrial plants. As a major greenhouse gas, CO_2 in atmosphere has risen greatly to 0.04% from historical 0.03% in last century mainly due to industrial releases. The anthropogenic CO_2 emissions may contribute to climate change and ocean acidification. In an industrial plant, flue gas passes through denitrification and desulfurization treatments to remove nitrogen oxides and sulfur oxides. The majority part of industrial flue gas that need to be treated is only CO_2. Recently, the use of microalgae to sequester CO_2 has been increasingly developed as their higher growth and turnover rates compared with terrestrial plants. Microalgae carbon capture methodology could save the energy typically consumed by a chemical absorption process. This methodology is energy sustainable because microalgal biomass can serve as an alternative to fossil fuels.

Microalgal biomass is rich in lipids, starches, proteins, beta-carotene, and other nutrients and can be used for biofuels, animal feed, and food products. Microalgae

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culture system integrated with CO_2 capture process will reduce the microalgal biomass production cost. The flue gas of industrial plants can provide a rich carbon source for microalgae. However, the CO_2 concentration of industrial flue gas is so high that it constrains the growth rates of most microalgae. The flue gas can be captured and purified to provide the microalgae culture with desired concentrations, which demands cost on flue gas delivery and distribution. The screening for tolerant microalgae species to directly treat flue gas is another option of the microalgae CO_2 capture methodology.

2 The Physiology Process for the Microalgal Carbon Capture

2.1 Photosynthesis as a Source of Energy

The 10^{22} kJ solar radiation energy will be stabilized as the chemical molecule through the photosynthesis of microalgae. During photosynthesis, CO_2 and H_2O molecules will be converted to carbohydrate and O_2, and radiation energy is reserved in the stable organic form. Photosynthesis consists of two parts: the light reactions and the Calvin cycle. The products of the light reactions, NADPH and ATP, are to drive the fixation of CO_2. Photosynthesis light reactions are mediated by photosystem I and photosystem II. Photosystem I uses light-derived electrons to create NADPH, and photosystem II is to generate electrons from H_2O molecule and transfer the electrons to plastoquinone. The electrons travel from photosystem II to cytochrome bf and to photosystem I which will generate H^+ gradient. The H^+ gradient across the thylakoid membrane drives the formation of ATP. The products of the light reactions, NADPH and ATP, are ready for the Calvin cycle (Berg et al. 2015).

2.2 The Entry of CO_2

The Calvin cycle of photosynthesis demands CO_2 molecule. The inorganic carbon transporters and accumulation components in eukaryotic algal cells include the CO_2 and HCO_3^- transporter systems, a stromal HCO_3^- pool, and various carbonic anhydrases (CAs), which bring the CO_2 to the pyrenoid compartment where RubisCO is localized (Badger et al. 1980; Badger and Price 1992, 1994).

The plasma membrane and the chloroplast inner envelope are the barriers for the transfer of the inorganic carbon. The CO_2 transfer system is composed of gas channels, which deliver CO_2 from the extracellular space into cell. CO_2 then diffuses through the thylakoid membrane to the reaction center of RubisCO. Only the CO_2 molecule can be utilized by RubisCO.

Alternatively, the entry of inorganic carbon can be transferred through HCO_3^-. HCO_3^- is converted into CO_2 molecule before or after the diffusion process with CAs. The inorganic carbon transfer for bicarbonate occurs at the plasma membrane and the chloroplast envelope. This energy demanding process is connected with the electron transfer chain and mitochondrial ATP (Tsuzuki et al. 1986; Badger et al. 1980; Badger and Price 1992, 1994; Colman et al. 2002; Giordano et al. 2005; Wang et al. 2011).

2.2.1 The Transporter System

2.2.1.1 CO_2 Channels

The identified possible CO_2 channels include RHP1 and RHP2. These 2 channels have 12 transmembrane domains. RHP1 localizes on plasma membranes, and its expression is induced by high concentration of CO_2.

2.2.1.2 HCO_3^- Transporters

Some efficient HCO_3^- transporters have been identified at the chloroplast envelopes, for instance, Na^+-independent transporter/Na^+-dependent transporter and ATPase-linked HCO_3^- transporter. Other possible HCO_3^- transporters include LCIA, CCP1, CCP2, and the LCIB protein family LCIB/LCIC/LCID/LCIE.

The H^+ transporters, such as CemA-dependent system, are considered to play a role in the HCO_3^- assimilation process. CemA is an integral membrane protein of the chloroplast envelope. The CemA-dependent system is considered to balance electrical and pH homeostasis.

2.2.1.3 HCO_3^- Pool

A HCO_3^- pool exists in the stroma (Badger et al. 1980). This is mainly because HCO_3^- is less permeable to lipid membranes and the alkaline stromal favors the acidic HCO_3^- form. LCIB can work with CAH6 to utilize the excess transferred carbon dioxide molecules.

2.2.1.4 Carbonic Anhydrases

Carbonic anhydrases catalyze the reversible reaction between HCO_3^- and CO_2. In the active center of the carbonic anhydrases, a key feature is a positively charged zinc center stabilized with 3 histone. This zinc center, with a hydrophobic patch, can bind a water molecule and a carbon dioxide. The zinc center will reduce the pKa of its binding water molecule from 15.7 to 7. This lower pKa can enhance the water

molecule deprotonation process. The OH^-, the end product of the deprotonation process, can serve as a nucleophile and attack the attached carbon dioxide (Badger and Price 1994; Moroney et al. 1985, 2001; Patel and Merrett 1986; Williams and Colman 1995). This catalytic process with an efficiency of kcat/Km of 8.3×10^7 accelerates the rate of CO_2 hydration dramatically (Berg et al. 2015).

2.2.1.5 External CAs

The extracellular carbonic anhydrases have been identified within the periplasmic space and the inner side of the cell wall, which are attached to the membrane periphery by ionic interactions. The activity of external CAs was reported to be repressed in microalgae grown on high CO_2 and at low pH (Williams and Colman 1995).

2.2.1.6 Internal CAs

The possible intracellular carbonic anhydrases, for instance, thylakoid lumen CAs encoded by the Cah3 gene, cytoplasmic CAs, and mitochondrial CAs, were identified. It is reported that CAH3 catalyzed the rapid dehydration process to enhance available CO_2 for RubisCO in thylakoid (Moroney et al. 1985; Patel and Merrett 1986; Williams and Colman 1995; Moroney et al. 2001; Swarnalatha et al. 2015). However, the functions of other CAs are still not clear.

2.3 CO_2 Fixation Through the Calvin Cycle

With the available NADPH, ATP, and CO_2 molecule, the Calvin cycle occurs in the matrix of the chloroplast. Through Calvin cycle, CO_2 molecules formed carbohydrates via ribulose 1,5-diphosphate carboxylase/oxygenase (RubisCO). The Calvin cycle creates the available energy sources for the creature of entire biosphere.

The Calvin cycle consists of three phases (Fig. 1–7):

1. Fixation of CO_2 by RubisCO
2. Hexose formation from 3-phosphoglycerate
3. Regeneration of ribulose 1,5-diphosphate

The first step in the Calvin cycle is the CO_2 fixation. Ribose-1,5-bisphosphate is converted to highly active enolate intermediates. The CO_2 molecule, the H_2O molecule, and the alkene glycol intermediate are condensed to form an unstable intermediate which then be hydrolyzed into two molecules of 3-phosphoglycerate. The reaction occurs at the surface of the matrix of the chloroplast thylakoid, where RubisCO stay. In fact, RubisCO is the richest enzyme and the most abundant protein on Earth. The role of RubisCO for the global biosphere is essential (Berg et al. 2015).

2.4 The Formation of Microalgal Biomass

The hexoses produced from photosynthesis are the building materials for the microalgal biomass. The corresponding microalgal pathways are mainly deduced from the established metabolism in bacteria and plants. There may be slightly difference among the pathways of species, but the information from bacteria and plants gives insights into the involved mechanisms of microalgae.

2.4.1 Carbohydrates

Microalgal carbohydrates are mainly stored as the disaccharide form, sucrose, and polysaccharide form, starches. After the photosynthesis process, sucrose is synthesized from the reaction between fructose-6-phosphate and the activated intermediate uridine diphosphate glucose (UDP-glucose).

Starches are composed of amylose (10–30%) and amylopectin (70–90%). Amylose is the unbranched macromolecule connected with α-1,4-linkages. The difference between amylopectin and amylose is that amylopectin has branched α-1,6-linkages. The α-1,4-linkages and α-1,6-linkages ratio in amylopectin is around 30 (Berg et al. 2015). Amylopectin and amylose are ready to be hydrolyzed into glucose by amylase. Specially, some α-1,3-linkages were reported in the *Phaeophyceae* and *Bacillariophyceae*.

Starch synthesis occurs in chloroplasts. The amylopectin synthesis includes four steps: substrate activation, chain elongation, branching, and debranching, while the synthesis of amylose is only involved in the first two steps. Fructose-6-phosphate from photosynthetic is converted to glucose-6-phosphate by a phosphoglucose isomerase and to glucose-1-phosphate through a plastidial phosphoglucomutase. The building block of starch is ADP-glucose, and it is formed via pyrophosphorylase. Then, the elongation of α-1,4-glucans is catalyzed with starch synthases. The ADP-glucose is transferred to the nonreducing end of the polyglucan. The elongation step continues until a certain number of glucans have been added. In the branching step, a branching enzyme hydrolyzes and transfers an α-1,4 linkage to α-1,6 linkage. Finally, in the debranching step, the pullulanase and isoamylase will catalyze the pre-loosely structure into tightly packed amorphous region (Bernfeld 2006; Sadovskaya et al. 2014).

For the degradation of starch, the glucan residues are treated to be easily accessible to β-amylase through a phosphorylate step on the surface of the starch granule. Phosphoglucan-water diakineses and glucan-water dikinases are involved to phosphorylate ATP to the C-6 and C-3 position of a glucan. This step is considered to disrupt the crystalline structure of the amylopectin helices. Then, β-amylase can easily cleave the α-1,4-bonds of starch. Starch can be treated to be branched and linear. These loose forms are further trimmed into maltose and glucose through β-amylase and starch phosphorylase. Maltose and glucose are then transported from

chloroplast to cytosol. The detailed mechanism for the degradation of starch in microalgae needs to be further studied.

Another major microalgal carbohydrate is cellulose, which usually is a structural component of microalgal cell wall.

Cellulose is an unbranched glucan polymer in β-1,4-linkages. Cellulose has a rigid and supportive structure form, with its long and straight chains compacted with hydrogen bonds (Berg et al. 2015).

2.4.2 Lipids

The algal lipids can exist as structural component of membrane, fuel molecules, signal molecules, and signal messengers. The membrane lipids are composed of phospholipids, glycolipids, and cholesterol. Phospholipids are composed of a platform function group, fatty acids chains, a phosphate, and an alcohol. Glycolipids are molecules attached with sugar moieties. Cholesterol is the molecule with a steroid platform. The lipids are served as energy store molecules mostly as the form of triacylglycerols. Triacylglycerols are uncharged esters of fatty acids with glycerol. Fatty acids are long hydrocarbon chains with a carboxylic acid end. They have different degrees of unsaturation. The carbon numbers of fatty acid usually range from 6 to over 22 (Berg et al. 2015). The carbon chain can have different degree of unsaturation. It is reported that lipid synthesis is likely very similar and simplified in unicell microalgae compared with the multicell plants (Hajra and Bishop 1982; Rangan and Smith 2002; Schnurr et al. 2013; Siaut et al. 2011).

For the synthesis of fatty acids, in microalgae, it occurs mainly in the chloroplast, plasmid, and cytoplasm. Fatty acid synthase are composed of the acetyl-CoA carboxylase and phosphopantetheinyl transferase, ketosynthase (KS), ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains.

Algal fatty acid synthesis consists of four steps. Fatty acid synthesis starts with an activated acyl CoA and a malonyl-CoA. The formation of a malonyl unit is the limited step of fatty acid synthesis which is catalyzed by acetyl-CoA carboxylase (ACCase), when acetyl-CoA is converted into malonyl-CoA. Malonyl-CoA is then attached to the acyl carrier protein. The acyl carrier protein goes through different conformational structures via fatty acid synthase enzymes, and the carbonyl group is reduced to a methylene group through dehydration and reduction. The product of the reduction is butyryl-CoA. This process is repeated until a desired length of fatty acid is synthesized. The molecule added at each round is malonyl-CoA. Finally, fatty acyl ACP thioesterase cleaves the bond between the fatty acyl chain and its carrier protein (Spalding and Prigge 2010; Berg et al. 2015).

Interestingly, the degradation of fatty acids is the opposite of the synthesis. It occurs in the mitochondria. This is an oxidative process that degrades the long fatty acid into activated acetyl-CoA. After an activation step, the two end carbons of fatty acid are cut down and oxidized to acetyl-CoA in each cycle. During the cycle, the methylene group in fatty acid goes through a double bond, a hydroxyl group, finally to a keto group (Berg et al. 2015).

The keto group is cleaved by coenzyme A to yield acetyl-CoA. Acetyl-CoA enters into the citric acid pathway. The fatty acids with the odd number of carbon will produce a molecule of propionyl-CoA in the final cycle. Propionyl-CoA needs to be further converted into succinyl-CoA before entering into the citric acid pathway.

Fatty acid synthesis is described as Fig. 3 (Berg et al. 2015).

2.4.3 Amino Acids

Glutamate and glutamine are nitrogen donors for most amino acids. The reaction process is catalyzed by glutamate dehydrogenase and glutamine synthetase. The amine of most amino acids is derived from α-aminoglutamic acid transamination. Glutamine, another major nitrogen donor, contributes to the nitrogen in side chain of tryptophan and histidine (Berg et al. 2015).

The carbon skeletons of amino acids come from glycolytic intermediates, the pentose phosphate pathway, or the citric acid cycle (Berg et al. 2015).

2.4.4 Nucleic Acids

Nucleotides biosynthesis pathways are divided into two categories: de novo synthesis and salvage pathways.

In the de novo synthesis pathway, the nucleotide bases are assembled. The framework of the pyrimidine base then adhere to the ribose. The backbone of the purine base is directly linked with the ribose.

In the salvage pathways, bases will be recycled and reconnected to the ribose end. The distinction between thymine and uracil methyl synthesis is only at the last step. Ribonucleotides are mainly relying on de novo synthesis (Berg et al. 2015).

Thus, the development of an efficient CO_2 capture technology will reduce anthropogenic CO_2 emissions. The CO_2 capture is meaningful for mitigating climate change and maintaining the stability and resilience of the ecosystem. Microalgae are the autotrophic and aquatic organisms. The microalgal cell is the delicate energy factory which converts the CO_2 into biomass. It is the most potential organism on the planet for a biological carbon capture process, as it possesses the higher exponential growth rate and photosynthesis capability as compared with other terrestrial crops. This study investigated the key scientific issues of carbon dioxide capture process: screening high CO_2-tolerant microalgae and its corresponding CO_2 conversion mechanisms.

3 The Microalgae Responses to the High CO_2

Microalgae species can develop adaptive mechanisms to mitigate the stress caused by a high CO_2 environment (Solovchenko and Khozin-Goldberg 2013; Baba and Shiraiwa 2012; Wang et al. 2011; Giordano et al. 2005; Solovchenko et al. 2015). The reported responses to the high CO_2 concentrations included decreasing CO_2 concentrating mechanisms (CCMs) operation; suppressing the photorespiration, rearrangements, and transitions of membrane proteins; and triggering gametogenesis. In *Chlamydomonas reinhardtii*, the CCMs would be inhibited through the bicarbonate transporters and carbonic anhydrases (CAs) in the high concentrations of CO_2. Giordano et al. suggested the high CO_2 concentrations might affect nitrogen assimilation through the photorespiratory nitrogen cycle, to satisfy intracellular C:N ratio (Giordano et al. 2005). Similarly, the high CO_2 concentrations might reduce intracellular nitrogen by suppressing possible nitrogen transporter activities and thus inhibiting nitrogen availability in the chloroplast (Baba and Shiraiwa 2012). Matsuda et al. described that the intracellular nitrogen level, for instance, ammonia, could induce gametogenesis, a sexual reproduction state to form mature haploid gametes (Matsuda et al. 1992). Based on response mechanisms, transcription, translation, and metabolite regulatory approach have been developed for distinct proteins, expressed genes, and the metabolic network under varied CO_2 inputs.

However, when it comes to microalgae CO_2 capture methodology, further studies are required to understand the responses of a tolerant microalgae resistant to CO_2 of industrial flue gases. The most reported studies were conducted with *C. reinhardtii* whose photosynthesis activity was low in the high CO_2 concentrations, and little information can be obtained when examining the molecular acclimation mechanisms for a tolerant species (Baba and Shiraiwa 2012; Wang et al. 2011; Giordano et al. 2005). Ying et al. study the cellular physiology adjustment to high CO_2 concentrations with metabolic analysis. A total of 432 molecules were isolated from microalgae cells and identified by GC-TOF-MS analysis. This chapter will discuss the molecular mechanism that allows microalgae to tolerate industrial flue gases.

In Ying's study, out of 432 identified metabolites, 37 metabolites showed a significant change between the elevated and ambient CO_2 treatments. These metabolites were related to carbon metabolism, amino acid metabolism, lipid metabolism, metabolism of cofactors and vitamins, energy metabolism, transporters, biosynthesis of amino acid tRNA, and biosynthesis of other secondary metabolites (the related pathways are described in Fig. 4–2). The responsive lipids and its derivatives included cerotinic acid, 2-monopalmitin, lipoic acid, sitosterol, phytol, and farnesal. The responsive monosaccharide and its derivatives included gluconic acid, xylitol, fucose, D-fructose 2,6-biphosphate, 1,2-cyclohexanedione, and myoinositol. The responsive metabolites of citrate cycle components included malate, furnarate, and oxamate. The photorespiration-related responsive metabolites included glyceric acid and glycine. The responsive metabolites of protein amino acids and nonprotein amino acids included 4-aminobutyric acid (GABA) and putrescine (diamine metabolism); lysine, 2,6-diaminopimelate, carnitine (lysine metabolism); noradrenaline; alanine; cysteine; and histidine. Under the stress of high concentration of carbon dioxide, the responses of metabolites would represent the transferred acclimation

information that is highlighted and maintained through generations. It is highly possible that some of these responses are passive regulation, and high concentrations of carbon dioxide stress increase or decrease the concentrations of many metabolites. Alternatively, some metabolites might be volunteer regulation molecules; those molecules can maintain a healthy intracellular environment (Table 18.1 and Fig. 18.1).

4 Metabolomics Analysis of Microalgae for the Cellular Physiology Adjustment to High CO_2

The cellular physiology is exhibited with two different molecular classes. The macromolecules, like polysaccharide, proteins, and nucleic acids, and the small molecules, like amino acids, hexose, and guanine, that is, metabolites. The macromolecules serve as structural components, the genetic information storage, and biological process catalyst. The macromolecules exist but may not play the role, for instance, latent genes and inactive enzymes. The cellular physiology state resulted from bundlers of biochemical reactions. Among different organisms, for certain biological purpose, those biochemical steps share critical features. The biochemical reactions start, intermediate, and end with metabolites. The metabolites can thus clearly display the current physiology conditions. Through the metabolic analysis, the intensity and activity of a physiology state can be addressed.

The understanding the functional metabolites will address the difficulty faced in genetic approach. In turn, improvement in current genomics and proteinic analysis makes it possible to study the process mechanism involving metabolic molecule. The EST (expressed sequence tag) databases and genetic engineering technology enhance the specificity and accuracy of transgenic microalgae species. These genetic engineering methods include high-throughput microRNAs and RNA interference in gene knockdown strategies and gene editing tool CRISPR-Cas9. CRISPR-Cas9 has been developed efficiently to make precise, targeted genome modifications. Through the tools achievement, there are over 30 microalgae species that have been successfully transformed. Transformation has fulfilled the goal for the expression of transgene; however, the difficulty is that the expression of transgenes is not always stable, and in some cases only transient expression is obtained. The understanding of the process mechanisms of macromolecular function requires detailed information on its involved metabolic molecules. The knowledge of metabolomics will be helpful for understanding the unstable expression issue.

The metabolism of the microalgae cell is complicated, and the physical and chemical information of metabolic molecules is very rich. The detected approach for the metabolites needs to be with great sensitivity and specificity. The identification of the metabolic molecules calls for a high-throughput GC-MS technique (Lisec et al. 2006; Laurens 2013). In order to understand the process mechanism of the developed high CO_2-tolerant microalgae cells, this chapter examines the metabolites in microalgae by combining the former results from metabolic analysis and GC-MS.

Table 18.1 Identified metabolites of significantly different concentrations between ambient (0.04% CO_2) and elevated (15% CO_2) treatments

Metabolites	Retention time Min	Relative content (0.04% CO_2)	Relative content (15% CO_2)	VIP	P-value	Description
Putrescine	16.4	1.28 ± 0.99	0.33 ± 0.25	1.8	0.03	Alkylamines
Phytol	20.8	0.04 ± 0.036	0.40 ± 0.38	1.7	0.05	Lipids
D-glyceric acid	11.4	0.02 ± 0.019	0.13 ± 0.11	1.8	0.04	Sugar acids
Myoinositol	20.0	0.05 ± 0.05	0.23 ± 0.17	1.7	0.03	Sugar derivatives
L-malate	13.5	0.39 ± 0.30	1.35 ± 0.96	1.7	0.04	Carboxylic acids
4-Aminobutyric acid	14.0	4.6E-08 ± 2.7E-08	0.18 ± 0.17	2.0	0.03	Amino acids
Lysine	18.4	0.13 ± 0.10	0.67 ± 0.45	1.9	0.02	Amino acids
Guanine	20.3	0.04 ± 0.03	0.008 ± 0.005	1.8	0.02	Purines
Alanine	8.2	1.38 ± 1.35	6.49 ± 4.77	1.8	0.03	Amino acids
Furnarate	11.7	0.01 ± 0.009	0.11 ± 0.08	1.9	0.03	Carboxylic acids
Phosphate	10.6	0.32 ± 0.24	1.42 ± 1.01	1.8	0.03	Phosphate
Methyl phosphate	9.2	0.02 ± 0.014	0.13 ± 0.10	1.8	0.03	Phosphate*
Xylitol	15.9	0.02 ± 0.01	0.005 ± 0.003	1.8	0.02	Sugar alcohols
Gluconic acid	19.1	9.8E-06 ± 2.8E-06	0.001 ± 0.001	2.1	0.01	Carboxylic acids
Glucose-1-phosphate	16.6	0.19 ± 0.15	0.02 ± 0.03	2.0	0.01	Sugar acids
Beta-alanine	12.7	0.0007 ± 0.0005	0.01 ± 0.006	2.1	0.01	Alanine *
N-methyl-DL-alanine	9.2	0.005 ± 0.005	0.03 ± 0.02	1.9	0.03	Alanine *
L-cysteine	14.3	0.03 ± 0.03	0.006 ± 0.005	1.7	0.04	Amino acids
Fructose	16.2	0.02 ± 0.01	0.008 ± 0.006	1.6	0.05	Sugars
Cerotinic acid	27.4	0.005 ± 0.003	0.001 ± 0.002	1.8	0.03	Fatty acids
L-glutamic acid	14.0	0.05 ± 0.04	0.15 ± 0.10	1.6	0.04	Amino acids
2-monopalmitin	23.8	0.002 ± 0.002	0.006 ± 0.005	1.6	0.03	Fatty acids
Aminooxyacetic acid	12.0	0.003 ± 0.002	0.01 ± 0.01	1.7	0.04	Amino acids
Sitosterol	29.6	0.0008 ± 0.0008	3.7E-08 ± 2.7E-08	1.7	0.03	Amino acids

Fructose 2,6-biphosphate degr prod	21.1	0.002 ± 0.002	0.0005 ± 0.0005	1.6	0.05	Sugar acids
Carnitine	10.0	0.0006 ± 0.001	0.004 ± 0.003	1.8	0.04	Amino acids
Glycine-d5	11.1	0.002 ± 0.002	3.7E-08 ± 2.7E-08	1.6	0.03	Amino acids
Butyraldehyde	10.7	0.002 ± 0.002	0.01 ± 0.01	1.8	0.04	
Noradrenaline	20.6	0.03 ± 0.03	0.002 ± 0.002	1.6	0.04	Amino acids
N(alpha),N(alpha)-dimethyl-L-histidine	17.5	0.007 ± 0.004	0.002 ± 0.002	2.0	0.01	Amino acids
Oxamate	10.3	4.6E-08 ± 2.7–08	0.001 ± 0.001	2.0	0.02	Carboxylic acid
2-Deoxy-D-galactose	17.0	0.0006 ± 0.0005	0.002 ± 0.002	1.5	0.05	Sugars
Nicotinic acid	10.7	0.0008 ± 0.0008	3.2E-05 ± 8.4–05	2.0	0.02	Coenzyme
Farnesal	17.1	0.001 ± 0.001	3.7E-08 ± 2.7E-08	1.9	0.03	Lipids
2,6-Diaminopimelate	17.7	0.02 ± 0.02	0.09 ± 0.06	1.7	0.03	Amino acids
1,2-Cyclohexanedione	12.5	0.004 ± 0.003	0.0009 ± 0.0007	1.7	0.03	Sugar derivatives
Lipoic acid	19.0	0.0007 ± 0.00095	0.003 ± 0.002	1.4	0.02	Fatty acid derivatives

The relative content (0.04% CO_2) represents the *mean value* ± *SD* of eight-independent experiments, and the relative content (15% CO_2) represents the *mean value* ± *SD* of seven-independent experiments. The combined PCA and PLS-DA model was identified with variable importance in the projection, VIP, >1 and p-value <0.05 to select the metabolites of significant change between two treatments

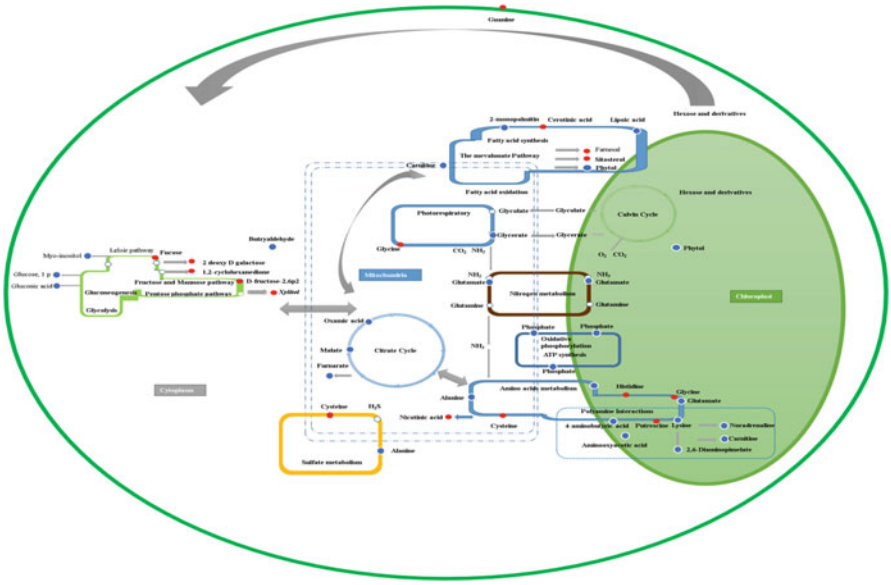

Fig. 18.1 The related pathways of response metabolites

4.1 Citrate Cycle: Malate, Furnarate, and Oxamic Acid

Under the high concentrations of carbon dioxide, the concentration of malic acid and fumaric acid in microalgae cells increased significantly. The citric acid cycle was the second stage of aerobic respiration of microalgae cells. Malic acid, fumaric acid, and oxaloacetic acid are the last three steps in the citric acid cycle. Oxamic acid may be converted from oxaloacetic acid.

The intermediates in citric acid cycle can be recharged with related biochemical reactions. When the intermediate of the citric acid cycle is consumed, the filling reaction can replenish in real time and maintain stability of the whole system.

The beginning of citric acid cycle is acetyl-CoA, and it is closely related to the carbohydrate, lipid, and amino acid metabolism. Acetyl-CoA is the starting material for carbohydrates, lipids, and most of the amino acids. Acetyl-CoA is also the degradation end product of the fatty acids; amino acids such as valine, leucine, and isoleucine; and pyruvate. Under the high concentration of carbon dioxide, the content of fumaric acid, malic acid, and **oxamic acid** increased significantly. Our estimation is that fumaric acid, malic acid, and **oxamic acid** are the smart molecules to store the extra carbon dioxide through their carbonyl groups.

4.2 Photorespiration: Glycerate and Glycine

RubisCO is difficult to distinguish between the structure of carbon dioxide and oxygen. Phosphoglycolate and 3-phosphoglycerate are the end products of RubisCO oxygenase reaction. For the recover carbon of phosphoglycolate, the photorespiration pathway needs to be conducted. Thus, another important response of microalgae in the high concentration of carbon dioxide environment is the photorespiratory process. One significant effort of photorespiratory is that it can regulate the balance of intracellular carbon and nitrogen.

In the photorespiratory process, there will be a loss of one molecule of carbon dioxide and a formation of one molecule of ammonia ion. RubisCO catalyzes the formation of one molecule 2-phosphoglycolate and 3-phosphoglycerate from one molecule of oxygen and one molecule of 5-diphosphate ribulose. 3-Phosphoglycerate needs to be converted to 2-phosphoglycolate through the following steps. Firstly, 3-phosphoglycerate enters peroxisomes to form glyoxylate. Glyoxylate and one molecule of ammonia are converted to glycine. Glycine then enters mitochondria. In mitochondria, two molecules of glycine form serine and generate a molecule carbon dioxide and a molecule ammonia ions. The ammonia ions can be used as a nitrogen source for the glutamine synthetase/glutamate synthase cycle in the chloroplast, which can participate in the biosynthesis of other amino acids. The ammonia molecule to form glycine can be regenerated when serine is converted back to 2-phosphoglycolate through glycerate.

Besides, glutamic acid is related to fumaric acid, putrescine, and aminobutyric acid. These corresponding pathways include arginine synthesis, the synthesis of polyamines. Glutamate can be decarboxylated to form aminobutyric acid, and aminobutyric acid can be converted to succinate, an intermediate of the citric acid cycle.

In the high concentration of carbon dioxide, the concentration of glycine decreased and the glycerate increased.

4.3 Oxidative Phosphorylation Pathway and Kinase Phosphorylation

The energies released from oxidative phosphorylation pathway, glycolysis, fatty acid oxidation, and citric acid cycle are stored in nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2).

When the active electrons of NADH and FADH2 eventually migrate to oxygen molecules through a series of transporters, the proton gradient is formed across the mitochondria membrane. The proton gradient can drive the ATPase utilize adenosine diphosphate and phosphoric acid to form adenosine triphosphate.

Phosphoric acid and nicotine acid are the component of NADPH and NADP. NADPH can provide a reducing force for the synthesis of lipids, fatty acids, and nucleotides. Intracellular NADP (NADPH) or its derivatives can effectively reduce the concentration of active oxides. NADPH can be applied to the carbon dioxide fixation process.

Another important role of phosphoric acid in cell is phosphorylation and dephosphorylation through kinases and phosphatases. The phosphorylation and dephosphorylation are the essential steps for life. The two reverse steps can regulate the activity of enzymes, the metabolites transfer process and the signal transduction process. Under high concentration of carbon dioxide, the concentration of phosphoric acid increased.

4.4 Carbohydrates Metabolism

4.4.1 Pentose Phosphate Pathway Gluconic Acid, Glyceric Acid, Xylitol

The site of phosphate pentose pathway is at cytoplasm. The main function of this pathway is as follows: generate nicotinamide adenine dinucleotide phosphate (NADPH) and create different sugar scaffold for triose, tetrose, pentose, hexose, and heptose intermediates. Xylitol can be converted from ribose frame. Gluconic acid and glycolic acid are intermediates of the pentose phosphate pathway.

Under high concentration of carbon dioxide, xylitol concentration decreased, and gluconic acid and glycerol acid concentration increased.

4.4.2 Fructose and Mannose Pathway and Leloir Pathway Fucose, D-Fructose-2,6p2, 1,2-Cyclohexanedione, Myoinositol

Fucose, fructose 6 is the intermediate of fructose and mannose pathway and Leloir pathway. The site of those pathways is in microalgae cytoplasm. Most of the hexose and their derivatives can be produced, for instance, allose and its derivatives, sorbose and its derivatives, rhamnose, and galactose. Fructose and mannose metabolic pathways are coupled with glycolytic and galactose metabolism through fructose 1,6 diphosphate and glucose, respectively. Fucose is involved in the metabolism of amino sugars and nucleic acids.

Fucose is the component of glycoprotein. Glycoproteins are composed with proteins and sugars. The sugar compositions of the glycoprotein are usually mannose, galactose, and fucose. The most notable efforts of oligosaccharide chain are its rich structural information; it serves as receptor sites for enzymes and other macromolecules.

Inositol is hexose derivatives. Inositol phosphate serves as the secondary messengers in the signal transduction process. In addition, inositol can be synthesized structural lipid phosphatidylinositol and its derivatives.

In the high concentration of carbon dioxide conditions, fucose, 2,6 fructose, and 1,2-cyclohexanedione concentration decreased; and inositol concentration increased.

4.5 Lipids

4.5.1 Long-Chain Fatty Acid Molecular Metabolism Cerotinic Acid, 2-Monopalmitin, and Lipoic Acid

Long-chain fatty acids cerotinic acid and 2-monopalmitin can be extended in microalgae mitochondria. The precursors of lipoic acid are octanoic acid, which are synthesized by octanoyl transferase and sulfuric acid synthase.

Under high concentrations of carbon dioxide, the concentrations of 2-monopalmitin and lipoic acid increased, while the concentration of cerotinic acid decreased.

4.5.2 Synthesis from Five-Carbon-Unit Precursors, Sitosterol, Phytol, and Farnesal

The starting units of the sitosterol phytol and farnesal molecules might be five-carbon-precursors. The five-carbon precursors are isopentane pyrophosphate and its derivatives. The five-carbon-precursors can be obtained from two metabolic pathways. The first is the mevalonate pathway, and the second is via the pentose phosphate pathway by a pentacontone unit.

Take the synthesis of farnesol, for instance. Three five-carbon structural unit will be condensed into farnesyl pyrophosphate. Then, farnesol pyrophosphate can be hydrolyzed to Farnesol. In microalgae cells, farnesol can be oxidated to farnesal and farnesoic acid with alcohol dehydrogenase and aldehyde dehydrogenase. It is reported that farnesal and farnesol are signaling molecules. They regulate a lot of cellular functions, including cell proliferation and apoptosis. Farnesyl transferase can act on the farnesyl group to modify the C-terminal cysteine of the target protein, during signal transduction and vesicle transport.

Sitosterol is an important component of membrane lipids and can increase glucose uptake and reduce triglyceride and cholesterol intake (Hwang et al. 2008).

The hydrophobic 20 carbon alcohol, phytol, is an important component of chlorophyll.

In the high concentration of carbon dioxide, the concentration of phytol increased, while the concentration of sitosterol and farnesal decreased.

4.6 Amino Acid Metabolism

Amino acid molecules can be divided into protein and nonprotein amino acids. Protein amino acid is the basic material for protein. Protein molecules can serve as structural molecules, enzyme molecules, transporters, and metabolic regulatory molecule. The keto groups in amino acid can be linked with fumaric acid and citric

acid cycle and then coupled with the fatty acids and saccharide metabolism. The amino groups can be related by steps such as transamination, oxidative deamination, combined deamination, and nonoxidizing deamination.

4.6.1 4-Aminobutyric Acid (GABA), Glutamic Acid, and Putrescine

4-Aminobutyric acid and putrescine are synthesized through arginine. Arginine can be hydrolyzed to the urea and ornithine. Ornithine and arginine can be converted to putrescine. Putrescine can be converted to 4-aminobutyric acid. The arginine is formed through arginine synthesis pathway, briefly, one molecule ammonia and one molecule of carbon dioxide form carbamoyl phosphate, consumed two molecules of ATP in the mitochondrial matrix. Then, carbamoyl phosphate transfers to the ornithine to form citrulline. Citrulline and glutamic acid form arginine succinic acid in cytoplasm. Arginine succinic acid can be cleaved into arginine and fumaric acid, while ornithine can enter the mitochondria and then participate in the arginine cycle.

Putrescine is the precursor of diamine molecules such as spermine and spermidine. The putrescine is mainly synthesized from arginine after L-ornithine synthesis.

4.6.2 Lysine, Diaminopimelate, and Carnitine

2,6-Diaminopimelate is the precursor of lysine. Lysine is one of the precursors of L-carnitine.

The RubisCO active site requires an ε-amino of lysine to bind carbon dioxide molecule. Lysine molecules can be modified to hydroxyl lysine. Hydroxyl lysine is an important component of the secretory protein-labeled glycopeptide chain.

The concentrations of diaminopimelic acid, lysine, and L-carnitine are increased in high concentrations of carbon dioxide conditions. Lysine and its derivatives are enhanced mainly because of the following:

1. Binding carbon dioxide molecules
2. Maintain pH environment
3. Biological activity

4.6.3 Alanine

Alanine can be involved in carbon dioxide fixation in microalgae cells. CO_2 and phosphoenolpyruvate (PEP) can form oxaloacetic acid. Oxaloacetic acid can be converted to malic acid and aspartic acid. Malic acid or aspartic acid can produce CO_2 and pyruvate or alanine. The carbon dioxide can be stored in malic acid and aspartic acid.

Another role of alanine is in the nitrogen storage performance. Amino groups can be combined with pyruvate to form alanine; alanine can be combined with

deamination step to generate pyruvate and free ammonia. Pyruvate is coupled through gluconeogenesis and glycolysis pathway. The intracellular ammonia is stored by amino acids.

The concentration of alanine increased at high concentrations of carbon dioxide.

4.6.4 Cystine

Sulfur is the essential elements for microalgae. Sulfur is necessary component of coenzyme, such as coenzyme A, biotin. In the methionine cycle, microalgae cells will take sulfate ions through the sulfur transporter and convert it into sulfur-containing amino, methionine, and cysteine. S-Adenosyl methionine is the precursor of cysteine, and cysteine can produce methionine.

The concentration of cysteine decreased at high concentrations of carbon dioxide.

4.6.5 Histidine

Histidine is synthesized from 5-phosphate ribose. The imidazole side chains of histidine can be protonated and deprotonated. Thus, histidine-composed enzymes are involved in many acid-based catalysis processes. Histidine serves as the active sites of many enzymes or the ligands in metal proteins. Interesting, histidine is an important component of the carbonic anhydrase.

The concentration of histidine decreased at high concentrations of carbon dioxide.

4.6.6 Nicotinic Acid

Aspartic acid and tryptophan can produce quinolinic acid, and quinolinic acid can produce niacin. Nicotinic acid is a coenzyme of NAD/NADH and NADP/NADPH.

The concentration of nicotinic acid decreased under high concentrations of carbon dioxide.

4.6.7 Noradrenaline

Noradrenaline belongs to the dopamine class. It is similar to the plant hormone (auxin family) and has plant hormonal functions, such as slowing down chlorophyll degradation, serving as antioxidant, resisting to the environmental stress, and affecting reproductive process.

The related KEGG pathways for metabolic analysis (blue dots refer to increased metabolites, and red dots refer to decreased metabolites in the elevated (15% CO_2) treatments) are as appendices illustrate. Metabolic analysis showed how the responses of *Desmodesmus armatus* to 15% CO_2 were tuned by metabolic molecules. This approach provided unbiased metabolic information of the acclimation to

the high concentrations of CO_2. The understanding metabolic acclimation mechanisms would be helpful to regulate the responsive enzymes and the conducted genetic expressions. These approaches overall can control the activity of key components in the carbon capture process such as the RubisCO, carbonic anhydrase, CO_2 channel, and bicarbonate transporters.

4.7 Signal Transduction

One of the interesting responsive pathways was the signal transduction. The signal transduction-related responsive metabolites included guanine, fucose, inositol, farnesol, 4-aminobutyric acid, and phosphoric acid. Guanine is a component of nucleic acid, and it attends intracellular signal transduction process. The cell surface of the largest receptor family is conjugated by G protein (Berg et al. 2015). Similarly, fucose is involved in glycoprotein formation of oligosaccharide chain, which might be the recognition site of many biomacromolecules on the surface of lipid membrane (Berg et al. 2015). Inositol phosphate serves as secondary messenger in the signal transduction process (Berg et al. 2015). Farnesol is possible signal molecules (Hall et al. 2011). 4-Aminobutyric acid may change the geometry and polarity of signaling molecules or promote the expression of those molecules (Berg et al. 2015, Löscher et al. 1991). In addition, phosphoric acid can participate in the signal transduction through phosphatase and kinase (Berg et al. 2015).

4.8 Nutrition for a Tolerant Species

The increased identified metabolites under the high CO_2 concentrations such as L-malate, fumarate, oxamate, and 1,2-cyclohexanedione can be considered as the possible decarboxylation metabolites which can store and release CO_2 in the cytosolic environment (Berg et al. 2015).

Specifically, glyceric acid, glutamic acid, alanine, and glycine can play a role in nitrogen metabolism and involve in the recycling of ammonia through the photorespiration N cycle (Brueggeman et al. 2012; Boyle and Morgan 2009; Chang et al. 2011; Dishisha et al. 2014; Renberg et al. 2010; Tripp et al. 2010; Ramazanov and Cárdenas 1994). The photorespiration process can regulate the balance of intracellular carbon and nitrogen, as there will be a loss of one molecule of CO_2 and a formation of one molecule of NH_4^+ during the process. The nitrogen assimilation is mainly related to amino acids and nonprotein amino acids metabolism. The intracellular NH_4^+ levels may be insufficient as putrescine with two amines decreased. However, the C:N ratio could not be easily satisfied by simply increasing the extracellular nitrogen source, as the remaining NO_3^- concentration was still really high. We argue that the activities of the nitrogen transporters such as LCIA, or NAR1.2, in chloroplasts need to be further enhanced in the high concentrations of CO_2 (Solovchenko and Khozin-Goldberg 2013; Baba and Shiraiwa 2012; Brueggeman et al. 2012; Wang et al. 2011, Giordano et al. 2005; Ramazanov and Cárdenas 1994).

Besides the limited nitrogen assimilation, as the essential ingredient, the phosphate and methyl phosphate accumulated and the sulfur relay system might be affected by the changing concentrations of cysteine and alanine (Berg et al. 2015).

The sufficient assimilation of carbon, nitrogen, sulfur, and phosphate could be considered as the benchmark of the tolerant species to stand the high concentrations of CO_2 (as Fig. 4-2 illustrates).

4.9 Intracellular pH for a Tolerant Species

The injected high concentrations of CO_2 decreased medium pH. The developed *Desmodesmus armatus* had the response that allowed microalgae to mitigate the pH environment (Raven 2010; Solovchenko and Khozin-Goldberg 2013). Fukuda et al. reported that another microalgae species *E. huxleyi* had alkalization activity to compensate for acidification (Fukuda et al. 2011). In our study, *Desmodesmus armatus* developed alkalization activity as well, as from 48 to 72 h the medium pH started to rise to the range of 6.81–7.00.

Interestingly, the responsive metabolites have a wide range of pKa, such as phosphate (pKa 2.12, 7.21, 12.67), and 2,6-diaminopimelate (pKa 1.85, 9.83) increased. The increasing of these metabolites can serve as the potential buffer to compensate the acidification of cytosolic environment.

The increased identified metabolites under the high CO_2 concentrations, such as D-glyceric acid (pKa 3.52), L-malic acid (pKa 3.4, 5.11), fumaric acid (pKa 3.03, 4.44), gluconic acid (pKa 3.86), oxamic acid (pKa 1.25, 4.14), and lipoic acid (pKa 4.52), have a pKa which ranges below the medium pH 6.5.

Specially, putrescine (pKa 9.7, 11.2); noradrenaline (pKa 8.58), with primary amines; and pKa above 6.5 decreased.

We considered those three groups of metabolites as the possible active or passive pH metabolites resulted from the cytosolic pH environment. These metabolites carried different charges, in the intracellular environment due to their unique molecular structures. Metabolites of different charges could affect activities through binding with the nucleic acids, phospholipid or protein molecules of the cell membrane, and ion channels or pump (such as carbonic acid channel, proton pump, calcium pump, and potassium pump) (Solovchenko and Khozin-Goldberg 2013; Baba and Shiraiwa 2012; Wang et al. 2011; Giordano et al. 2005).

Besides the above metabolic acclimation mechanisms, these responsive metabolites were reported to occur in other pathways. Phytol is a constituent of chlorophyll. The ε-amino group in lysine is an important functional group for RubisCO to bind CO_2 (Berg et al. 2015). Sitosterol is a key pre-ingredient for membrane lipid and reported to enhance the glucose uptake and reduce the concentration of triglycerides and cholesterol (Berg et al. 2015). Histidine serves as the active sites of carbonic anhydrase, as imidazole side chains are involved in the acid-base catalysis process (Berg et al. 2015). Nicotinic acid is a coenzyme of NAD/NADH and NADP/NADPH (Berg et al. 2015). Carnitine is the conjugated transporter for activated long-chain fatty acids across the inner mitochondrial membrane (Berg et al. 2015).

Lipoic acid was reported as organosulfur cofactor for enzyme complexes during oxidative and one-carbon metabolism (Berg et al. 2015). 2-Deoxy-D-galactose was reported to induce phosphate trapping (Starling and Keppler 1977). 2,6-Diaminopimelic acid and phosphate play the regulation role for the mass transfers across cell wall and membrane (Mason and White 1971). 1,2-Cyclohexanedione functions as the modification of the receptor in the metabolism of lipoprotein (Packard et al. 1985). Noradrenaline belongs to the dopamine class and may have hormone functions (Ekblad et al. 1984).

Metabolic analysis showed how the responses of *Desmodesmus armatus* to 15% CO_2 were tuned by metabolic molecules. This approach provided unbiased metabolic information of the acclimation to the high concentrations of CO_2. The understanding metabolic acclimation mechanisms would be helpful to regulate the responsive enzymes and the conducted genetic expressions. These approaches overall can control the activity of key components in the carbon capture process such as the RubisCO, carbonic anhydrase, CO_2 channel, and bicarbonate transporters.

Currently, studies for cellular physiology regulatory can be conducted via the metabolite approach besides the transcription and translation methods. For example, the addition of polyamines can be mainly applied to enhance nitrogen assimilation process and to improve the resistance to external stress. With the deepening understanding of metabolic research, it is expected to explore the key metabolic molecules to precisely control the intracellular environment of microalgae cells (Moinard et al. 2005).

5 Conclusion

Metabolic analysis shows the responses of metabolites that represent the transferred acclimation mechanisms for the resistance of high concentrations of CO_2. This approach provides unbiased information about metabolites and will be helpful to explore the key metabolic molecules to precisely control the intracellular environment of microalgae cells.

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Chapter 19
Employment of Wastewater to Produce Microalgal Biomass as a Biorefinery Concept

Alfredo de Jesús Martínez-Roldán and Jorge Ibarra-Berumen

Abstract Microalgae have been proposed for use in the production of biodiesel because oil from microalgae has characteristics similar to those of vegetable oils. Nevertheless, the production of microalgal biomass only for oil is not economically feasible because its costs are higher than those of vegetable crops. One option to improve the economic balance of the process is a biorefinery concept that consists of the complete exploitation of the biomass and capitalization of all possible products. The biorefinery includes the employment of wastes such as wastewater, residues from food industries, or even residual wastes for biomass production. In particular, wastewaters have high amounts of nitrogen and phosphorus, and microalgae culture can remove them very quickly because both are essential biomass constituents. The employment of wastewater to grow microalgae reduces the risk of eutrophication of water bodies; in a biorefinery concept, it permits the reduction of the cost of nutrient addition, increasing the economic feasibility. The development of the biorefinery concept requires innovations in many steps of the biomass production process, such as cell disruption, biomass recuperation, and extraction techniques, in order to ensure the complete use of the biomass. The cost-effectiveness is not favorable if only the production of biomass is considered, but with the biorefinery concept, the possibility of obtaining high-value subproducts such as carotenoids, polyunsaturated fatty acids, or even active molecules permits a positive economic balance and drives the development of this type of process.

Keywords Microalgae biomass capitalization · Waste water · Economic balance

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1 Introduction

Microalgae are a group of unicellular organisms with the ability to carry out oxygenic photosynthesis. They are microscopic in size, and they have a very diverse distribution including in seas, in freshwater bodies, in salt water, on rocks, and in soils. Microalgae are the primary producers in many ecosystems, and they contribute to almost 50% of all oxygen production; moreover, they have an essential role in many biogeochemistry cycles (Chapman 2013; Venkatesan et al. 2015; Barsanti and Gualtieri 2006). They have different reactions to culture conditions because of their great diversity, and consequently, they have many applications in several industrial sectors. Some of these applications include the production of active molecules with potential uses in the pharmaceutical industry, in animal feed, etc. From microalgae, it is possible to produce oil (to obtain biodiesel), polyunsaturated fatty acids (PUFAs) (ω3, ω6, and ω9) (for dietary supplementation), and carotenoids (chlorophylls, carotenoids, xanthophylls, etc.), and microalgae can also be used in the treatment of wastewaters (Abdel-Raouf et al. 2012; Williams and Laurens 2010; Huang et al. 2010; Arias-Peñaranda et al. 2013a; Flórez-Miranda et al. 2017). Nevertheless, the production of every bioproduct depends on many factors, such as trophic condition, nutrient supplementation, temperature, pH, carbon source, salinity, etc. (Arias-Peñaranda et al. 2013a; Shi et al. 2007; Pal et al. 2011; Martínez-Roldán et al. 2014).

One aspect of the metabolic diversity of microalgae is related to their capability to use different carbon and nitrogen sources. The carbon can be inorganic (CO_2) or organic (e.g., acetate, glucose, glycerol, etc.) (Liang et al. 2009). When the culture uses CO_2 as carbon and light as energy, the culture is autotrophic; if the microalgae instead employ an organic source of carbon and energy, the culture is heterotrophic. Additionally, there is a case mixing both metabolisms, which is called mixotrophic (Flórez-Miranda et al. 2017). It is important to highlight the fact that only a few strains are capable of indefinite growth in heterotrophic conditions because some metabolic routes need light to be expressed. The consumption of organic matter by microalgae is an advantage that can be exploited for the treatment of wastewater. The use of microalgae for wastewater has two advantages, namely, the oxygenation of the effluent and the reduction of the DBO and DQO.

These kinds of microorganisms have particular needs, and the bioreactors used for their growth therefore have specific designs. One such design provides a continuous supply of light; these bioreactors are called photobioreactors (PBRs) (Martínez-Roldán and Cañizares-Villanueva 2015). Under autotrophic conditions, microalgae can grow in very different configurations of PBRs, such as ponds, high-rate algal ponds (HRAPs), flat panel reactors, tubular reactors (vertical or horizontal), thin-layer reactors, etc. (Martínez-Roldán and Cañizares-Villanueva 2015; Norsker et al. 2011). Any PBR aims to increase productivity by improving the mass and light transfer. For heterotrophic cultures, a fermenter configuration similar to that employed for bacteria is typically used; this is because the culture must be axenic (Xu and Miao 2006).

2 Bioproducts from Microalgae

The composition of the microalgal biomass includes different types of biomolecules, such as carbohydrates, lipids, proteins, pigments, etc. Any of these biomolecules has potential applications in industrial products such as cosmetics, pharmaceuticals, feed, biofuels, and others.

2.1 Carbohydrates

Many species of microalgae can accumulate large amounts of carbohydrates, reaching values close to 50% of the dry weight (Ho et al. 2012). The principal carbohydrates from microalgae include starch, cellulose, hemicellulose, glucose, and different types of polysaccharides (Table 19.1). Today, the focus on the development of renewable and environmentally friendly sources of energy drives the research into carbohydrate production from microalgae; this is because these sugars can be fermented to produce low-molecular-weight alcohols such as methanol or ethanol that can be used as biofuels or in different industries (Zhu 2015; Chew et al. 2017; Nurra et al. 2014; Ho et al. 2013). The carbohydrates in the biomass may be constituents of the cell wall (e.g., lipopolysaccharides, peptidoglycan, cellulose, hemicellulose, etc.) or storage compounds (typically starch or paramylon) (Chen et al. 2013). These sugars are easily hydrolyzed to produce a mix of different low-molecular-weight monosaccharides including xylose, mannose, glucose, galactose, and rhamnose (Markou et al. 2012). These monosaccharides can then be fermented to obtain small alcohols (Ho et al. 2013) (Table 19.1).

Nevertheless, in the carbohydrate fraction, it is possible to obtain some polysaccharides with biological activity (Arad and Levy-ontman 2010). These polysaccharides are normally sulfated, and many authors have demonstrated their antioxidant, antitumor, anticoagulant, anti-inflammatory, and antiviral activities. Microalgae such as *Rhodella* (Chen et al. 2010), *Chlorella*, *Phaeodactylum* (Guzmán et al. 2003), *Gyrodinium* (Kim et al. 2012), *Porphyridium* (Matsui et al. 2003), and *Haematococcus* (Park et al. 2011) produce this kind of sugar. It is

Table 19.1 Specific content of diverse genera of microalgae and cyanobacteria (Chen et al. 2013; González-Fernández and Ballesteros 2012)

Genera	Carbohydrate content
Chlorella	9–55
Chlamydomonas	17–60
Chlorococcum	26–32.5
Scenedesmus	10–53
Nannochloropsis	8–50
Tetraselmis	8–26
Porphyridium	40–57
Spirulina	13–20
Synechococcus	15

important to consider the fact that while the carbohydrates used to produce ethanol have low market prices, sulfated sugars can reach prices of more than \$1000 kg^{-1}. This fact is essential in the development of processes focused on integrated exploitation of biomass; the obtaining of subproducts with higher market prices can permit the cost-effectiveness of a process when the primary product is cheap, as biofuels are. This extra input can allow biofuels from microalgae to compete with fuels obtained from petroleum (Chew et al. 2017; Nurra et al. 2014; Trivedi et al. 2015).

2.2 *Lipids*

The lipids in the microalgal biomass are long-chain fatty acids (FAs) with 12–20 carbon atoms (Yen et al. 2013). These FAs are mainly saturated and monounsaturated; nevertheless, some genera can produce FAs with 2–5 unsaturations with ω3, ω6, and ω9 configurations (Borowitzka 2013; Gouveia and Oliveira 2009). Under standard culture conditions, the specific content of lipids in the biomass is almost constant (20–30%), but by employing stress conditions, this content can achieve values close to 60% of the dry weight (Arias-Peñaranda et al. 2013a; Martínez-Roldán et al. 2014; Chisti 2008).

The oils obtained from biomass have different uses depending on their specific characteristics. Oil that is rich in saturated and monounsaturated FAs is useful to produce biodiesel via transesterification. In contrast, if the amount of polyunsaturation is high, including FAs such as linoleic (18:2), linolenic (18:3), arachidonic (20:4), and eicosapentaenoic (20:5) acids, among others, the oil has the potential to be used for dietary supplementation (human and animal) because many of these FAs are essential and must be included in the diet (Arias-Peñaranda et al. 2013a; Flórez-Miranda et al. 2015).

Oil with high amounts of saturated FAs can be converted to biodiesel via transesterification with low-molecular-weight alcohol (e.g., methanol, ethanol, etc.). This reaction produces fatty acid methyl esters (FAME); this is formally biodiesel and can be used in an internal combustion engine as a substitute for the diesel obtained from petroleum (Chisti 2008).

2.3 *Other Substances with High Market Value Obtained from Microalgae*

There are other molecules produced by microalgae or obtained from its biomass with the potential for use in the pharmaceutical, cosmetic, and food industries (Borowitzka 2013; Borowitzka 1995). This is because many microalgae can produce

molecules with anti-asthma, antiviral, antifungal, anti-inflammatory, antidiabetic, antioxidant, and other biological activities (Mayer and Hamman 2005; Senevirathne and Kim 2011; Hu 2004). The level of progress of the production process for many of these compounds is on the laboratory scale; nevertheless, the market for natural carotenoids from microalgae is in development. Even the present production of beta-carotene, astaxanthin, chlorophyll, lutein, etc. employs cultures of different microalgae genera, such as *Muriellopsis*, *Scenedesmus*, *Chlorella*, *Dunaliella*, *Botryococcus*, *Phaeodactylum*, *Haematococcus*, *Spirulina*, etc. (Borowitzka 1995, 2013).

3 Biorefinery

The biorefinery concept has been developed in recent years and can be applied to production processes employing biomass as a raw material for the production of a specific product (biodiesel, ethanol, etc.). To frame a process as a biorefinery concept, it is necessary to add steps that permit the obtaining of different subproducts with high market value or that diminish the amount/number of residuals that the process produces (Mussgnug et al. 2010). For microalgae biomass, several subproducts can be obtained from the different fractions of the biomass (proteins, lipids, and carbohydrates) (Fig. 19.1).

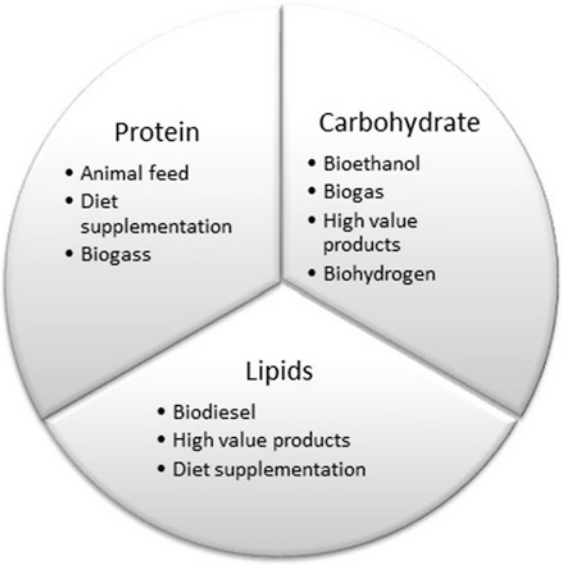

Fig. 19.1 Potential subproducts obtainable from microalgae biomass in a biorefinery process

3.1 Biorefinery from Microalgae

The biorefinery concept as applied to microalgae has many advantages compared with biorefineries employing fungi, bacteria, yeast, or vegetal biomass; some of these are the ease of cultivation, the lack of a requirement for an organic carbon source and fertile soil, and the ability to obtain nutrients from wastewater, among others (Vanthoor-Koopmans et al. 2013). Nevertheless, the actual challenges occur in the postharvest steps (Zhu 2015; Vanthoor-Koopmans et al. 2013).

For the application of the biorefinery concept, it is necessary to employ a low-consumption technique for the recuperation of the biomass and gentle cellular disruption and extraction strategies. The objective of these approaches is to exploit the full potential of the biomass and not just a fraction of the available types of compounds: this is the principal challenge of the biorefinery. For biomass recuperation, it is necessary to use a technique with high efficiency but low operational and energetic costs (Vanthoor-Koopmans et al. 2013).

3.2 Steps to Consider in a Biorefinery Process from Microalgae

3.2.1 Biomass Recuperation

The small size of the cell causes that the biomass recuperation costs can represent from 20% to 30% of the total production costs of the biomass (Molina Grima et al. 2003) and the fact that the cultures normally reach low biomass concentrations (<1 g L^{-1}) (Vanthoor-Koopmans et al. 2013). Several different innovative methods for biomass separation have been developed, employing filters, coagulants (chemical or organic), or techniques such as electrocoagulation or autoflocculation (Uduman et al. 2010; Vandamme et al. 2016).

3.2.1.1 Centrifugation

This process separates the biomass by the use of gravity, and its efficiency depends on the difference between the densities of the liquid and the solid particles suspended (Uduman et al. 2010). Centrifugation is typically highly effective for the recuperation of biomass, but its investment, operational, and energetic costs are very high. Additionally, it is less useful for large culture volumes, and in some cases, the shear stress can break the cells (Knuckey et al. 2006).

3.2.1.2 Flocculation

This process occurs when colloidal particles interact, forming aggregates (flocks) that sediment easily. There are compounds called flocculants that increase the efficiency and reduce the time involved in this process; the flocculant interacts with the cell wall and facilitates the agglomeration of the cells (Vandamme et al. 2016). Flocculants may be inorganic compounds, mainly salts of Fe(III) or Al(III), or organic polyelectrolytes, and the most common are chitosan, alginate, etc. (Shelef et al. 1984; Das et al. 2016). Autoflocculation may also occur; in this case, changes in pH can favor the spontaneous generation of flocks and its sediment (Uduman et al. 2010).

3.2.1.3 Filtration and Screening

This method employs a porous medium through which the microalgae culture is passed; the medium holds the biomass and lets the liquid through. A specific pore diameter should be used to ensure appropriate separation (Uduman et al. 2010); if a larger pore diameter is used, the flow can be higher, and the operational cost is diminished, but fewer cells can be retained. Moreover, the biomass concentration in the culture is an essential factor because if the concentration is too high, the filter can collapse, and the flow will stop (Wilde et al. 1991).

3.2.1.4 Flotation

Flotation is a physicochemical separation by the bubbling of air or gas into a biomass suspension. The gaseous particles interact with the solid fraction, enabling its flotation; after that, the particles float and accumulate on the surface, facilitating its separation (Uduman et al. 2010). The efficiency of the flotation depends on both the stability of the gas-solid particles and the size of the solid, and it is more useful when the particle size is <500 μm (Shelef et al. 1984; Matis et al. 1993).

3.2.2 Cell Disruption

The cellular disruption can be done mechanically or nonmechanically; some of the techniques include homogenization, high pressure, heating, microwave, osmotic shock, or chemical disruption. Current research is focused on the application of mild techniques that permit the integrated use of the biomass (Vanthoor-Koopmans et al. 2013; Amarni and Kadi 2010; Zheng et al. 2011).

3.2.2.1 Electric Pulsed Field

This is a technique that employs high electric pulses that disrupt the cell membrane or even the cell wall; this method is very effective but is not useful at commercial scale (Vanthoor-Koopmans et al. 2013). The actual principal use of the electric pulsed filed is in the pasteurization of beverages in the food industry because its use does not change the beverage flavor or chemical composition (Zulueta et al. 2007). Many authors confirm that it is possible to process from 400 to 2000 L h-1 of culture with this technology (Min et al. 2003a, b).

3.2.2.2 Ultrasound

The employment of ultrasound radiation causes cavitation inside the cells; this causes the formation of microbubbles that eventually explode and break open the cells. This technique is very practical for the extraction of oil from microalgae biomass (Vanthoor-Koopmans et al. 2013).

3.2.2.3 Enzymes

Some enzymes are useful for breaking the cell wall and typically are active under mild conditions (pH, temperature, etc.). Typically, the composition of the cell wall in microalgae includes cellulose, hemicellulose, and some glycoproteins and polysaccharides; these compounds are responsible for its rigidity (Zheng et al. 2011; Günerken et al. 2015). By employing lysozyme, cellulases, xylanases, etc., it is possible to break the cell and extract many high-value products without affecting the composition of residual biomass (Vanthoor-Koopmans et al. 2013; Günerken et al. 2015).

3.2.3 Extraction and Separation Techniques

The extraction of high-value compounds such as lipids, PUFAs, or carotenoids is typically carried out by employing organic solvents such as hexane, chloroform, ethanol, or methanol. These solvents have high extraction yields but make it impossible to use the remaining biomass (Arias-Peñaranda et al. 2013a; Zhu 2015; Amarni and Kadi 2010). Because of the necessity of the integral use of the biomass components, the biorefinery concept proposes the use of other extraction techniques that use mild conditions and do not modify the resulting biomass (Vanthoor-Koopmans et al. 2013; Draaisma et al. 2013).

3.2.3.1 Ionic Liquids

Ionic liquids include cations and anions with fusion points lower than 100 °C, are useful for the extraction of both hydrophilic and hydrophobic compounds, and can be easily recycled because their impact to the environment is low compared with those of classical solvents such as methanol, ethanol, hexane, or chloroform (Vanthoor-Koopmans et al. 2013).

3.2.3.2 Surfactants

The employment of surfactants is used mainly for the separation of proteins; the separation depends on the pH and ionic strength of the medium and the composition of the biomass. The amount of surfactant and the type of solvent should be carefully selected (Vanthoor-Koopmans et al. 2013).

3.3 Biorefinery Employing Wastewater as a Nutrient Source

There are some examples of biorefineries using microalgae at commercial scale; some of them have the production of active molecules as the primary objective. One of them is the biorefinery established in Tarragona, Spain, which is focused on obtaining lipids from *Nannochloropsis gaditana* in a 53 m^{-3} installation (Nurra et al. 2014). Nevertheless, for the production of high-value molecules, it is necessary to use freshwater, and this increases the operational costs of the process. Recently, the development of processes that employ wastewater as nitrogen source for the production of biofuels was proposed (Rawat et al. 2013a).

The use of wastewater is possible because the microalgae are among the most promising tools for the removal of nitrogen and phosphorus from different types of sewage; the consumption of N and P is very high and rapid because both these elements are employed for the production of many biomolecules. The are many advantages to the use of microalgae in wastewater treatment, including the oxygenation and consequent inactivation of pathogens and bacteria in the residues and the removal of N and P, reducing the risk of eutrophication when the water is discharged to a waterbody (Oswald et al. 1985).

The nitrogen in wastewater may be present in diverse forms, including inorganic molecules, such as NH_4^+, NO_3^{-1}, and NO_2^{-1}, or even organic molecules, such as polypeptides or amino acids (Bashaar 2004; Franco Martínez et al. 2017; Martínez-Roldán 2008). The assimilation of NH_4^+ is faster than that of other nitrogen sources, and it is typically consumed first; this is because it does not require modification for its use, nevertheless, the amount of ammonia in some types of wastewaters can reach inhibitory values. The ammonium tolerance depends on the strains, but for many

Table 19.2 Nitrogen removal from wastewater by microalgae cultures

Strain	Nitrogen source	Removal efficiency	References
Chlorella vulgaris	$N\text{-}NH_3$	$65.6 \pm 11.7\%$	Ruiz-Marin et al. (2010)
	Synthetic wastewater		
Scenedesmus obliquus	$N\text{-}NH_3$	$96.6 \pm 1.7\%$	
	Agua residual sintética		
Desmodesmus communis	$N\text{-}NO_3$	5 mg L^{-1} day^{-1}	Samorì et al. (2013).
	Real wastewater		
Chlorella vulgaris and *Scenedesmus*	$N\text{-}NH_3$	20 mg L^{-1} (nueve días)	Shi et al. (2007)
	Synthetic wastewater		
	$N\text{-}NO_3$	3 mg L^{-1} (cinco días)	
	Synthetic wastewater		
Chlorella vulgaris	$N\text{-}NH_3$	5–11 mg$N\text{-}NH_3$ L^{-1} day^{-1}	Franco Martínez et al. (2017)
	Real wastewater		
Spirulina platensis	$N\text{-}NO_3$	5 mg L^{-1} day^{-1}	Martínez-Roldán (2008)
	Real wastewater		

genera, the upper limit is 100–200 mg_{NH3} L^{-1} (Franco Martínez et al. 2017). The nitrogen consumption rate of microalgae cultures can reach very high values compared with those of cultures of other microorganisms and typically ranges from 2 to 10 mg L^{-1} day^{-1} (Table 19.2).

Phosphorus is another nutrient that is essential for energy metabolism; it is used for the production of nucleic acids, lipids, proteins, and energetic molecules such as ATP, GTP, and ADP (Barsanti and Gualtieri 2006). This nutrient should be supplied at an amount sufficient to allow good development of the culture because its limitation can cause decreases in the growth rate and the biomass concentration or even the deviation of some metabolic routes (Franco Martínez et al. 2017; Cai et al. 2013). The principal P sources are $H_2PO_4^-$ and HPO_4^{-2}, both of which are directly incorporated into the biomass and used in several metabolic routes, including energy transfer (Martinez et al. 1999). The P consumption routes of many strains have been studied, and some of them are shown in Table 19.3.

The use of wastewater as a nutrient source in a biorefinery process presents two principal impacts: the elimination of two contaminants that can cause eutrophication in receptor water bodies and the possibility of producing products with high market value as biofuels (e.g., biodiesel, biohydrogen, bioethanol) (Rawat et al. 2013a).

Microalgae need not only nitrogen and phosphorus but also carbon (mainly CO_2), sulfur, and micronutrients such as magnesium, manganese, calcium, silicon, cobalt, molybdenum, potassium, and sodium. Wastewater is a suitable culture medium for microalgae because it typically contains low concentrations of many of these micronutrients (Cai et al. 2013). It is important to highlight the fact that in some wastewaters (e.g., industrial), the concentrations of some heavy metals can reach

Table 19.3 Phosphorus removal from wastewater by microalgae cultures

Strain	P source	Removal efficiency	References
Chlorella pyrenoidosa	$P\text{-}PO_4$	80% ($C_0 = 10$ mg L^{-1})	Tam and Wong (1989)
	Real wastewater		
Phormidium spp.	$P\text{-}PO_4$	62% 5.3 mg P-PO4 L^{-1} day^{-1}	Pouliot et al. (1989)
	Tertiary treatment effluent		
C. vulgaris and *S. rubescens*	$P\text{-}PO_4$	Almost 100% ($C_0 = 3$ mg L^{-1})	Shi et al. (2007)
	Synthetic wastewater		
Chlorella vulgaris	$P\text{-}PO_4$	1–2.7 mg L^{-1} day^{-1}	Franco Martínez et al. (2017)
	Real wastewater		
Spirulina platensis	P-PO4	2–4 mg L^{-1} day^{-1}	Martínez-Roldán (2008)
	Synthetic wastewater		

high values, causing inhibition of the photosynthetic metabolism or even the death of the culture (Perales-Vela et al. 2006). Additionally, there is the potential for the displacement of the microalgae by other microorganisms present in the wastewater, such as fungi, bacteria, or even insects and protozoa.

Municipal and domestic wastewaters are the best options to be used as a nutrient source for microalgal cultures because they contain enough phosphorus and nitrogen (nitrate or ammonium) to support culture growth and the concentration of contaminants (mainly heavy metals) does not reach inhibitory concentrations (Franco Martínez et al. 2017; Pittman et al. 2011). Some studies have proven that the employment of wastewater for the production of biomass to obtain biofuels is a viable option or even highlight the necessity of including sewage to make the process feasible (Pittman et al. 2011; Lundquist et al. 2010). The final biomass concentration will depend on the amount of nitrogen and phosphorus present in the wastewater (Table 19.4).

Despite the potential of the wastewater to be a source of nutrients to microalgal culture, the amount of nutrients is typically not sufficient to maintain exponential growth for many days (Franco Martínez et al. 2017; Martínez-Roldán 2008). This is important if the batch culturing approach is selected because it is usually advisable to use culturing strategies that permit a constant supply of nutrients. This can be achieved by the use of repeated batch or continuous culture, but the dilution rate can be carefully selected to avoid the washing out of the biomass in a PBR (Martínez-Roldán 2008; Ethier et al. 2011). If the process employs continuous culture, it will probably be necessary to use continuous illumination to avoid biomass losses and reach the highest productivity (James and Al-Khars 1990). Theoretically, both continuous and repeated batch cultures can be maintained indefinitely, but the consecutive cellular division could cause the accumulation of genetic mutations and consequent modification of the microalga characteristics, so it is advisable to renew the culture every so often (Flórez-Miranda et al. 2017).

Table 19.4 Biomass production employing wastewater as nutrient source

Strain	Characteristics of the wastewater	Biomass production	References
Chlamydomonas reinhardtii	128.6 mg L^{-1} TKN	2 g L^{-1} day^{-1}	Kong et al. (2010)
	67 mg L^{-1} $N\text{-}NH_4$		
	120.6 mg L^{-1} TP		
Hindakia sp.	134.8 ± 6.8 mg L^{-1} TKN	275 mg L^{-1} day^{-1}	Zhou et al. (2011)
	91 ± 1.8 mg L^{-1} $N\text{-}NH_4$		
	212 ± 7.2 mg L^{-1} $P\text{-}PO_4$		
Botryococcus braunii	32.6–45.9 mg L^{-1} TKN	34 mg L^{-1} day^{-1}	Chinnasamy et al. (2010)
	17.58–25.85 mg L^{-1} $N\text{-}NH_4$		
	20.31–35.1 mg L^{-1} TP		
Chlamydomonas sp. and *Desmodesmus* sp.	38.4 mg L^{-1} $N\text{-}NH_4$	0.9–1.5 g L^{-1}	Wu et al. (2012)
	3.1 mg L^{-1} $N\text{-}NO_3$		
	44.7 mg L^{-1} $P\text{-}PO_4$		
Mixture of *Actinastrum*, *Scenedesmus*, *Chlorella*, *Spirogyra*, *Nitzschia*, *Micractinium*, *Golenkinia*, *Chlorococcum*, *Closterium*, and *Euglena*	Diluted wastewater	812 mg L^{-1} (3 days)	Woertz et al. (2009)
	39 mg L^{-1} $N\text{-}NH_4$		
	51 mg L^{-1} TKN		
	2.1 mg L^{-1} $P\text{-}PO_4$		
Botryococcus braunii	Piggery wastewater	7 g L^{-1} (10 days)	An et al. (2003)
Desmodesmus communis	84.62 ± 0.53 mg L^{-1} $N\text{-}NH_3$	3.83 ± 0.44 g L^{-1}	Samorì et al. (2013)
	1.73 ± 0.05 mg L^{-1} P		

4 Economic Analysis of the Microalgae Biorefinery Concept

Today, microalgae are considered a potential source of many biofuels (e.g., biodiesel, bioethanol) and of many high-value products such as carotenoids and PUFAs. Its potential is increased by the fact that it uses atmospheric CO_2 as a carbon source and does not need fertile soil for its production (Chew et al. 2017). Generally, in the

biodiesel process, the primary product is the lipids, but the production costs of the microalgal biomass are close to \$16 kg^{-1}, and because the specific lipid content is approximately 30%, the cost of the oil is almost three times the price of the biomass (Slegers 2014). This makes it impossible for the oil to compete with petroleum-derived oil, which has a market price lower than \$100 per barrel (Schenk et al. 2008).

The competition between biodiesel and petrodiesel can be evaluated from the perspective that biodiesel is environmentally friendly and does not contribute to global climate change; this is true, but there are other photosynthetic sources of lipids with which microalgae must also be compared. One of the most popular sources of lipids for biodiesel production is the palm *Elaeis guineensis*, whose oil has a market value close to \$0.6 kg^{-1}; thus, the oil from microalgae is also not competitive with palm oil because its cost is close to 100 times higher (Wolkers et al. 2011). For this reason, biodiesel production from microalgae must be developed into a biorefinery concept to be feasible.

First, the maximal productivity of both biomass and lipids must be ensured. This could be enabled by the employment of a particular PBR configuration that allows higher values of biomass productivity to be achieved, such as thin layer/cascades or flat panel reactors (Martínez-Roldán and Cañizares-Villanueva 2015; Torzillo et al. 2010). The use of these high-efficiency PBRs allows increases of ten times or even more in biomass concentration compared with less productive PBRs such as open ponds or raceways, but their investment costs are higher (Martínez-Roldán and Cañizares-Villanueva 2015).

Another aspect to consider is the cost of the nutrient supply. Food-grade salts are usually used to prepare the medium, but there are other options with lower prices, such as commercial fertilizer or even wastewaters (Trivedi et al. 2015). If it is possible to employ some residue as the nutrient supply, the only impediment is the presence of contaminants, but many wastewaters have low concentrations of nutrients. The effluent resulting from the anaerobic digestion of residues from agro-industries or livestock, or even municipal solid wastes, can be used (Rawat et al. 2013b).

From the perspective of the biorefinery, the ability to obtain certain molecules can increase the income from the process and can cause a drop in the price of the oil. These molecules include carotenoids, some of which have considerable demand and high market values. Genera such as *Muriellopsis*, *Scenedesmus*, *Chlorella*, *Dunaliella*, *Botryococcus*, *Phaeodactylum*, *Haematococcus*, and *Nannochloropsis* can produce different carotenoids and also accumulate lipids (Flórez-Miranda et al. 2017; Borowitzka 2013; Borowitzka 1995; Arias-Peñaranda et al. 2013b; Martínez-Roldán and Cañizares-Villanueva 2017). Therefore, the production of biodiesel and carotenoids can be coupled. The value of the biodiesel production process stimulated by including the production of carotenoids can be enormous; some carotenoids can reach market values close to \$2000 kg^{-1} (depending on purity) (Borowitzka 2013). Moreover, the market for carotenoids is growing and today is worth close to \$1.4 billion.

PUFAs are other subproducts that can be included in the microalgae biorefinery, and many of the microalgae used for the production of lipids include PUFAs in their oil. Strains such as *Nannochloropsis*, *Chaetoceros*, *Isochrysis*, and *Phaeodactylum* can produce large amounts of these fatty acids (Pal et al. 2011; Borowitzka 2013;

Martínez-Roldán and Cañizares-Villanueva 2017; Priyadarshani and Rath 2012), and many of the PUFAs produced by microalgae can reach high prices. It is estimated that the demand for ω3 PUFAs alone may reach 241,000 tons in 2020 and the market may be close to $4.9 billion (Industry Experts 2014). Nevertheless, a separation step is needed and should be very specific because these types of FAs (saturated FAs, monosaturated FAs, and PUFAs) are very similar; HPLC or gas chromatography is likely needed to separate them.

5 Conclusions

The use of wastewater for microalgae biomass production to obtain lipids/biodiesel is a process that is beginning to become real today. Nevertheless, many obstacles need to be overcome in the coming years. Some of these obstacles relate to process productivity, including operation conditions, the use of PBRs, the strain used, the nitrogen and phosphorus sources, and the light supplementation.

If the cost-effectiveness of the biodiesel production is the primary objective, the production of biodiesel by itself is not enough; this is because the prices of the alternatives, petroleum and palm oil, are very low, while the cost of biomass production remains high. An option to improve the economic balance of the process is to use a residual product as the nutrient source, but the residue must be carefully selected to ensure the optimal growth of the culture and the safety of the biomass (absence of contaminants in the biomass).

A further improvement to the cost-effectiveness can be made by the generation of high-value subproducts such as PUFAs and carotenoids and the commercialization of the remaining biomass for livestock feed. This is because the market prices of many of these products are higher than that of biodiesel and their demand is very large and growing. Nevertheless, the safety of these subproducts must be ensured, especially if they are to be used in the food, feed, cosmetic, or even pharmaceutical industries. This precludes the use of wastewater, but residues that do not contain heavy metals or biological agents probably can be used.

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Chapter 20
Microalgae and Wastewater Treatment: Advantages and Disadvantages

Beatriz Molinuevo-Salces, Berta Riaño, David Hernández, and M. Cruz García-González

Abstract Wastewater generation has concomitantly increased with the growth of world human population in the last century. The uncontrolled discharge of wastewater may result in serious social, environmental and health problems. At the same time, the use of microalgal-based systems has been widely studied for a variety of residual effluents treatment since the early 1950s. In this context, different technologies have been developed, and new strategies to cope with specific needs have been investigated worldwide. There are several advantages of microalgal-based systems compared to traditional wastewater treatment technologies, namely, (1) pollutants and pathogen decrease, (2) nutrient recovery in the form of valuable biomass, (3) energy savings and (4) CO_2 emissions reduction. In spite of all these advantages, there are still many challenges to overcome before attaining the real implementation of this technology. Those challenges include (1) land requirement, (2) effect of wastewater characteristics, (3) environmental and operational condition influence and (4) biomass harvesting and valorization. This chapter will explore and discuss the main advantages and limitations of using this green technology for wastewater treatment based on our expertise and the latest insights on this topic.

Keywords Microalgae · Wastewater · Advantages and limitations · Green technology

1 Introduction

Microalgae are a group of photosynthetic microorganisms mostly developed in aquatic habitats and capable of converting light energy and inorganic carbon sources (carbonate and CO_2) into biomass while releasing O_2 to the atmosphere. The term microalgae is generally considered as a general term and often includes cyanobacteria (blue-green algae) as both, microalgae and cyanobacteria, are

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commonly found in microalgal-based systems for wastewater treatment. However, it is important to underline that cyanobacteria are photosynthetic prokaryotes and microalgae are photosynthetic eukaryotes. In this chapter, the term microalgae will be referred to both groups.

Microalgae biotechnology has been developed for different commercial applications, but in recent years, development of microalgae-bacteria consortia for wastewater treatment has received more attention as an efficient alternative to conventional wastewater treatment plants, based on the avoidance of external oxygen supplementation for heterotrophic bacteria, decreasing energy costs and recovering nutrients as valuable biomass (Hernández et al. 2016). During photosynthesis, microalgae capture light using pigments (chlorophylls and carotenoids) as electromagnetic energy source to break down H_2O (light phase) and to reduce inorganic carbon to glucose (dark phase) through the Calvin cycle, releasing O_2. The photosynthesis-respiration process can be represented by the following equation (Eq. 20.1):

$$6\,CO_2 + 6H_2O + \text{Energy (light)} \rightarrow C_6H_{12}O_6 + 6O_2 \tag{20.1}$$

The presence of inorganic carbon forms (CO_2, H_2CO_3, HCO_3^- and CO_3^{-2}) in wastewater is governed by the following equations (Eqs. 20.2, 20.3 and 20.4) (Andersen 2002):

$$H_2O \leftrightarrow H^+ + OH^- \qquad pKW = 14 \tag{20.2}$$

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \qquad pK1 = 6.38 \tag{20.3}$$

$$HCO_3^- \leftrightarrow CO_3^{-2} + H_2O + H^+ \qquad pK2 = 10.37 \tag{20.4}$$

During the photosynthesis process, algae capture high amounts of CO_2, causing a gradual increment of pH. When microalgal biomass concentration is high, CO_2 concentration decreases, and carbonate/bicarbonate species dissociate into CO_2 with the subsequent alkalinity drop, so that culture medium losses stability. Therefore, the lack of CO_2 triggers carbon sequestration from atmosphere to the water. In this manner, microalgal biomass culture shows to be a suitable tool to capture carbon, fixing it in the form of valuable biomass.

As previously mentioned, mitigation of pollutants in photobioreactors is usually made by consortia of microalgae and bacteria. Interactions between both groups of microorganisms can support an efficient removal of organic and inorganic carbon, nitrogen, phosphorus, heavy metals and other pollutant compounds, as they play a complementary or competitive role in the consortia. Due to this interaction, organic matter mineralization by aerobic bacteria produces the inorganic carbon needed by the microalgae. In turn, the O_2 required for bacterial degradation is produced photosynthetically by microalgae (González-Fernández et al. 2011). In the case of nutrient removal, nitrogen assimilation into microalgal-bacteria biomass is the most common removal mechanism observed in microalgae-bacteria cultures. The

Table 20.1 Microalgae species in different wastewaters

Substrate	Microalgae	References
Swine manure	*Chlamydomonas* sp., *Microspora* sp., *Chlorella* sp., *Protoderma* sp., *Selenastrum* sp., *Oocystis* sp., *Ankistrodesmus* sp., *Nitzschia* sp., *Achnanthes* sp.	de Godos et al. (2009)
Digested swine manure	*Oocystis* sp., *Chlorella* sp., *Protoderma* sp., *Chlamydomonas* sp.	Molinuevo-Salces et al. (2010)
Fish processing wastewater	*Ankira* sp., *Chodatella* sp., *Microspora* sp., *Scenedesmus* sp., *Chroococcus limneticus*, *Cyanophyta cocal*, *Dactylococcopsis* sp., *Phormidium* sp., *Stigeoclonium* sp.	Riaño et al. (2011)
Slaughterhouse wastewater	*Chlamydomonas subcaudata*, *Teilingia* sp., *Anabaena* sp., *Phormidium tergestinum Pinnularia* sp., *Nitzschia* sp.	Hernández et al. (2016)

preferred N source in microalgal cultures is NH_4^+ as it is easily assimilated into amino acids to produce microalgae-bacteria biomass. However, NH_3 can result toxic and inhibit photosynthetic activity in some microalgal species (Park and Craggs 2010).

Microalgal species growing on wastewater treatment systems are especially tolerant to pollution. It has been reported that *Chlorella*, *Nitzschia* and *Scenedesmus* are the most tolerant genera with a high presence in wastewater systems (Muñoz and Guieysse 2006; de Godos et al. 2009). Other species such as *Ankira*, *Microspora*, *Chroococcus limneticus*, *Cyanophyta cocal*, *Dactylococcopsis* sp., *Phormidium* sp. and *Stigeoclonium* sp. have been reported in microalgal-based systems treating fish processing wastewater (Riaño et al. 2011); and *Chlamydomonas subcaudata*, *Teilingia* sp., *Anabaena* sp., *Phormidium tergestinum*, *Pinnularia* sp. and *Nitzschia* sp. have been reported in open ponds for slaughterhouse wastewater (Hernández et al. 2016) (Table 20.1).

As in other wastewater treatment systems, microalgae community composition is influenced by different variables that act as a key selection pressure. These variables produce changes in the community composition from the initially inoculated microalgae to the steady-state period, changing microalgae species diversity and its abundance. The main factors responsible for microalgae community structure are related to wastewater characteristics, species interaction, variations in the environmental conditions, photobioreactor configuration and operational conditions. Diverse species with differential interactions/competition also contribute to the system stability with enhanced biomass growth and efficient removal of nutrients (Hernández et al. 2016).

Microalgal cultivation can be carried out in fully contained photobioreactors or in open ponds and channels (Molina Grima et al. 2003). Open ponds, namely, raceway ponds or high-rate algae ponds (HRAPs), are the most widespread systems for microalgal cultivation. They consist of rectangular basins or channels where the wastewater is kept in constant motion with a powered paddle wheel. Closed systems

are mainly designed as a column or with tubular shape, although there are different designs and configurations seeking to combine high productivity and low-energy consumption for large-scale application (Gouveia 2011). Open and closed reactors present pros and constrains; some of them are the following, according to Gouveia (2011): open systems need more area-to-volume ratio, water loss through evaporation is high, gas transfer and light utilization efficiency are poor, harvesting cost is high and contamination by other microorganisms is high. In a closed system, the control of growth conditions is easy, capital investment and operating cost are high but harvesting cost is lower and scale-up technology for commercial level is more difficult than in open systems. As pointed out, in open systems, maintenance of microalgae population is complicated due to external contamination of small and rapidly growing microalgae; therefore, some authors have proposed the use of enclosed photobioreactors since they support more effective species control (Tredici 1999). In the case of wastewater treatment, mixed open ponds are the only large-scale implemented technology, probably due to the high-energy costs to operate closed photobioreactors.

Regarding to the scale-up of this technology, the main challenge is the recovery of the produced biomass, called the harvesting process. Biomass concentration in photobioreactors is usually low, between 0.5 and 5 g/L dry weight (Gouveia 2011); consequently it is necessary to remove water to concentrate and harvest the biomass for its further valorization. Harvesting is still considered one of the bottlenecks of wastewater treatment with microalgae, as these microorganisms' cells have a small size (5–20 μm) and they are very stable in colloidal suspension. Different harvesting methods have been explored, which include gravity sedimentation, centrifugation, filtration, flotation, coagulation and flocculation, as well as several combinations of them. The harvesting process is considered the major limiting factor for wastewater treatment development by microalgae (Molina Grima et al. 2003); for that reason, the choice of the harvesting method is of vital importance for the economic feasibility of microalgal-based wastewater treatment systems. The harvested biomass from wastewater treatment systems is mainly used for energy, biofertilizers and animal food production (Acien et al. 2017). Specifically, microalgae biomass grown in wastewater is characterized by high-protein content, being successfully used as protein source for rainbow trout feed (Tomás-Almenar et al. 2017; Tomás-Almenar et al. 2018; Larrán et al. 2017). This chapter will explore and discuss the main advantages and limitations of using microalgae-based technology for wastewater treatment, from the recovery of nutrients for biomass production, considered one of the main advantages of these systems, to the biomass harvesting and valorization processes; all are based on our expertise and the latest insights on this topic.

2 Advantages of Microalgae for Wastewater Treatment

2.1 Pollutants and Pathogen Decrease

2.1.1 Nutrient Removal During Wastewater Treatment by Microalgae-Bacteria Consortia

Most wastewaters are rich in ammonium, nitrates and phosphorus, and treatments are usually aimed at removing them. In conventional activated sludge treatment plants, carbon is oxidized to CO_2, nitrogen (N) is stripped to the atmosphere in the form of N_2 and phosphorus (P) is usually precipitated, avoiding nutrient valorization (Adav et al. 2008). Microalgae-bacteria consortia are capable to remove nutrients while producing valuable biomass, so that their use for wastewater treatment has been widely studied during the last two decades. During photosynthesis, microalgae liberate O_2 to the medium, which is used by the aerobic bacteria to degrade organic matter into CO_2, soluble phosphorus and different inorganic N sources (NH_4^+, NO_3^- and NO_2^-). Then, microalgae uptake inorganic carbon (CO_2) and solubilized macro- and micronutrients to grow, resulting in a clean effluent and a valuable biomass. Therefore, if compared to conventional wastewater treatment plants, the use of microalgae-bacteria consortia for wastewater treatment presents the advantage of nutrient recycling (Adav et al. 2008; Hernández et al. 2016).

Table 20.2 shows total nitrogen (TN) and total phosphorus (TP) removal efficiencies in microalgal-based systems treating different wastewaters. Wastewater characteristics and microalgae species determine nutrient removal efficiency in microalgal-based systems. The overall wastewater composition affects nutrient uptake, existing an optimal C:N:P ratio, which differs between microalgae species. Moreover, nutrient uptake also depends on environmental factors that affect microalgae growth such as pH, temperature, light intensity, turbidity and watercolour, among others. Therefore, a wide variation in TN and TP removal rates, in ranges of 22–100% and 20–98% of the initial TN and TP in the wastewater, respectively, has been reported (Table 20.2). According to the results presented in Table 20.2, the main genera present in photobioreactors used for wastewater bioremediation are *Chlorella* and *Scenedesmus*. In many occasions, a consortium of different microalgae (i.e. freshwater algae) is used to treat wastewater. Freshwater algae are composed of several species, being most of them unable to acclimate to wastewater polluting conditions and, subsequently, dying. On the contrary, depending on the particular characteristics of each wastewater, some genera grow and become the main species (Hernández et al. 2016).

Table 20.2 Removal of total nitrogen (TN) and total phosphorus (TP) using microalgae-based systems for different wastewater treatments

Main microalgae	Wastewater used	TN initial concentration (mg/L)	TN removed (%)	TP initial concentration (mg/L)	TP removed (%)	Reference
Benthic algae	Digested dairy manure	225	39	25	51	Wilkie and Mulbry (2002)
Chlorella vulgaris	Artificial wastewater	22	97	4	96	Feng et al. (2011)
Chlorella sp.	Digested manure	100–240	76–83	15–30	63–75	Wang et al. (2010)
Chlorella sorokiniana	Potato processing wastewater	3.4	>95	0.4	81	Hernández et al. (2013)
Chlorella sorokiniana	Secondary pig manure	3.2	83	5	58	Hernández et al. (2013)
Chlorella vulgaris	Dairy industry wastewater	3–36	30–95	112	20–55	González et al. (1997)
Coelastrum microporum	Municipal wastewater	40	88	5.3	89	Lee et al. (2015)
Oocystis sp.	Fish processing wastewater	14–17	>95	3–11	42	Riaño et al. (2011)
Phormidium tergestinum	Slaughterhouse wastewater	149	83	1.4	91	Hernández et al. (2016)
Scenedesmus obliquus	Municipal wastewater	27	79–100	12	47–98	Ruíz-Marín et al. (2010)

Scenedesmus dimorphus	Dairy industry wastewater	36.3	>90	112	20–55	González et al. (1997)
Mucidosphaerium pulchellum	Domestic wastewater	64–79	79	4.6–7.2	49	Sutherland et al. (2014)
Freshwater microalgae	Coffee processing wastewater	77–766	80	6–60	n.d.	Posadas et al. (2014)
Freshwater microalgae	Fish processing wastewater	8–42	45–85	0.6–3	n.d.	Posadas et al. (2014)
Freshwater microalgae	Carpet mill effluents	33–46	>95	5–14	>95	Chinnasamy et al. (2010)
Freshwater microalgae	Primary domestic wastewater	200–850	70	25–60	85	Posadas et al. (2013)
Freshwater microalgae	Municipal wastewater	100–4530	22	10–500	26	Boelee et al. (2011)

2.1.2 Organic Pollutants Removal During Wastewater Treatment by Microalgae-Bacteria Consortia

Microalgae and bacteria symbiotically carry out organic pollutants' elimination. In the presence of light, microalgae (autotrophs) produce the O_2 required by heterotrophs to oxidize the organic pollutants. In this way, high organic matter removal rates have been reported for different wastewaters treated by microalgae-bacteria consortia. For instance, 62, 85 and up to 92% of total chemical oxygen demand (TCOD) was removed when treating piggery effluents, potato processing waste and slaughterhouse wastewater, respectively. The initial TCOD concentrations in these wastewaters corresponded to 616, 1536 and 1621 mg TCOD/L, respectively. Different removal rates were attributed to variations in the biodegradability of the different wastewaters (Hernández et al. 2013, 2016). Moreover, the symbiotic relationship between bacteria and microalgae has resulted in an efficient bioremediation of oil spills in marine environments or in the increase water quality in aquaculture hatcheries (Paniagua-Michel 2017). Even though heterotrophic activity has been traditionally associated to bacteria, the occurrence of a mixotrophic algal metabolism and a key role of microalgae during organic matter removal have been recently highlighted (Olguín et al. 2015).

2.1.3 Pathogen Removal During Wastewater Treatment by Microalgae-Bacteria Consortia

Wastewaters often contain a variety of microorganisms such as *Escherichia coli*, which can potentially contribute to disease transmission. In fact, the absence of *E. coli* and faecal coliforms after wastewater treatment is included as an indicator parameter for effluent discharge to public water bodies. Pathogen removal in microalgae-bacteria systems is mainly determined by dissolved oxygen (DO) and pH in the culture broth (Posadas et al. 2015, 2018). The photosynthetic activity of microalgae results in an increase in the DO and pH of the cultivation broth. High temperature and pH reduce pathogen survival, while high DO concentrations promoted photo-oxidative damage of cells, resulting in pathogen removal. Moreover, sunlight may inactivate bacteria cells due to both the UV-B radiation, which causes damage on the bacterial DNA structure, and the UV-A radiation, which results in the damage of cell organelles (Al-Geethi et al. 2017), boosting pathogen removal. Finally, the excretion of inhibitory metabolites by microalgae to compete with bacteria also contributes to pathogen removal. For example, Mezrioui et al. (1994) reported high removal efficiency of *Vibrio cholerae* due to the toxic products secreted by *Chlorella* sp. when treating domestic wastewater. Although pathogen removal during wastewater treatment by microalgae-bacteria systems has been widely reported in literature, there is little information about the removal mechanisms and the survival of pathogens such as viruses or intestinal parasites.

2.1.4 Heavy Metals and Organic Pollutant Removal During Wastewater Treatment by Microalgae-Bacteria Consortia

During wastewater treatment by microalgae-bacteria consortia, heavy metals are removed from wastewater, being assimilated into the biomass. However, the efficiency of this process is determined by the wastewater characteristics, since the activity of microorganisms can be diminished due to the presence of certain heavy metals. Bacteria are often much more tolerant to these toxic compounds than microalgae, which are severely inhibited in the presence of a few milligrammes per litre of toxicants. More specifically, heavy metals in wastewater may inhibit photosynthesis of microalgae, since metals are able to substitute the metal atoms in the prosthetic groups for specific photosynthetic enzymes.

Microalgae and bacteria are able to accumulate heavy metals from polluted effluents, being a cost-effective and sustainable wastewater treatment alternative to traditional methods (Paniagua-Michel 2017). The main heavy metals in wastewater are Cu, Cd, Cr, Hg, Zn, Pb and Ni. The principle of metal removal from wastewater is mainly based on the relationship between heavy metals and negatively charged groups contained in the carbohydrates and exopolysaccharides of the bacteria and microalgae cell surface (Subashchandrabose et al. 2011). The microorganisms carry out these removal mechanisms as a response of the presence of heavy metals in their growth media (i.e. wastewater). Different mechanisms including adsorption, ion exchange, covalent bonding or heavy metal precipitation have been reported for heavy metal removal in wastewaters (Ozturk et al. 2014; Chojnacka et al. 2005; Posadas et al. 2018). Simultaneously, those metals are bioaccumulated (i.e. bioabsorpted) in cell vacuoles, by a metabolically active biological process of diffusion (Pereira et al. 2013; González et al. 2017). In this way, several commercial biofilms have been developed based on the microalgae capacity for accumulating heavy metals. Some examples are (1) ALGASORB™, produced by Bio-Recovery System, Inc. (USA). It consists in *Chlorella vulgaris* immobilized in silica gel polymer matrix. It can be used for a wide range of heavy metal concentrations (1–100 mg g^{-1}) and (2) BV-SORBEX™, produced by BV Sorbex, Inc. (Canada). This adsorbent contains *Sphaerotilus natans*, *Ascophyllum nodosum*, *Halimeda opuntia*, *Palmyra pomata*, *Chondrus crispus* and *Chlorella vulgaris*, and it is able to recover up to 99% of metal in the solution.

2.2 Nutrient Recovery in the Form of Valuable Biomass

Microalgae need a great amount of nutrients (N and P) to successfully grow. According to Oswald (1988), microalgal biomass is composed by $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$. Thus, N and P are essential elements for their growth. The use of commercial fertilizers as nutrient source for microalgae growth would lead to an increase in cost production, making food market unstable (Chisti 2008). In this

context, the use of agro-industrial wastewaters, rich in nutrients, is an interesting alternative as nutrient source to produce microalgal biomass according to life-cycle analysis studies (Christenson and Sims 2011). Removal efficiency is mainly related to microalgal productivity; thus, the higher amount of N and P removed from the wastewater, the higher biomass productivity. This biomass has been proposed for different applications like biofuel production, biofertilizer and feed additive in the commercial rearing of many aquatic animals, both freshwater and marine (Mata et al. 2010; Larrán et al. 2017; Tomás-Almenar et al. 2017).

2.2.1 Nitrogen Recovery

Nitrogen has a key role in amino acids, nucleic acids and pigment synthesis (Richmond 2008). During the last years, many studies have evidenced that when using microalgal-based systems, the assimilation of N is the main mechanism to remove this nutrient from wastewater. Other mechanisms, such as ammonia stripping or denitrification, have less importance in the nitrogen balance in microalgal-based wastewater systems (Cai et al. 2013). The preferred inorganic nitrogen source for microalgae is NH_4^+, although they are also able to use NO_3^- and, in a lesser extent, NO_2^- (Jia and Yuan 2016). The assimilation processes need active transport to incorporate N forms to the cell, but since a reduction to N_3^- state is required before assimilation, the energetic cost for NO_3^- and NO_2^- assimilation is higher than the one for NH_4^+ (Cai et al. 2013). The assimilation of inorganic nitrogen is governed by the equation represented in Fig. 20.1, where "Fd" corresponds to the enzyme ferredoxin (Richmond 2008).

The presence of bacteria in the wastewater presents several advantages for nitrogen assimilation by microalgae. When microalgae are used for agro-industrial wastewater bioremediation, aerobic bacteria oxidize proteins and nucleic acids to NH_4^+, which is assimilated by microalgae. However, when NH_4^+ concentration is higher than 100 mg/L and pH is higher than 8, the proportion of NH_4^+ that turns to

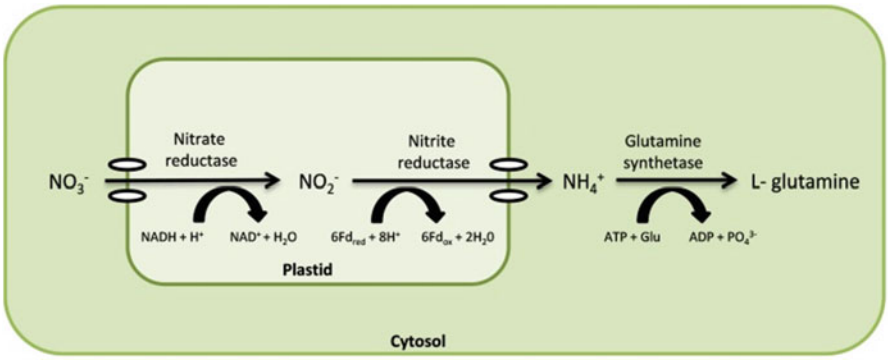

Fig. 20.1 Assimilation of different inorganic nitrogen sources in eukaryotic algae

NH_3 could result toxic for algae inhibiting their growth (Park and Craggs 2010). Moreover, under these conditions, an important part of NH_3 could volatilize to the atmosphere, diminishing nitrogen recovery. On the other hand, nitrifying bacteria helps to avoid NH_3 stripping and therefore contribute to mitigate nitrogen losses by volatilization. Nitrifying bacteria comprise ammonium-oxidizing bacteria (AOB) and nonoxidizing bacteria (NOB) (Eqs. 20.5, 20.6 and 20.7). They grow slower than microalgae and aerobic bacteria present in the wastewater; thus, they require higher HRT than typical operational conditions in microalgal-based systems, which usually ranges between 2 and 10 days. According to de Godos et al. (2014), when the hydraulic retention time (HRT) ranges from 2 to 10 days, most AOB and NOB are washed out, and the nitrification process does not take place. In order to enhance AOB and NOB growth, and help aerobic bacteria to avoid NH_3 stripping, microalgae-bacteria sludge retention time should be controlled and the settled biomass continuously pumped to the HRAP to increase retention time, allowing to grow and to nitrify to the AOB and NOB organisms, so that nitrification process only occurs when hydraulic retention time (HRT) is higher than 10 days or when settled biomass is recirculated into the system (de Godos et al. 2014).

$$NH_4^+ + \left(\frac{3}{2}\right)O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{20.5}$$

$$NO_2^- + \left(\frac{1}{2}\right)O_2 \rightarrow 2NO_3^- \tag{20.6}$$

$$NO_3^- + \left(\frac{1}{3}\right)CH_3OH \rightarrow NO_2^- + \left(\frac{1}{3}\right)CO_2 + \left(\frac{2}{3}\right)H_2O \tag{20.7}$$

Finally, the activity of denitrification bacteria (Eq. 20.8) is avoided by the presence of oxygen in the media, since it is an anoxic process. The concentration of dissolved oxygen is usually higher than 1 mg/L, as microalgae release oxygen during photosynthesis. In this way, nitrogen recovery by microalgae is favoured.

$$NO_2^- + \left(\frac{1}{2}\right)CH_3OH \rightarrow \left(\frac{1}{2}\right)N_2\,(\text{gas}) + \left(\frac{1}{2}\right)CO_2 + \left(\frac{1}{2}\right)H_2O + OH^- \tag{20.8}$$

2.2.2 Phosphorus Recovery

Phosphorus is an essential element for microalgae, necessary for metabolic activities, energy transfer and phospholipid and DNA synthesis (Richmond 2008). In conventional wastewater treatment plants, P is chemically removed through precipitation. In microalgal-based systems, P removal occurs simultaneously to nitrogen assimilation, so that P is recovered within the biomass. Moreover, under specific operational conditions, microalgae can be induced to further accumulate polyphosphates inside

the cell structure independently of the biomass productivity. Thus, when microalgae are exposed to "P excess – P starvation – P excess" conditions or under certain light supply and temperature conditions, the accumulation of polyphosphates inside the cell (luxury uptake) allows high P removal efficiencies (Brown and Shilton 2014). On the other hand, microalgae growth under P limitation results in a build-up of carbohydrates and/or lipids. The accumulation of one or another macromolecular compound depends more on the microalgae species than on the operational conditions. Most species including *Chlorella*, *Scenedesmus*, *Chlamydomonas* or *Spirulina* accumulate lipids inside the cell (Wang et al. 2010). Carbohydrate accumulation is frequent in *Spirogyra*, *Ulva*, *Gelidium*, *Laminaria* or *Saccharina*, among others (Buck and Buchholz 2004; Kraan 2013).

2.2.3 Nutrient Uptake Efficiencies for Different Wastewaters

Nitrogen uptake rates found in literature vary from 0.1 to 65 mg of total nitrogen per litre of photobioreactor per day for different microalgae like chlorophyte (mainly composed of *Chlorella* and *Scenedesmus*), cyanobacteria (*Arthrospira* and *Oscillatoria*), diatom and haptophyte (Cai et al. 2013). Phosphorus uptake rates can reach up to 40 g of soluble P per kg of produced biomass under luxury uptake. However, the general demand of P is in the range of 10–15 g per kg of microalgae (Powell et al. 2009). The optimization of the configuration of the reactors and the operational conditions result essential to maximize nutrient recovery during wastewater treatment by microalgae-bacteria consortia. In this manner, diverse studies have reported final biomass with very variable concentrations ranging, 0.11–0.70 g/L and 5.5–35 g/m^2 day.

2.3 Energy Savings

Microalgae biomass is often associated with the production of feedstock for biofuel production or high added-value products, and therefore, their energy consumption and production costs are associated with these processes. There are few works exploring and assessing microalgae energy consumption and production costs from a global point of view; this means considering the sum of energy used for cultivation, harvesting and drying. In the same way, the data for LCA (life-cycle assessment) studies must be extrapolated from laboratory-scale systems, as no industrial-scale process (for biofuel production) exists yet (Slade and Bauen 2013). Some other studies pointed out that energy production (i.e. biofuels production) from microalgal biomass will only be commercially feasible if it is coupled with an algae-based wastewater treatment system (Mulbry et al. 2008; Gouveia 2011), which is related to input energy cost. Therefore, recycling of N and P from wastewater will provide some of the nutrients for microalgae growth in combination with wastewater remediation, hence contributing to reduce energy cost.

Microalgae-based system feasibility is highly related to its energy demand, and it is mostly focused on biomass production for its further valorization. According to Slade and Bauen (2013), in raceway ponds, the most important contributions to the energy demand come from the electricity, required to circulate the culture, and the embodied energy in pond construction, as well as the energy embodied in the nitrogen fertilizer. However, microalgae-based systems for wastewater remediation can compete with activated sludge processes, as energy demand for these systems is approximately 500 Wh per m^3 of wastewater treated and the energy required for mixing conventional HRAPs ranges from 1.5 to 8 Wh per m^3 (Mendoza et al. 2013). In these order to evaluate energy demand of microalgae-based systems for wastewater treatment, empirical data of the performance of these specific systems is necessary.

2.4 CO_2 Emission Reduction

Current global warming has triggered international awareness concerning greenhouse gas emissions. Greenhouse gas emissions were 5.25×10^7 kt CO_2eq/year in 2014 (FAO 2014). Different techniques have been studied for CO_2 capture, which may be divided in geological sequestration, chemical processing or absorption and bioprocessing from photosynthetic organisms (Morrissey and Justus 2000; Chisti 2007). The geological sequestration consists of the storage of liquid or gaseous CO_2 underground in a geological formation or in deep ocean storages (Lal 2004). There is a major concern about the possibility of the escape of huge amounts of CO_2 towards surface waters that can lead to their acidification and the subsequent disturbance to aquatic ecosystems. Chemical processing/absorption processes require alkaline reagents, which are energetic intensive and expensive. Meanwhile, natural bioprocesses remove close to 50% of anthropogenic CO_2 emissions per year from atmosphere (Benemann 1993). Moreover, photosynthetic organisms have a double-positive environmental impact as they capture high amounts of inorganic carbon and release O_2 to the atmosphere. Microalgae present several advantages compared with other photosynthetic organisms for carbon capture (i.e. fixation), namely, (1) they are able to grow tenfold higher than terrestrial plants; (2) their growth is independent from arable lands, attenuating thus food and feed competition; and (3) they may not require nutrient supplementation when they grow in agro-industrial wastewaters, especially those rich in N and P (Rittman 2008; Stephens et al. 2010). Moreover, photosynthesis conversion efficiency in microalgal cells is remarkably higher than in superior plants due to the absence of structures, differentiation and lining structures, among others. When agro-industrial wastewater is treated using algae-bacteria consortia, microalgae capture the CO_2 produced after organic matter degradation by aerobic bacteria, thus reducing CO_2 emissions when compared with other aerobic wastewater treatments. The resulting biomass may also be valorized in the form of a carbon neutral renewable fuel like biodiesel, bioethanol or biogas (Chisti 2008;

Hernández et al. 2015, 2016). Hence, the use of photosynthetic biomass for energy production would diminish the dependence of oil-based fuels.

Carbon capture with microalgae has been widely studied during the last decades (Benemann 1993; Aresta et al. 2005; Park and Craggs. 2010). During the photosynthesis process, algae capture high amounts of CO_2 to produce organic molecules. When microalgae productivity is high, the lack of CO_2 triggers carbon sequestration from atmosphere to the water, and O_2 is released to the atmosphere. In this manner, microalgal biomass culture shows to be a suitable tool to capture carbon, fixing it in the form of valuable biomass. However, when light supply is scarce, an important amount of mixotrophic microalgae turns from autotrophic to heterotrophic metabolism. Microalgae are thus able to oxidize organic matter from wastewater releasing CO_2 that may be subsequently captured by photosynthetic algae. In this vein, although photosynthesis is more efficient from a carbon capture point of view, when heterotrophic behaviour occurs, most of the carbon is still captured in the form of valuable algal biomass.

Emissions of CO_2 in a microalgae-bacteria wastewater treatment plant are remarkably lower than those produced in conventional aerated activated sludge processes, and a negative balance can also occur when flue gas rich in CO_2 is injected into the process. Thus, from the CO_2 emissions point of view, the use of microalgal-based systems for wastewater treatment is always a better alternative than conventional active sludge treatment, since microalgae assimilate 1.8 tonnes of CO_2 per ton of algal biomass, while in activated sludge treatments, all the sludge produced must be managed and may not be valorized. When industrial CO_2-enriched air is efficiently injected to the photobioreactor and captured by microalgae, biomass productivity remarkably increases. The injection of CO_2 leads to a decrease in culture medium pH, and therefore, injection rate must be controlled in the ponds. Scientific literature has already presented many different optimal CO_2 concentrations and injection rates to maximize biomass production, but differences between them are high due to the great heterogeneity of microalgal species, culture mediums used, types of photobioreactors employed and operational conditions tested (light intensity, salinity and temperature). In this manner, it should be kept in mind that CO_2 feed rate must be optimized to the previously selected algae and to the operational conditions. The optimization of these parameters will maximize biomass production and thus carbon sequestration.

3 Challenges of Microalgae for Wastewater Treatment

3.1 Land Use

Most studies indicate that replacing conventional activated sludge wastewater treatments by high-rate algal ponds (HRAP) with microalgae would reduce GHG emissions and operational costs. However, the installation of these ponds would require enormous land areas, and the impacts derived of the land use change (LUC)

should be addressed, due to its potential environmental damage and the changes in soil carbon stocks. In most of the cases, LUC and suitability of land close to the wastewater producers are not taking into account for the studies of wastewater treatment by HRAP (Searchinger et al. 2008). In fact, many studies indicate that nonarable or marginal lands may be used to place enormous HRAP. As an example, a recent study comparing the potential land competition between microalgae and terrestrial feedstock production in the USA reported that there is little conflict with each other. In this way, microalgae production in HRAP in the USA could be located in areas outside the key agricultural-producing regions (Langholtz et al. 2016). However, the majority of the wastewater is produced close to urban centres where the price of land is expensive and wastewater transport to a further place is not an option, due to high costs.

Furthermore, if most of wastewater is treated using microalgae, most of the existing biomass in the landscape would be removed, including grasslands, croplands and forestlands. The impacts that are considered for life-cycle analyses during the installation of the ponds of microalgae systems are (1) loss of soil carbon, (2) removal of carbon below the original vegetation, (3) change in the surface albedo and (4) change in GHG emissions from the original vegetation in comparison to the microalgae ponds (Fortier et al. 2017). However, there is little information concerning these impacts and therefore the GHG emissions related to LUC in large-scale microalgae cultivation. Hander et al. (2017) reported that forests and grasslands/croplands also forego ongoing carbon sequestration and a significant CO_2 penalty is related to LUC in microalgae cultivation for energy production. More specifically, this penalty is estimated in 4–8 and over 40 g CO_2eq/MJ for grassland/croplands and forest, respectively. Moreover, terrestrial feedstock and microalgae production may compete for the same land. In this context, the LUC only results convenient when it increases carbon capture compared to conventional use. Hence, carbon sequestration accounting must reflect the net impact on the carbon benefit and not only the assimilation by microalgae. In this vein, many studies have failed in accounting emissions related to LUC. However, to substitute conventional treatments to implement large-scale HRAP for wastewater treatments, a deep economic and environmental impact is required, even with generous government subsides.

Previous studies have shown that LUC may result in enormous differences between different locations (e.g. Everglades, Florida and Tamaulipas, Mexico), despite these locations were selected due to their similar irradiance and temperature during the year (Fortier et al. 2017). Hence, in order to evaluate this impact, an analysis focused in each particular place must be previously performed. There is no doubt that a good progress in wastewater treatment with microalgae-bacteria using pilot-scale HRAP has been made during the last years (Park and Craggs 2010; Hernández et al. 2016). There is also a better understanding by the industry of the need to use a more sustainable and eco-friendly technology, and at the same time, the resulting biomass may result an income source. However, higher effort must be made to determine the economic and the GHG emission impact due to the LUC in order to obtain a deeper view of this key aspect.

3.2 Culture Conditions: Influence of Wastewater Characteristics

The characteristics of wastewater highly determine the efficiency of microalgal-based systems for wastewater treatment. These characteristics include pollutant and nutrient concentration, turbidity, colour and wastewater pH.

3.2.1 Pollutant and Nutrient Concentration

The ratio C:N:P in wastewater is an important factor affecting the overall efficiency of the system. In Table 20.3, a summary of the general characterization of municipal and industrial wastewater is presented. The lack of some essential nutrients or their low bioavailability in some types of wastewaters could negatively affect the performance of microalgal-based systems in terms of pollutant removal efficiencies and biomass production (Posadas et al. 2013; Markou et al. 2014). In addition, some compounds present in wastewater or produced during its treatment could cause inhibition in microalgal activity. The most usual toxic compound during wastewater treatment is NH_3, whose concentration concomitantly rises when increasing the pH values in the photobioreactor because of photosynthesis. Ammonia concentration that results inhibitory is species dependent. Free ammonia concentrations of up to 51 g/m^3 have been proved to diminish photosynthetic rates by 90% in dense culture of the microalgal species *Scenedesmus obliquus*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* (Azov and Goldman 1982; Sutherland et al. 2015). Ammonia toxicity should be taken into consideration when treating wastewater with high nitrogen concentration such as livestock wastes or anaerobically digested agro-industrial effluents. For this type of wastewaters, a strategy to avoid microalgal inhibition, such as pH control, a previous dilution step or the operation at low loading rates, is required (Markou et al. 2014). Toxic effects of nitrite, an intermediate product from the oxidation of ammonium to nitrate, have been also reported at high concentrations (Markou et al. 2014). In addition, some heavy metal ions that

Table 20.3 Characterization of municipal and industrial wastewater (Adapted from Hernández 2015)

Type of industry	TSS (mg/L)	COD (mg/L)	BOD_5 (mg/L)	N (mg/L)	P (mg/L)
Municipal	100–350	250–1000	100–400	85–20	4–15
Potato processing	700	10,000	3000	150	200
Fish processing	200–3000	500–4500	400–4000	1–20	5–90
Slaughterhouse	200–5000	750–350,000	500–5350	48–750	10–90
Pig manure	46,000–76,000	52,000–73,900	3500–61,000	3500–5400	3200–6200

TSS (total suspended solids), *COD* (chemical oxygen demand), *BOD_5* (biological oxygen demand), *N* (total nitrogen), *P* (total phosphorus)

can be present in industrial wastewater affect the survival of specific microalgal strains (Zeraatkar et al. 2016). The presence of organic acids, phenols and pesticides also decreases microalgal activity (Guldhe et al. 2017). Nevertheless, some microalgae species such as *Chlorella*, *Nitzschia* and *Chlamydomonas* present a high tolerance to pollutant concentration, which can explain its predominance in microalgal-based wastewater treatment systems (Muñoz and Guieysse 2006; de Godos et al. 2009).

3.2.2 Turbidity, Colour and pH

Light availability is a major factor affecting microalgal performance and, therefore, the overall wastewater treatment efficiency. In this sense, both industrial and domestic wastewaters usually present high concentrations of suspended solids that result in wastewater turbidity. These solids, together with the presence of coloured dissolved compounds in wastewaters, can absorb light and reduce its penetration in culture broth. Consequently, biomass production in microalgal-based systems could diminish. A physical-chemical pretreatment of the wastewater before feeding the photobioreactors would decrease turbidity and the concentration of coloured compounds. Dilution of feeding stream is also required in some cases for highly polluted wastewaters. Nevertheless, this issue might not be very important if the wastewater contains organic carbon and the microalgae species can grow in heterotrophic or mixotrophic mode (Markou et al. 2014). Finally, the pH of wastewater highly influences on the microalgae-bacteria systems. In this way, wastewaters that present a pH outside of the optimal range for their treatment in microalgal-based systems are hardly biodegraded without any pretreatment (Posadas et al. 2018).

3.3 Culture Conditions: Environmental and Operational Aspects

The efficiency of microalgal-based wastewater treatment will depend on a combination of factors including environmental and operational conditions. In this section, the main environmental and operational aspects affecting the performance of microalgal systems are briefly described. Temperature, light availability, pH, concentration of O_2 and CO_2 in the culture medium and hydraulic retention time (HRT) are described as the main factors affecting microalgal-based systems.

3.3.1 Temperature

The effect of temperature on microalgae growth is well known. Increasing temperature enhances microalgal growth until achieving an optimum value. Once this

optimum value is reached, biomass productivity dramatically declines with the increment in temperature (Larsdotter 2006). Although the optimum value of temperature for microalgal growth is genera and strain dependent, it usually lies between 20 and 30 °C (Umamaheswari and Shanthakumar 2016; de Godos et al. 2017). Operating at favourable temperature conditions led to greater nutrient removal efficiencies as well as higher biomass productivities (Molinuevo-Salces et al. 2016). Under lower temperatures, metabolic rates of microalgae diminish, attaining lower growth rates and diminishing nutrient removal efficiency. As an example, Cho et al. (2015) reported a biomass productivity reduction of approximately tenfold when treating raw municipal wastewater, from summer (temperatures up to 30 °C) to winter (temperatures near 5 °C). On the contrary, higher temperatures above the optimum range can cause oxidative stress and a decrease of photosynthetic activity (Posadas et al. 2018). Reactor overheating is thus a problem, especially in humid climates where evaporation is inhibited (Larsdotter 2006). Supplying cooling water on the surface of the reactor and the regulation of air temperature by refrigerated air conditions units are two of the strategies that can be applied to prevent overheating (Lavens and Sorgeloos 1996; Umamaheswari and Shantakumar 2016), but both alternatives significantly increase operational costs.

3.3.2 Light Availability

In a similar way as described for temperature, a positive correlation between light availability and biomass productivity occurs. Microalgae growth increases when increasing light intensity until reaching an optimum value. Above this optimum value, too much light may decrease photosynthesis rate (Park et al. 2011). Photosynthetic activity is saturated at relatively low irradiances ranging from 100 to 200 $\mu E/m^2$ day (Acien et al. 2017). Since solar radiation is several times higher than this saturation level, an excess of solar radiation can lead to microalgal photoinhibition. A strategy to overcome the influence of the excess of irradiance is to operate under higher culture densities and a proper mixing regime (de Godos et al. 2017). In fact, mixing is one of the most important parameters during microalgal-based system operation, providing turbulence and a degree of vertical mixing through the pond depth that ensures that microalgae are intermittently exposed to light (Park et al. 2011; Posadas et al. 2018). Strong mixing could result in shear stress and in cell rupture (mainly in cyanobacteria), negatively affecting microalgae growth.

3.3.3 pH

Because of microalgal photosynthesis and respiration, pH oscillates over the day in microalgal-based systems (Sutherland et al. 2015), as seen in Fig. 20.2. pH values above 9 can be commonly achieved in photobioreactors, especially when operating at low organic and nutrient loading rates and at favourable conditions (Riaño et al. 2012; Hernández et al. 2016). Most microalgae usually tolerate wide pH intervals, but out of this interval, the growth is greatly reduced. More specifically, pH values

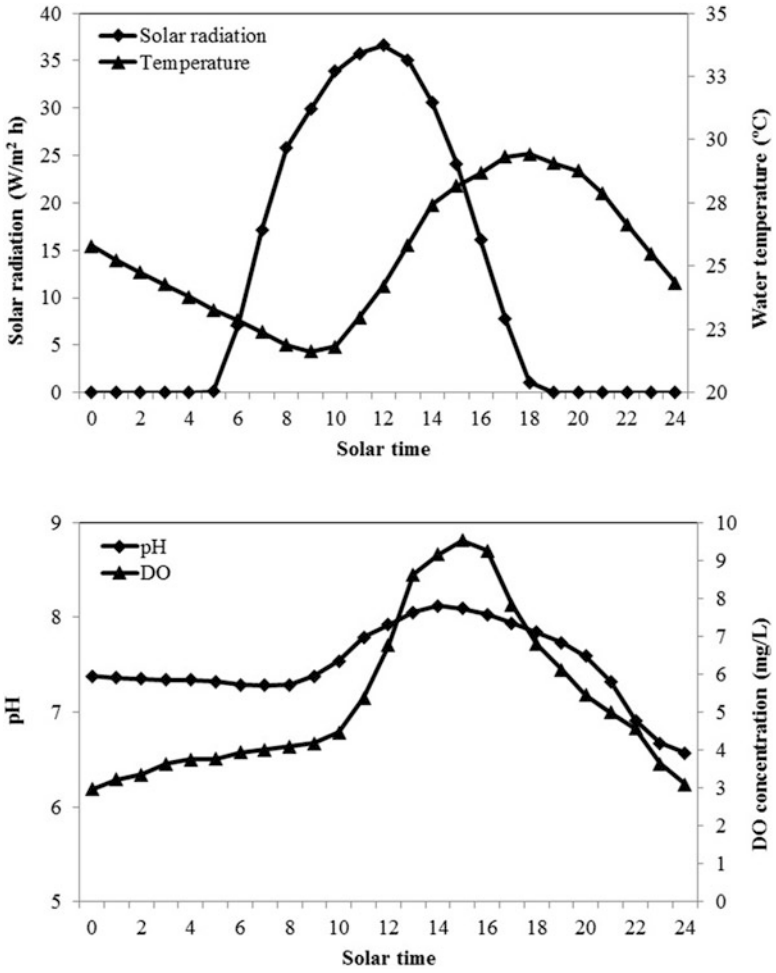

Fig. 20.2 Daily variation in solar radiation on a high-rate algal pond (HRAP) treating swine manure and the culture parameters (temperature, pH and dissolved oxygen) as a function of solar time

higher than 9 negatively limit microalgal activity, since the capacity to absorb CO_2 is dramatically reduced and the cell's ability to maintain the activity of the RuBisCO enzyme is interfered (Sutherland et al. 2015). Moreover, high pH in the culture broth also causes the dissociation of NH_4^+ ion, increasing free NH_3 concentration that can significantly inhibit microalgal growth and increase nitrogen losses by volatilization. The pH control allows reducing NH_3 volatilization and enabling greater nitrogen recovery into microalgae-bacteria biomass. Injection of CO_2 into the culture broth is the most common strategy to reduce pond culture pH; however, nowadays few studies have evaluated the performance of wastewater supplied by CO_2 addition at semi-industrial or industrial scale.

3.3.4 O_2 Concentration

The evolution of dissolved oxygen concentration in photobioreactors is characterized by an increase during the day and a decline during the night, according to the cycle of photosynthesis and respiration of microalgae (Fig. 20.2). Although more research is required regarding toxicity of O_2 for microalgae, O_2 concentrations above 20 mg/L are believed to negatively affect microalgae growth, favouring photorespiration and O_2 radical formation and causing thus partial inhibition of photosynthesis (de Godos et al. 2017). Bacteria activity such as the organic matter oxidation and the nitrification during wastewater treatment involves a decrease in the oxygen concentration, preventing microalgae inhibition. For instance, reported O_2 concentrations in microalgal-based systems for several agro-industrial wastewater treatments were lower than 14 mg/L (Riaño et al. 2011; Hernández et al. 2013, 2016). Nevertheless, CO_2-enriched air supply has been again defined as a strategy for degassing the culture broth in high-rate algal ponds to enhance nutrient assimilation while decreasing the dissolved oxygen concentrations (de Godos et al. 2017).

3.3.5 CO_2 Concentration

During microalgal-based wastewater treatment, non-photosynthetic microorganisms produce the CO_2 needed for microalgae growth during organic matter degradation (Molinuevo-Salces et al. 2010). However, C:N ratios in most agro-industrial wastewaters are lower than the optimal reported ratio of 100:18 (Posadas et al. 2018). These lower C:N ratios are correlated with lower biodegradability, resulting in a reduction of removal efficiencies and biomass production (Posadas et al. 2014). The injection of inorganic carbon (via flue gas or biogas resulting from anaerobic digestion) has been proposed as an alternative to enhance wastewater treatment, preventing CO_2 competition between the autotrophic communities present in the culture broth (Alcántara et al. 2015) and also a change from autotrophic to heterotrophic metabolism that will suppose a decrease on productivity. Nevertheless, it is worth mentioning that the presence of volatile fatty acids in wastewater can be also used as carbon source in mixotrophic cultures (Olguín et al. 2015).

3.3.6 Hydraulic Retention Time (HRT)

HRT (defined as the volume of the photobioreactor divided by the flow rate) is a key design parameter that affects the performance of microalgal-based systems for wastewater treatment. It has been already demonstrated that organic and inorganic contaminant removal from wastewaters in natural treatment systems increases at longer HRTs due to the enhancement of biodegradation, photodegradation and sorption processes (Matamoros et al. 2015). The operation at longer or shorter HRTs also allows or prevents biomass accumulation (Sutherland et al. 2015).

Most typical values of HRTs are in the range between 2 and 10 days (Muñoz and Guieysse 2006; Posadas et al. 2018). Longer HRTs are required in colder seasons due to the decrease in the metabolic activity and to the low microalgal growth rates.

3.4 Biomass Harvesting and Valorization

3.4.1 Biomass Harvesting

Biomass harvesting is one of the major challenges for large-scale production of microalgae in wastewater treatment systems, accounting for 20–30% of the total operational costs (Molina-Grima et al. 2003; Vandamme et al. 2013). For most of the microalgae species (exception made for *Spirulina*, due to its filamentous nature), biomass is suspended in water due to the small size of microalgae cells (5–20 μm) coupled to their colloidal stability in suspension. In this way, biomass concentrations in the range of 0.5 g microalgae/L have been reported for HRAPs, which are mostly used to treat wastewater (Benemann 1993). Biomass harvesting consists of separating microalgae biomass from water. The low biomass content needs to be concentrated to final values of 150–250 g microalgae/L. Prior to the final concentration values (i.e. biomass thickening), a harvesting step, where initial biomass concentration is increased to 10–50 g microalgae/L, is usually carried out (Muylaert et al. 2018). Since microalgae are a very heterogeneous group, the harvesting process should be adjusted to the microalgae species and the culture conditions (both wastewater characteristics and operational parameters). Moreover, the choice of the harvesting technology is also determined by the valorization strategy of the final biomass, which should not compromise both biomass and final effluent qualities (Muylaert et al. 2018). In this way, low-cost technologies are preferred for microalgae harvesting in the case of biomass obtained from wastewater systems.

Table 20.4 shows the main advantages and disadvantages of different microalgae biomass harvesting methods. These methods comprise sedimentation, flotation, centrifugation, filtration, coagulation-flocculation as well as several combinations of them. Sedimentation is easy to perform; it requires low-cost equipment and low-energy demand. For instance, for harvesting a biomass concentration of 1–15 g dry microalgae/L, the sedimentation process requires 0.1 kWh/m^3 using lamella separators (Milledge and Heaven 2013). It is a slow process (retention times of 1–2 days) that can lead to some deterioration of the biomass. It may be a good harvesting alternative for filamentous microalgae as *Spirulina* or for algae-forming aggregates, like *Scenedesmus*. In the case of wastewater treatment, biomass sedimentation is favoured by the high amount of bacteria and nutrients in the media (Alam et al. 2017). This technology usually needs to be combined with another technology (e.g. coagulation-flocculation) to further concentrate the biomass. Opposite to sedimentation, when using flotation as harvesting technology, microalgae are collected from the surface of the tank. Air is bubbled into the microalgae solution, so that the bubbles are attached to the microalgae cells and both move to the surface.

Table 20.4 Main advantages and disadvantages of different microalgae biomass harvesting methods

	Sedimentation	Flotation	Centrifugation	Filtration	Coagulation-flocculation
Technology investment cost	1	1	3	4	2
Requirement of skilled operators	1	2	1	3	2
Power consumption during harvesting	1	3	5	3	3
Retention time	5	3	1	2	3
Risk of biomass damage	4	2	1	1	2
Dewatering efficiency	2	3	5	4	3
Algae species dependency	4	2	1	4	4
Feasibility as a preconcentration step	5	3	1	1	3
Need of chemicals	3	2	1	1	3

1, 2, 3, 4 and 5 correspond to very low, low, medium, high and very high, respectively

Flotation is a relatively fast separation method where biomass is not damaged. However, the aeration sometimes results in high-energy costs, and the interaction between cells and bubbles is not always good. Similar to sedimentation, chemical addition is often needed to increase flotation efficiency. In this way, it has been seen that the addition of surfactants; the use of ozone as gas carrier, instead of air; or the combined flotation-flocculation method may achieve promising results for microalgae biomass recovery in the floating cakes. However, the economic feasibility of flotation is still a challenge. The increase in the biomass recovery yield depends either on an expensive coagulant or on high-energy requirements (in the case of electrical-based systems), increasing in both cases the operational costs (Laamanen et al. 2016).

On the other hand, centrifugation is a fast and easy technology that produces a high-quality thick biomass paste. A microalgae biomass concentration up to 250 g TSS/L can be achieved in a single step. It is currently the most used technology both for harvesting of high-value biomass and as a second step for thickening of low-cost biomass. However, the high-energy consumption (50–75 kWh) (Milledge and Heaven 2013) coupled with the high investment costs still makes this technology economically unfeasible as a single step for harvesting microalgae biomass for low-cost purposes (low-cost biomass), which is the case of biomass obtained from wastewater treatment. In the case of filtration, water and microalgae biomass are separated by means of a selectively permeable medium. The high concentration efficiency together with the no need of chemical addition makes this technology an interesting option. However, some drawbacks such as membrane or filters clogging or high investment cost for membranes have been reported. In general, filtration results an economically unfeasible method for harvesting low-cost microalgae

biomass. However, filtration by means of low-cost nylon screens has been demonstrated as an effective method for harvesting large-sized microalgae such as *Spirulina* (Toyoshima et al. 2015). Finally, by coagulation-flocculation, single cells are aggregated to form flocs, which are easy to harvest. It has been highly used due to the low-cost and high separation efficiency (Alam et al. 2017). Microalgae cells are negatively charged, and chemical addition to neutralize those charges is often required. Different chemicals have been successfully tested for microalgae harvesting, including metal salts, inorganic polymers and biopolymers. The high cost of the chemicals together with a possible decrease in the quality of the harvested biomass is the main drawback for not scaling up this technology. On the contrary, electrocoagulation and bio-flocculation are two of the most economically feasible methods for microalgae harvesting, due to the high quality of the resultant biomass. Bio-flocculation refers to a spontaneous microalgae flocculation due to the action of other microorganisms such as bacteria or fungi. In the case of wastewater treatment by microalgae, bio-flocculation is a promising alternative since it spontaneously occurs quite often. In some cases, auto-flocculation can be achieved when pH increases in the culture media. This is due to the precipitation of Ca and P salts, which act as flocculants and can be naturally present in wastewater. This strategy involves some advantages since these salts are not as toxic as metal salts. The separation efficiency of both bio-flocculation and auto-flocculation is strongly dependent on the media composition and the microalgae species, so that they cannot be applied to any kind of wastewater.

The development of a low-cost method for microalgae harvesting is still a challenge. According to recent studies (Barros et al. 2015; Alam et al. 2017; Muylaert et al. 2018), a two-stage process would be necessary to concentrate microalgae biomass. Flocculation followed by sedimentation is proposed, being bio-flocculation the best alternative, since it would reduce chemical costs while not compromising biomass quality. Although there is a wide variety of investigations at lab-scale reporting promising results, more large-scale studies are required in order to demonstrate the different harvesting technologies proposed. Finally, the harvesting method should be adapted to microalgae species and to the final use of the obtained biomass.

3.4.2 Biomass Valorization

Microalgae biomass can be used for different applications. These applications include energy production (i.e. biofuels), products for agriculture (biofertilizers, biopesticides etc.), animal feed and products for human consumption (foods and pharmaceuticals). When biomass is obtained as a by-product after wastewater treatment, low-cost applications are preferred, since only microalgae recognized as safe (GRAS) can be sold for human consumption. Therefore, the most common uses are energy, biofertilizer and animal food production (Acien et al. 2017).

The use of microalgae as feedstock for biofuels production, such as biodiesel and bioethanol, presents several advantages over other biomass feedstock such as corn or

vegetable oil, but it is noteworthy that microalgae production, harvesting and conversion into biofuels are expensive, compromising the economic feasibility of the process. However, a recent bioeconomy study about biofuels production from microalgae biomass reported promising results. More specifically, positive net present values (NPV) were achieved both for economy studies for ethanol and for biodiesel production, meaning that these technologies are worth to invest in (Peng et al. 2018). In the case of biogas production, anaerobic hydrolysis of microalgae appears to be the limiting step to reach an economically feasible process. A deeper knowledge of anaerobic digestion, hydrolytic bacteria and microalgae cell wall composition are needed to overcome that bottleneck and increase methane yields (González-Fernández et al. 2015). Microalgae grown on wastewater present an enormous potential as biofertilizer. The ability of microalgae to uptake nutrients such as C, N and P from wastewater results in an enhancement of nutrient availability for plant systems. Microalgae present a chemical composition, including macronutrients such as N, P and K and micronutrients as Ca^{+}, better than available organic fertilizers (Mahapatra et al. 2018). Even though microalgae are a source of proteins, lipids and carbohydrates, there are few studies regarding microalgae supplementation to animal diets. Microalgae biomass grown in wastewater is generally characterized by high-protein contents, so that a possible valorization way is its use as protein source for animal feed. For instance, microalgae were included in rainbow trout diets in percentages of 12.5%, 25% and 50%. The results evidenced that an inclusion higher than 12.5% resulted in nutritional deficiencies in trout (Dallaire et al. 2007).

4 Conclusions

The use of microalgae-based systems for wastewater treatment has been widely studied due to the enormous potential of this technology as an alternative to traditional wastewater treatment systems. This potential comprises the high bioremediation efficiency for a variety of wastewaters, the contribution to CO_2 emission reduction and the remarkable energy savings (including the potential valorization of the produced biomass as energy). However, there are still some challenges to overcome before a real implementation of this technology. These issues include land use competition, wastewater variability, the influence of environmental conditions and high-cost biomass harvesting. Although there is little conflict for land competition between microalgae and terrestrial feedstock, GHG emissions related to land change use should be addressed when installing open ponds for large-scale microalgae cultivation. Secondly, wastewater characterization is of major importance for an efficient treatment. The lack of essential nutrients, the presence of toxic compounds or the low bioavailability in some wastewaters could negatively affect the performance of microalgal-based systems. Moreover, the proposed strategies as wastewater dilution to ensure light availability or the pretreatments to control wastewater pH are energy demanding, so that an economic study with different scenarios for each specific case should be carried out before large-scale implementation. In the third place, environmental and operational aspects as temperature, light

availability, pH, O_2 and CO_2 concentration in the culture medium and hydraulic retention time (HRT) determine microalgae productivity and, consequently, wastewater treatment efficiency. For instance, pH control could be done by coupling a CO_2 emission source with a microalgae-based wastewater treatment facility. Finally, low-cost technologies are preferred for microalgae harvesting in the case of biomass obtained from wastewater streams as culture media. A two-stage process would be necessary to concentrate microalgae biomass. Flocculation followed by sedimentation is proposed, being bio-flocculation the best alternative, since it would reduce chemical costs while not compromising biomass quality. The harvesting method should be adapted to microalgae species and to the final use of the obtained biomass, being energy, biofertilizer and animal food production the most common uses.

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Chapter 21
Design Considerations of Microalgal Culture Ponds and Photobioreactors for Wastewater Treatment and Biomass Cogeneration

Truong Giang Le, Dang-Thuan Tran, Thi Cam Van Do, and Van Tuyen Nguyen

Abstract Despite highly potential feedstock for biofuel production, high microalgal biomass production cost has been a major obstacle for commercialization of microalgal bioenergy. Coupling cultivation of microalgal in wastewater for simultaneous nutrients/pollutants removal and biomass cogeneration has been considered as a feasible solution for reducing microalgal production cost. Microalgae are photosynthetic microorganisms which require large amount of nitrogen and phosphorus for their growth and releases oxygen during photosynthesis process. Nevertheless, it is hard to maintain pure cultures of these algae in wastewater treatment processes. Therefore, the utilization of natural and artificial microalga consortia including either microalgae solo or microalgae and bacteria has been studied by several groups. Whatever the mode of culture of microalgae such as single or poly-culture of algae, algae-bacteria, algae-yeast, algae-fungi in wastewater, its production is based on the sample principles such as light availability, appropriate mass and heat transfer, and adequate control of operational parameters. This chapter is aimed at taking consideration of these principles in designing microalgal culture ponds and photobioreactors for wastewater treatment and biomass production. Different emerging designs and important factors and the parameters influencing their performance are reviewed. Mechanism of microorganism interactions and reactor designs used for polyculture cultivation in wastewaters to achieving win-win benefit are also discussed.

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1 Introduction

Although microalgae production has been tested at large scale in last decades, it is still evaluated that potential challenges to wide-scale production is nutrients, water requirements, algae productivity, and energy needed for downstream processing (Brennan and Owende 2010; Pienkos and Darzins 2009). The essential nutrients for microalgae growth in either phototrophic or mixotrophic modes are carbon, nitrogen, and phosphorous. Wastewaters present considerable concentration of these nutrients; therefore, they are promising nutrient sources for microalgae growth. In addition, cultivation of microalgae in wastewater attracts global attention because long-term utilization of chemical fertilizers will become unsustainable, particularly in the generation of lipid-rich biomass for biofuels production as commodity chemicals (low-cost products). Furthermore, assimilation of nitrogen and phosphorous in microalgae could be recycled via production of biofertilizers from microalgal biomass, or the resulted biomass can be utilized for the production of bioenergy, food, animal feed, and pharmaceuticals; while simultaneously obtaining oxygenated effluent is discharged into the water bodies. It has been reported that high efficiencies (80–100%) of nitrogen and phosphorous removal was achieved by utilization of microalgae on different wastewater sources such as industrial (Safonova et al. 2004; Tarlan et al. 2002), domestic (Posadas et al. 2013; Yang et al. 2011), agricultural (Hernandez et al. 2013), leachate (Lin et al. 2007; Mustafa et al. 2011), and refinery (Chojnacka et al. 2004).

Nevertheless, it is hard to maintain pure cultures of these algae in wastewater treatment processes. Therefore, the utilization of natural and artificial microalga consortia including either microalgae solo or microalgae and bacteria for wastewater treatment and biomass cogeneration has been studied by several groups (Rawat et al. 2011; Munoz and Guieysse 2006; Olguin 2012; Subashchandrabose et al. 2011; Chen et al. 2015; Ramanan et al. 2016; Johnson and Admassu 2013; Unnithan et al. 2014). Combination of different algal species having different metabolic activities can allow to develop polycultures which may possess widely adaptive capability to environmental conditions and nutrient load (Johnson and Admassu 2013; Boonma et al. 2015; Fouilland 2012). Supportive interaction established between algal-algal and algal-bacteria can result in higher nutrient uptake (Renuka et al. 2013).

Microalgae have conventionally cultured in open photobioreactors (e.g., open raceways) because of their simple design and low cost. However, these photobioreactors are limited in controlling operational conditions as well as the algae cultures are easily contaminated. In contrast, closed photobioreactors allow good control of operation conditions, making culture reproducible and avoiding contamination. Whatever the bioreactors used, they must be designed and operated

to appropriate culture conditions required for the particular microalgae strain, algal-algae, and algae-bacteria consortia. Its production is based on the sample principles such as light availability, appropriate mass and heat transfer, and adequate control of operational parameters. This chapter is aimed at taking consideration of these principles in designing microalgal culture ponds and photobioreactors for wastewater treatment and biomass production. Different emerging designs and important factors and the parameters influencing their performance are reviewed. Special design for polycultures for win-win benefit for different microorganism as well as mechanism are also discussed.

2 Wastewaters Characteristics as Nutrients Source

Human activities and industries are the main sources of discharging different types of wastewaters; each has different chemical composition and volumetric production. Table 21.1 summaries an overview of several types of wastewaters and their content of N and P, which are mainly generated from domestic, agriculture, urbanization,

Table 21.1 Wastewater sources and their N and P concentration

Wastewater source	N (mg/L)	P (mg/L)	N/P (molar ratio)
Municipal			
Domestic sewage	20–85	5–20	11–13
Landfill leachate	112–192	7–9	16–21
Sewage	24–220	1–12	18–24
Animal manure			
Pigs	800–2300	50–320	12–17
Beef cattle	63	14	10
Dairy cattle	185	30	4
Poultry	800	50	32
Industrial			
Coke production	757	0.5	3000
Tannery	273	21	29
Paper mill	11	0.6	41
Textile	90	18	11
Winery	110	52	5
Starch	49–115	50–385	1–4
Olive mill	530	182	2.9
Anaerobic-digested wastewater			
Food waste	1600–1900	300	
Dairy manure	1279–1961	240	6–8
Poultry manure	1380–1580	370–382	3.6–4.3
Sewage sludge	427–467	134–321	–

Data adopted from (Cai et al. 2013; Gonçalves et al. 2017; Christenson and Sims 2011)

and industries. As indicated in Table 21.1, wastewater composition strongly depends on its source. The three major sources of wastewater are domestic wastewater, wastewater generated from animal manure, and industrial wastewater. Generally, nitrogen and phosphorous contents in anaerobic-digested effluent and wastewaters of animal manure (e.g., pigs, poultry) are higher than these in municipal wastewaters. On the other hand, wastewaters releasing from winery, starch, dairy industries, and food-processing contain high concentration of carbon materials (e.g., COD, BOD). Although carbon, nitrogen, and phosphorous are all abundantly present in wastewaters, their composition should be considered prior to the use of microalgae for nutrients removal and treatment of wastewaters, because nitrogen-to-phosphorous molar ratio (N/P) greatly influences microalgal biomass production as well as nutrient uptake. The average elemental composition of microalgae biomass could be estimated via stoichiometric formula of $C_{18}H_{181}O_{45}N_{16}P$ (Oswald 1988), resulting an N/P molar ratio in microalgal biomass of about 16:1. Therefore, the N/P molar ratio in wastewater lower than 5:1 may result in nitrogen limitation, whereas N/P molar ratios higher than 30:1 result in limitation phosphorous (Larsdotter 2006). According to Table 21.1, the most appropriate wastewaters for microalgal growth are domestic sewage (N/P = 11–13), landfill leachate (N/P = 16:21), sewage (N/P = 18–24), pigs manure and beef cattle (N/P = 10–17), and textile (N/P = 11), tannery (N/P = 29), and dairy manure (N/P = 6–8) as N/P molar ratios were reported between 5 and 30. Whereas, dairy cattle (N/P = 4), starch-processing wastewater (N/P = 1–4), olive mill wastewater (N/P = 2.9), and poultry manure (N/P = 3.6–4.3) are N-limiting medium and coke wastewater (N/P = 3000) and paper mill wastewater (N/P = 41) are P-limiting medium, indicating that phosphorous found in wastewaters is rarely limiting algal growth, but nitrogen may be more often (Borowitzka 1998). However, as wastewaters likely exposes the microalgae to nutrient concentration up to three orders of magnitude higher than under natural conditions, growth of the microalgae is more likely limited by carbon and light (de la Noüe et al. 1992).

3 Microalgae Consortia in Nutrients Removal

Although cultivation of microalgae in different wastewaters have been successfully tested for nutrients removal and biomass production, it is hard to maintain a monoculture for reproducible production. Recently, microalgal consortia constituted by either microalgal-microalgal or microalgal-bacteria have been studied to explore the advantages of the combined cultures over single-species cultures. Wastewaters are complex media, which may be difficulty degraded by single-species but polyculture can implement biodegradation/assimilation of contaminants better. Moreover, single culture is easily suffered from contamination, invaded by other species and unable to resist fluctuation of environmental conditions (Paerl and Pinckney 1996). Microalgae consortia can be naturally occurred in the environment or be artificially engineered by combination of microorganisms that do not

necessarily occur, generating polycultures that overcomes the drawbacks which are associated with single cultures (Jagmann and Philipp 2014). The consortia of microorganisms can be established by combination of microalgal-microgalgal which are comprised of photosynthetic microalgae, microalgal-bacteria consortia, which are constituted by photosynthetic microalgae and heterotrophic bacteria, algae-yeast, and algae-fungi (Santos and Reis 2014; Magdouli et al. 2016). The biotrophic interactions between algae and other organisms can be established by mutualism, commensalism, and parasitism, among them (Santos and Reis 2014; Fuentes et al. 2016). The following sections present the type of interactions of these consortia and their capability in improvement of nutrients removal and biomass cogeneration.

3.1 Microalgae-Bacteria Consortia

Microalgal-bacteria consortia have been studied well in the literature, all demonstrating that bacteria play an important role in mutualistic relationships with algae for cycling of carbon (Cho et al. 2015; Grossart et al. 2006; Ask et al. 2009), nitrogen (Le Chevanton et al. 2016), sulfur (Segev et al. 2016; Durham et al. 2015), and phosphorus in aquatic ecosystems (de-Bashan et al. 2004; Grover 2000). That means the mutualism in an algae-bacteria consortium occurs when micro- and macronutrients needed for cell growth are exchanged (Cho et al. 2015; Durham et al. 2015; Croft et al. 2005; Kazamia et al. 2012; Amin et al. 2009; Watanabe et al. 2005; González et al. 2000; Kim et al. 2014; Hernandez et al. 2009; de-Bashan et al. 2002, 2011, 2016; De-Bashan et al. 2008a; Bashan and de-Bashan 2010; Leyva et al. 2015; Leyva et al. 2014; De-Bashan et al. 2008b; Choix et al. 2012; Choix et al. 2014; Meza et al. 2015; Rivas et al. 2010; Cheng et al. 2013; Palacios et al. 2016; Amavizca et al. 2017). In wastewater treatment, the consortia of microalgae-bacteria have been widely utilized to remove nutrients from different wastewater sources. Typically, a *C. vulgaris*-bacteria consortium established by Zhao et al. (2014) was cultivated in landfill leachate for carbon fixation and lipid production. Experimental data showed that removal efficiency of NH_4^+ and PO_4^{3-} reached over 95% from the leachate diluted with 10% water. The lipid productivity obtained under the established condition was 24.07 mg/L·d.

Similarly, cyanobacteria can be symbiotically associated with microalgae, resulting in higher biomass growth rate and improved nutrient and pollutants uptake (Subashchandrabose et al. 2011). However, the metabolic mechanism regulated by the interaction is rarely documented. A native co-culture of *Chlorella vulgaris* (a microalgae) and *Leptolyngbya* sp. (a cyanobacterium) was constructed by Silaban et al. (2014) to examine mutualism interaction between them on dextrose and sodium acetate-based media provided at different C:N ratios (15:1 and 30:1) under heterotrophic (dark) and mixotrophic (400 μmol/m^2·s) modes. Microalgal growth rates were recorded to compare with those under autotrophic conditions. It was reported that the carbon source and C:N ratio were found to impact both growth rate

and biomass productivity. Mixotrophic cultures with sodium acetate (C:N = 15:1) resulted in the highest mean biomass productivity (134 g/m^3·d) and neutral lipid productivity (24.07 g/m^3·d) compared to the autotrophic growth (66 g/m^3·d and 8.2 g/m^3·d, respectively). Moreover, lipid content of algal biomass achieved by the co-culture was also significantly higher for mixotrophic growth with sodium acetate addition (18.2%) compared to autotrophic growth (8.7.%). Thus, based on this experiment, mixotrophic growth with sodium acetate (C:N = 15:1) was found to be the preferred cultivation condition to improve biomass and lipid production by the co-culture.

3.2 Microalgae-Yeast Consortia

The interest in microalgae co-cultivation with other microorganisms has also been extended to yeasts due to the known ability of these single-cell eukaryotic organisms to produce wide range molecules that promote microalgal growth and productivity. It has been demonstrated that yeast-microalgae co-culturing improved the yield of high value products, and resulted in high growth rate and biomass concentration (Cai et al. 2007; Cheirsilp et al. 2011). The benefits of mutualistic algae-yeast interaction include CO_2 production by yeast that is used by algae for photosynthesis and the utilization of O_2 produced by algae for heterotrophic metabolism of yeast. *R. glutinis* and the microalgae *Chlorella vulgaris* were co-cultured in industrial wastewater to enhance lipid production in both algae and yeast (Cheirsilp et al. 2011). When the yeast was cultivated in monoculture, it grew slower and the lipid production was lower than when cultivated with the alga. The growth of *Chlorella* in monoculture was also slower than that in co-culture. In the co-culture, *C. vulgaris* acted as an O_2 generator for the yeast to utilize while *R. glutinis* produced CO_2 needed for the alga growth resulting in an overall enhanced lipid production in both algae and yeast cells. Synergic use of CO_2 (released by the yeast and taken up by the alga) and O_2 (released by the alga and taken up by the yeast) averted the accumulation of higher concentration of both gases that can become deleterious for the two organisms. The same mechanism was responsible for the enhanced accumulation of total biomass and total lipid yield when *R. glutinis* was co-cultured with the microalga *Spirulina platensis* (Xue et al. 2010), which reached biomass concentration and COD removal efficiency of 1.6 g/L and 73%, respectively, on monosodium glutamate wastewater. Similar to the results obtained in the previous study, when the oleaginous yeast *Rhodotorula glutinis* was co-cultured with *Scenedesmus obliquus* in a 5 L photobioreactor, it resulted in an increase in 40–50% of biomass and 60–70% of total lipid when compared to the single culture batches (Yen et al. 2015). Cai et al. (2007) evaluated the mixed culture of the alga *Isochrysis galbana* and the yeast *Ambrosiozyma cicatricosa* for cell growth performance and biochemical composition. Significantly higher specific growth rates were achieved in the mixed culture as compared to the monocultures during the same growth phases. The final biomass concentration in the mixed culture was significantly higher than those obtained in

monocultures. Overall, the latter study demonstrated improved growth performance and similar biochemical compositions in mixed culture as compared to monocultures.

3.3 Microalgae-Fungi Consortia

Fungi-alga consortia established by combination of *Chlorella vulgaris* and two isolated fungus strains *Aspergillus* spp. were utilized to form palletization-assisted biofloculation for harvesting microalgae (Zhou et al. 2012; Zhang and Hu 2012). These pellets also showed a promising capability in removal of ammonium, total nitrogen, and total phosphorous from diluted swine manure wastewater at efficiency of 23.23, 44.68, and 84.70%, respectively (Zhou et al. 2012). The developed co-culture can be regarded as a form of commensalism of microorganism since the benefit of the mixed culture can be exploited at commercial level. Recently, Rajendran and Hu (2016) developed a novel biofilm platform technology using filamentous fungi (*Mucor* sp.) and microalgae (*Chlorella* sp.) to form a lichen-type "mycoalgae" biofilm on a supporting polymer matrix (polymer-cotton composite) with 99% algae attachment efficiency. It was reported that the mixed-culture of *Mucor* sp. and *Chlorella* sp. produced higher amount of biomass than the axenic cultures of fungi and algae under the similar experimental conditions. These results demonstrated that algae can be grown on a bio-augmenting fungal surface, biofilm, with high attachment efficiency, making biomass harvested readily as biofilm for product development.

3.4 Microalgae Consortia

While microalgae-bacteria interaction has been studied well (previous section), the interaction of microalgal-microalgal in their consortia is deficiently documented. In reality, growing of photosynthetic microalgae in a consortium could be established via either cooperative or competitive interactions (Qin et al. 2016). The consortia of microalgae can be formed through the exchange of metabolites, leading to improvement of biomass productivities as well as nutrients removal efficiency (Qin et al. 2016). On the other hand, co-culturing of microalgae may result in the excretion of second metabolites, which are known as allelochemicals, exhibiting a negative impact on the combined algal poly-cultures (Cembella 2003; Bacellar Mendes and Vermelho 2013). For instance, chlorellin, a fatty acid generated by *C. vulgaris* considerably inhibited *Pseudokirchneriella subcapitata* when these two microalgae combined in a consortium (Fergola et al. 2007). The utilization of microalgal-microalgal consortia in simultaneous nutrients removal and wastewater treatments owns advantages of enhancement of the overall nutrient uptake (Renuka et al. 2013), high resistance to contaminants and predators as well as environmental factors, and

capable development of settleable module by using one of the strains in the consortia as a bioflocculating agent (Hu et al. 2017). Microalgal consortia applied for wastewater treatment can be formed by both native and artificial strains. Typically, combination of *Chlorella* sp. and *Scenedesmus* sp. was examined in nitrogen and phosphorous removal from a primary municipal wastewater by Koreivienė et al. (Koreivienė et al. 2014). It was reported that removal efficiencies of total nitrogen and total phosphorous were evaluated in the range of 88.6–96.4 and 99.9–99.9%, respectively. Interestingly, the engineered consortium was able to accumulate about 0.65–1.37 g CO_2/L·d in their biomass via CO_2 sequestration. A native microalgal consortium was developed by Chinnasamy et al. (2010) to treat a carpet mill industry effluent and biomass production. A high biomass and lipid productivity of 9.2–17.8 ton/ha·year and 6.82%, respectively, as well as high NO_3^- and PO_4^{3+} removal efficiency of between 96.6 and 99.8%, was reported. The consortium formed by the combination of artificial or native unicellular and filamentous microalgae resulted in self-flocculating culture in treatment of a primarily-treated sewage, achieving NO_3^-, NH_4^+, and PO_4^{3+} removal efficiencies of 81.5–83.3, 100, and 94.9–97.8%, respectively.

4 Mechanisms Involved in Microalgae-Based Nutrients Removal Processes

The main nutrients in culturing media including wastewaters are carbon-, nitrogen-, and phosphorous-based compounds which are assimilated by microalgae for their growth. In order to enhance efficiency of wastewater remediation processes using microalgae, it is extremely important to comprehend the mechanisms involved in nutrients removal.

4.1 Carbon Source

Carbon sources can be originated from inorganic form (mainly CO_2 from atmosphere and flue gas, CO_3^{-2}, HCO_3^-). Microalgae can uptake soluble carbonates as a source of CO_2 through photosynthesis process that is dependent on the pH of the culture media. In the pH range of 5.0–7.0, CO_2 uptake occurs via diffusion. On the other hand, when pH of culturing medium raises to higher than 9.0, CO_2 is turned into HCO_3^-, that is, the most common form of inorganic carbon present in solution, which enables the external carbonic anhydrase and promotes active transport of this carbon source into microalgal cells (Picardo et al. 2013; Sayre 2010; Sydney et al. 2014). When HCO_3^- penetrated into the cells, it is converted into CO_2 that can be fixed by rubisco (ribulose biphosphate carboxylase oxygenase), producing two molecules of 3-phosphoglycerate (Sayre 2010; Sydney et al. 2014). Although

microalgae are mainly autotrophic, many microalgae are heterotrophic, which only consume organic carbon such as acetate, glucose, glycerol, and ethanol as carbon sources, whereas others are mixotrophic, using facultatively CO_2 and organic carbon source (Neilson and Lewin 1974). In the latter case, growth of microalgae follows both respiratory and photosynthetic mechanism (Morales-Sánchez et al. 2015).

4.2 Nitrogen Source

Nitrogen presents mostly in the form of nitrate, nitrite, nitric acid, ammonia, molecular nitrogen, nitrous oxide, nitric oxide, and nitrogen dioxide (Cai et al. 2013; Barsanti and Gualtieri 2005). Microalgae play an important role in nitrogen cycle on the earth via nitrogen fixation and assimilation. Cyanobacteria are able to fix atmospheric molecular nitrogen (N_2) and transform it to ammonia (NH_3) using nitrogenase as a catalyst (Eq. 21.1), which can be constituted into amino acids and proteins or excreted to the environment (Barsanti and Gualtieri 2005).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \tag{21.1}$$

In contrast, eukaryotic microalgae are able to assimilate nitrogen forms including NH_4^+, NO_3^-, and NO_2^-. The nitrogen sources enter microalga cells through active transport at the plasma membrane. Nitrate form is commonly found in aquatic environments as it is thermodynamically more stable than ammonium (Barsanti and Gualtieri 2005; Grobbelaar 2007). However, assimilation of this nitrogen source by microalgae requires prior reduction into ammonium in a two-step processes enzymes nitrate reductase and nitrite reductase (Barsanti and Gualtieri 2005; Czarena et al. 2012; Hellebust and Ahmad 1989). Firstly, nitrate is reduced to nitrite by nitrate reductase catalyst using nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent (Eq. 21.2). The nitrite formed is further reduced to ammonium by nitrite reductase using ferredoxin in the second step (Eq. 21.3) (Barsanti and Gualtieri 2005; Hellebust and Ahmad 1989).

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \tag{21.2}$$

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O \tag{21.3}$$

The resulting NH_4^+ actively incorporated into microalgal cells is directly converted into amino acids via the glutamine synthetase-glutamate synthase pathway, where glutamine synthase catalyzed glutamine formation from glutamate and adenosine triphosphate (ATP) via Eq. 21.4 (Barsanti and Gualtieri 2005; Czarena et al. 2012; Hellebust and Ahmad 1989).

$$\text{Glutamate} + NH_4^+ + ATP \rightarrow \text{Glutamine} + ADP + P_i \tag{21.4}$$

Because assimilation of NH_4^+ does not require previous reduction steps, this is a preferred nitrogen form for microalgae. However, no significant difference of microalgal productivity was observed when separated forms of nitrogen NH_4^+ and NO_3^- were used for cultivation (Grobbelaar 2007). Another physicochemical process contributing to nitrogen removal is volatization of NH_4^+ when pH and temperature of culture media increased (Aslan and Kapdan 2006; Delgadillo-Mirquez et al. 2016; Abdel-Raouf et al. 2012).

4.3 Phosphorus Source

Phosphorous is an extremely important element that is utilized for energy transfer and nucleic acid synthesis in microalgal metabolism. The nutrient enters microalgal cells through active transport at the plasma membrane in the forms of $H_2PO_4^-$ and HPO_2^-. Transformation of PO_4^{3-} to organic compounds is performed following serial processes: (i) phosphorylation at the level substrate; (ii) oxidative phosphorylation; and (iii) photophosphorylation. The principal reaction can be presented as (Eq. 21.5) (Abdel-Raouf et al. 2012; Martínez et al. 1999; Gonçalves et al. 2017):

$$\mathrm{ADP} + \mathrm{P}_i \rightarrow \mathrm{ATP} \tag{21.5}$$

As shown in Eq. 21.5, ATP is produced from adenosine diphosphate (ADP) and energy input, which can be obtained from the oxidation of the respiratory substrates or from the electron transport system of the mitochondria (in the case of the first two processes) and from light energy transformation (in the case of the third step). The removal of PO_4^{3-} can also be influenced by environmental conditions such as pH and dissolved oxygen concentration. For example, when pH of culture media raises above 8.0 and oxygen concentration is high, phosphorous precipitation may be occurred (Aslan and Kapdan 2006; Delgadillo-Mirquez et al. 2016; Abdel-Raouf et al. 2012; Su et al. 2012).

4.4 Biological Fundamental and Biochemical Reactions Involved in Microalgae-Bacteria–Based Wastewater Treatment Processes

Although specific strains can be inoculated, wastewater treatment using microalgae is realistically performed by consortia of microalgae and bacteria; those composition varies depending on wastewater composition (Fouilland et al. 2014), environmental, and operation conditions (Posadas et al. 2014). Information reporting about variation of algae and bacteria population as function of culturing conditions and wastewater fluctuations are rarely found in the literature. Regarding to microalgae, the top

5 species *Euglena viridis, Nitzschia palea, Oscillatoria limosa, Scenedesmus quadricauda, and Oscillatoria tennis* have been ranked as the most-tolerant microalgae in wastewater treatment system due to their high adaption to variation of irradiance and temperature at the locally experimental field (Palmer 1969). Diverse population is usually found in wastewater treatment systems (Posadas et al. 2014; Morales-Amaral et al. 2015a; Park et al. 2011a), as very few culture was reported to be dominant by single strain (e.g., 90% of total microalgae population) (Fouilland et al. 2014). The presence of bacteria is necessary and indeed beneficial to microalgae growth due to symbiotic relationship created between bacteria and microalgae. It was reported that bacteria population presented in wastewater treatment system are regulated by wastewater types. For example, *Pseudomonas stuzeri*, *Labrenia*, *Hoefflea*, and *Sulfitobacter* were mainly found in digestate (Vasseur et al. 2012), while *Verrucomicrobium*, *Firmicutes*, and *Proteobacteria* (*Gammaproteobacteria*) were dominant in pig manure (Ferrero et al. 2012).

In terms of biochemical aspect, bacterial presence in wastewater is responsible for oxidation reaction of organic matter to inorganic compounds (e.g., CO_2, NH_4^+, PO_4^{3-}), while microalgae carry out photosynthesis utilizing solar energy and consuming inorganic compounds to produce O_2 and biomass. This simple metabolism have been reported elsewhere (Munoz and Guieysse 2006; Gonçalves et al. 2017); however, the real biochemical reactions are more complex, revealing different and simultaneous metabolism of microalgae and bacteria. For the microalgae-based process, the phototrophic (consuming CO_2 and sunlight) and mixotrophic/heterotrophic growth (up-taking small organic molecules) of microalgae could take place at the same time. On the other hand, aerobic growth and denitrification by anoxic growth of heterotrophic bacteria, nitrification by the aerobic growth of autotrophic bacteria, and biomass decay are included in the bacteria-based phenomena. Additionally, bicarbonate reactions, ammonification of soluble organic nitrogen, and hydrolysis of organic matter are also occurred. Based upon the proposed simple metabolism, an equilibrium between microalgae and bacteria in O_2/CO_2 production/consumption or COD/N-P decomposition/assimilation could be reached. However, Vasseur et al. (2012) figured out that the carbon content associated with microalgae is often higher than that of bacteria by a ratio of 10–20 in batch cultivation using centrate as a nutrient source. Furthermore, carbon content associated with microalgae increases during the entire course of the batch culture, whereas carbon contents associated with bacteria increases only in the first 2–3 days, followed by decreased period to below 10 mg/L. At equilibrium state, photosynthesis by microalgae is expected to produce an amount of oxygen which must be equal to the oxygen demand by bacteria to perform oxidation of organic matter, and that the dissolved oxygen concentration must be constantly remained. However, experimental data harvested by Acien et al. (2016) confirmed that the dissolved oxygen concentration in the cultivation system is a function of operating and environmental conditions. Moreover, laboratory measurements of photosynthetic/respiration demonstrated that the oxygen production rate (mainly by microalgae) (Eq. 21.6) is much

higher than heterotrophic oxygen requirement (Eq. 21.7) or the nitrification (Eq. 21.8) during daytime (Acien et al. 2016).

$$CO_2 + H_2O + NH_4^+ + PO_4^{3-} \rightarrow \text{Microalgae biomass} + O_2 \quad \text{(Photosynthesis)} \tag{21.6}$$

$$\text{COD} + O_2 + \text{nutrients} \rightarrow \text{Bacterial biomass} + CO_2 \quad \text{(Heterotrophic metabolism)} \tag{21.7}$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad \text{(Nitrification)} \tag{21.8}$$

Therefore, it is recently stated that contribution of bacteria to the overall performance of microalgae-bacteria–based wastewater treatment is minor, and that the process is regulated by microalgae (Fig. 21.1). The bacterial population is regulated by organic matter originated from wastewater source, which are degraded by a low bacterial biomass concentration, making metabolism by microalgae become a rate-limiting step in consuming the degraded inorganic materials. The proportion of microalgae and bacteria population is varied depending on wastewater composition; for instance, initial wastewater rich in organic materials contains the high population of bacteria, but overall steady state is usually reached whatever the wastewater type used. In principal, it is indeed to understand these phenomena in order to adequately design and operate microalgae-bacteria–based wastewater treatment system.

Based upon current evident, the performance of microalgae-bacteria consortia-based wastewater treatment is fundamentally regulated by controlling growth conditions to be adequate for microalgae. In this system, bacteria digest the organic matter very rapidly and decompose complex organic matter to produce CO_2, NH_4^+, and PO_4^{3-}, which

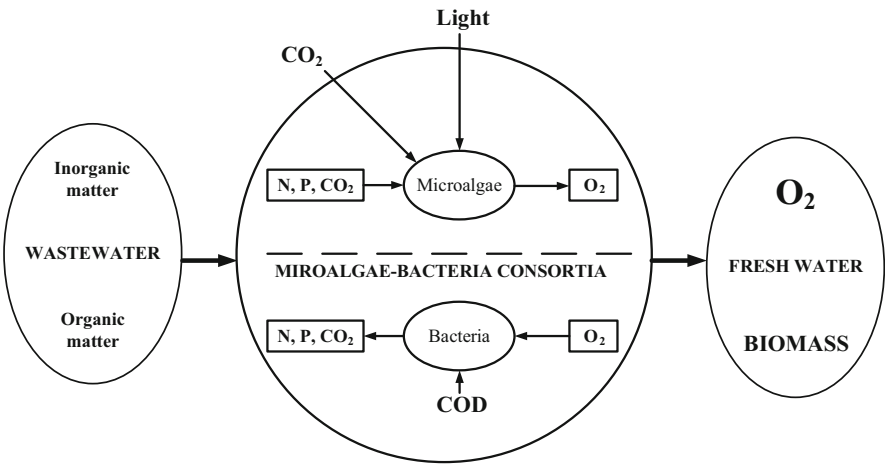

Fig. 21.1 Mechanism of microalgae-bacteria–based wastewater treatment with microalgae metabolism in a rate-limiting step

then must be assimilated by microalgae. In case algae-cultivating conditions are not appropriate for microalgae, the CO_2 is releasing to the air instead of being consumed or stored in the water as bicarbonate buffer. Moreover, NH_4^+ could be oxidized to NO_3^- and then denitrified to N_2 (denitrification), or directly stripped to the air as NH_3 when pH is raising to over 9.0. In addition, PO_4^{3-} can be precipitated under alkaline conditions as calcium salt. Therefore, to maximize the assimilation of organic compounds, microalgae culture conditions have to be optimized, leading to optimal microalgal productivity and thus optimal nutrients-removal capacity of the microalgae-bacteria–based wastewater treatment system. Overall, reactor design and system operation has to be adequate for microalgae.

5 Design Parameters for Open Ponds and Photobioreactors

5.1 Design Considerations

5.1.1 Irradiance

Light energy is the most important factor stimulating the productivity of photosynthetic microalgae; hence, supplying of irradiance is a key design challenge for efficient photobioreactors (PBRs). Photosynthetic efficiency of microalgae is influenced by the source of light, spectral quality, and light intensity. The photosynthesis rate (P_{O2}) is a function of the irradiance (I) to which the cells are exposed. Irradiance is the total amount of radiation reaching a point from all direction of every wavelength in space; however, photosynthetic microalgae can only utilize light spectrum of 400–700 nm range, known as photosynthetically-active radiation (PAR). Photosynthesis is attained saturation at irradiances from 100 to 500 μE/m^2·s (Vejrazka et al. 2011, 2012, 2013). Higher light intensity (e.g., 10,000 μE/m^2·s) could cause photo-oxidation or photo-inhibition in most microalgal strains, but strongly depended on specific strain. Therefore, the effect of light on the photosynthesis rate of any particular strain had to be studied in each case. Models describing the correlation of photosynthesis rate and irradiance, including photo-inhibition consideration, have been empirically reported elsewhere (Zonneveld 1998; Posten 2009; Yoshimoto et al. 2005; Rubio et al. 2003; Brindley et al. 2011). Whatever simulation of the cell behavior under any light regime supplied for algae culture, full light integration and local light use are popularly considered. In reality, the irradiance inside the culture varies as a function of incident radiation on the culture surface, position, and light attenuation by the biomass. As a result, algal cells are usually exposed to different irradiances and so different photosynthesis rates. Additionally, microalgal cells frequently move according to fluid dynamics of algae culture inside the reactor; therefore, the overall synthesis rate will be a function of the average irradiance (Grima et al. 1994) to which the cells are exposed. The average irradiance concept is developed to estimate net light availability inside dense cultures from which a net photosynthesis rate taking place that is more

reliable. In summary, photosynthesis rate is regulated by the light regime at which cells are exposed, but in large photobioreactors operated under similar fluid dynamic conditions, the algae growth rate can be modeled as a function of average irradiance inside the reactors (Takache et al. 2010, 2012; Cornet and Dussap 2009). Dark zones presented inside reactors are popularly existence, in which cells are performing respiration instead of photosynthesis, leading to reduction of the algae yield. Therefore, reducing the volume of dark zones (improving the light regime) to reach light integration model is preferred. This is heavily relevant to the design of photobioreactors, because a photobioreactor basically contains an illuminated outer zone and a darker core. The microalgal cells are unavoidably subjected to fluctuating illumination according to the movement of fluid between the illuminated zone and dark interior; fortunately, the higher the frequency of movement the higher the yield of the culture (Brindley et al. 2011).

5.1.2 Mass Transfer and Hydrodynamics

Mass transfer is the most critical aspect in designing photobioreactor for microalgae cultivation. The photosynthetic process of microalgae is simultaneously accompanied by the production of oxygen and the uptake of carbon dioxide, resulting in constant alternation of culture medium and the pH. Photosynthesis of many microalgae can be inhibited by oxygen levels of above air saturation (e.g., 7.2 mg O_2/L), whereas severe photo-oxidation may occur when elevated levels of oxygen combined with high levels of irradiance, reducing the yield of algae culture. Thus, avoiding oxygen build-up to inhibitory levels via establishing combinations of mass transfer capacity and photosynthesis rate need to be taken into consideration in designing photobioreactors for scale-up production of microalgae (Posten 2009; Rubio et al. 1999). The volumetric mass transfer coefficient k_La is the most critical parameter for assessing the performance of photobioreactor to obtain optimum microalgal cell growth. Mass transfer inside PBRs involves three phase mass transfer system: gas (CO_2)-liquid (culture medium)-solid (microalgal cells). The volumetric mass transfer coefficient (k_La) is the product of mass transfer coefficient (k_L) and the interfacial area per unit volume of aerated reactor. Therefore, it is affected mainly by superficial gas velocity, sparer type, agitation rate, temperature, etc. (Ugwu et al. 2008; Chen et al. 2011; Kumar et al. 2011). Mass transfer from gas phase to liquid phase can be expressed by the following equation.

$$\frac{\mathrm{d}C}{\mathrm{d}t} = k_La(C^* - C) \tag{21.9}$$

where $\frac{\mathrm{d}C}{\mathrm{d}t}$ is the mass transfer rate (mol/m^3·s), k_L is the mass transfer coefficient (1/m^2·s), a is the interfacial area (m^2), C^* is the equilibrium gas concentration at the interface of the gas and liquid (mol/m^3), and C is the gas concentration in the liquid (mol/m^3).

k_La is used to describe the overall volumetric mass transfer coefficient in photobioreactor. The k_La increases linearly with increase in superficial gas velocity up to a certain limit after which this trend starts to decline due to coalescence of bubbles that changes the interfacial area per unit volume of gas. Mass transfer is greater in bubble column and flat panel reactors which are pneumatically agitated than the horizontal tubular reactor which has plug flow kind of system and results in oxygen build-up.

Desorption rate of oxygen Q_{O_2} of photobioreactors can be calculated as:

$$Q_{O_2} = k_L a_{O_2} \left(C_{O_2} - C^*_{O_2} \right) V_{mt,O_2} \tag{21.10}$$

$$C^*_{O_2} = H_{O_2} P_T y_{O_2} \tag{21.11}$$

where V_{mt,O_2} is the volume of the mass transfer unit (m^3); $k_La_{O_2}$ is volumetric mass transfer coefficient of oxygen (1/s); $C^*_{O_2}$ is the equilibrium O_2 concentration at the interface of the gas and liquid (mol/m^3), and C is the O_2 concentration in the liquid (mol/m^3); H_{O_2} is Henry's constant for diluted gases under ideal conditions ($mol/m^3 \cdot Pa$); P_T is the total pressure (Pa); and y_{O_2} is the molar fraction of O_2 in the gas phase.

Assuming that photosynthesis takes place in the entire reactor volume V then the required mass transfer coefficient which could avoid excessive dissolved oxygen accumulation can be calculated as (Eq. 21.12):

$$k_L a_{O_2} = \frac{P_{O_2} V}{\left(C_{O_2} - C^*_{O_2} \right) V_{mt,O_2}} \tag{21.12}$$

In contrast to O_2, microalgae require CO_2 as the carbon source; hence, this must be supplied at reasonable concentration to avoid limiting their growth. Doucha et al. (2005) reported that the concentration of CO_2 should be higher than 3.3 mg CO_2/L (0.076 mol/m^3), which is corresponding to the convertibly equivalent to partial CO_2 pressure of 0.2 kPa. However, the partial pressure of CO_2 in the atmosphere is only 0.04 kPa, indicating that pure air is not sufficient for CO_2 supply and that an enriched gas mixture with higher fraction of CO_2 is required. To support product formation or microalgae cultured under high light intensities, higher concentration of CO_2 may be necessary (Yoo et al. 2010). Supplying of pure CO_2 can constitute up to 30% of the overall microalgae production cost (Acien et al. 2012); hence, flue gases should be explored for algae culture.

Similar to oxygen, the overall mass transfer coefficient value required to supply enough CO_2 when photosynthesis is taking place in the entire reactor volume V can be calculated as (Eqs. 21.13, 21.14 and 21.15):

$$Q_{CO_2} = k_L a_{CO_2} \left(C_{CO_2} - C^*_{CO_2} \right) V_{mt,CO_2} \tag{21.13}$$

$$C^*_{CO_2} = H_{CO_2} P_T y_{CO_2} \tag{21.14}$$

$$k_L a_{CO_2} = \frac{P_{CO_2} V}{\left(C_{CO_2} - C^*_{CO_2}\right) V_{mt,CO_2}} = \frac{-P_{O_2} V}{\left(C_{CO_2} - C^*_{CO_2}\right) V_{mt,CO_2}} \tag{21.15}$$

where Q_{CO_2} is the desorption rate of CO_2 (m^3), $V_{mt,\ CO_2}$ is the volume of the mass transfer unit (m^3); $k_L a_{CO_2}$ is the volumetric mass transfer coefficient of CO_2 (1/s); $C^*_{CO_2}$ is the equilibrium CO_{22} concentration at the interface of the gas and liquid (mol/m^3), and C is the CO_2 concentration in the liquid (mol/m^3); H_{CO_2} is Henry's constant for diluted gases under ideal conditions ($mol/m^3 \cdot Pa$); P_T is the total pressure (Pa); and y_{CO_2} is the molar fraction of CO_2 in the gas phase.

Combination of Eqs. 21.12 and 21.15 yields Eq. 21.16 which expresses a relationship between mass transfer coefficient and overall volumetric mass transfer coefficient for oxygen and carbon dioxide to avoid excessive oxygen accumulation or carbon dioxide limitation.

$$\frac{k_L a_{O_2}}{k_L a_{CO_2}} = -\frac{\left(C_{CO_2} - C^*_{CO_2}\right) V_{mt,CO_2}}{\left(C_{O_2} - C^*_{O_2}\right) V_{mt,O_2}} \tag{21.16}$$

From Eq. (21.16), it is possible to calculate the predicted production of oxygen and uptake of carbon dioxide via the fundamental photosynthesis equation and prediction methodology proposed by Rubio et al. (1999). Thus, any photobioreactor design can be estimated for different sections on which it can be divided and optimized.

Hydrodynamics is generated by flow of liquid phase inside reactors via mechanical mixing or bubbling. Light penetration, mass transfer, mixing, and shear stress are strongly associated with the hydrodynamics of the algal cultivation system (Mirón et al. 1999). Mixing and gas hold-up are considered as important hydrodynamics parameters of PBRs and tightly dependent on flow regime of the reactor. Whereas, mixing time is defined as the time taken to achieve a homogeneous mixture after injection of tracer solution (Ugwu et al. 2008), a very important parameter in designing PBRs for improving mass transfer inside the reactors as well as enhancing CO_2 sequestration and avoiding O_2 hold-up. Poor mixing results in oxygen build-up leading to inhibitory to microalgal cells and cause biofouling etc., which are contrastive to enhanced results (improves mass transfer, reduces photo inhibition, increases biomass yield, etc.) achieved when good mixing is implemented (Qiang and Richmond 1996). Mixing and bubbling create shear effects, which increase growth rate of microalgae to a certain level (optimum level of turbulence), but growth of microalgae may decrease with any further increase of superficial gas velocity that is attributed by cell damage due to bubble rapture at the cell surface (Silva et al. 1987).

Gas hold-up can be calculated as:

$$\varepsilon = \frac{A_G}{A} \tag{21.17}$$

where A_G and A are the actual or true cross-sectional area (m^2) for gas flow and the total cross section of the gas-liquid flow channel, respectively. The relationship between ε and volumetric mass transfer coefficient can be estimated for any PBRs as:

$$k_L a_L = \psi \frac{\varepsilon}{1-\varepsilon} \tag{21.18}$$

$$a_L = \frac{6\varepsilon}{d_B(1-\varepsilon)} \tag{21.19}$$

where k_L is the mass transfer coefficient (m/s), a_L is the interfacial area per unit liquid volume (1/m), ψ is a constant (1/s), ε is the overall gas hold up, and d_B is the mean bubble diameter (m). The hydrodynamics of flow in horizontal, vertical columns, and raceway ponds are very different. Horizontal tubular reactors or raceway ponds are almost free of gas or bubbles, whereas gas sparged airlift and bubble columns have greater has hold-up. Consequently, flow in vertical reactors is more turbulent and chaotic (Karemore et al. 2015).

5.2 *Heat Transfer*

Temperature is a very important factor that affects growth rate of microalgae. Optimal temperature range for microalgae is between 20 and 30 °C, and below 20 °C, the growth rate decreases, whereas above this range, algal cells damage can be occurred (Carvalho and Malcata 2003; Sandnes et al. 2005, Sanchez et al. 2008). Although microalgae can be classified as thermophilic (grows at high temperature) and mesophilic, marine microalgae are less tolerant to high temperatures (growth rate reducing at above 28 °C and causing death at above 30 °C), but freshwater microalgae have optimal temperatures in the range of 25–35 °C range and can be serviced at up to 40 °C (Acién Fernández et al. 2013).

During the daylight course, the average temperature culture is close to optimal; hence, temperature control only has a small effect, but when tempeature gets too low, particularly in night time or winter time, then controlling temperature for algae culture has a significant effect (Bosma et al. 2007). Heating culture can be applied by natural sunlight in the morning when algae cultivation takes place in countries having abundant solar. Several researchers reported that 60% of daily algal biomass productivity can be improved to 28% by heating the culture for 2 h in the morning (Vonshak et al. 2001). Photosynthetic process of microalgae and biochemical reactions taking place in microalgae cultures only cause heat loss to or from the environment by

convection, but do not modify the temperature of the culture. The rising of algae culture temperature is mainly achieved by heat absorption from solar radiation of the culture (outdoor cultivation). Heat balance is usually applied to estimate the power requirements for temperature control in microalgae cultivation system (Acién Fernández et al. 2013). In a laboratory scale, maintaining stable temperature for algae system is usually performed by equipping heat supply or heat removal devices which work at the same rate, but costly. Outdoor reactors should be designed to avoid temperature gets loo low or too high which may cause death of the cultures. Therefore, some types of cooling systems are used, which are water spray on the surface of reactor or internal heat exchangers inserted inside reactors columns to remove heat during summer days. In winter time, waste heat or raw energy can be used to heat algae cultivation system to sustain production in pilot plant (Quinn et al. 2012). However, large-scale performance of this technique is limited as the results of resource availability and the economics associated with raw energy.

5.3 *Factors Affecting Nutrient Removal and Algal Production*

Beside reactor design parameters which were discussed previously, other important factors that affect microalgae productivity, which should be considered, are nutrient supply, CO_2 supply, O_2 removal, and other environmental parameters including pH, temperature, salinity, etc.

In primary growth stage, nutrients supply is necessary for photosynthetic microalgae. Media usually consists of carbon source in the form of CO_2, HCO_3^-, mineral salts of nitrogen, phosphorous, calcium, magnesium, hydrogen, sulfur, etc., micro-elements (iron, boron, copper, cobalt, manganese, nickel, etc.), and water. Although biochemical composition of microalgal biomass is strongly dependent on species, general molecular formula of microalgae can be given as $CH_{1.83}O_{0.48}N_{0.11}P_{0.01}$ to which the requirement of nutrients can be determined (Grobbelaar 2007). Since carbon can be supplied from CO_2 and hydrogen and oxygen from water, the rate limiting nutrients are nitrogen and phosphorous. Molar ratio of N/P is a crucial factor for microalgae growth and the optimal ratio globally applied is 11:1 (N/P). While deficiency of any critical nutrients (N and P) may decrease growth rates, the biomass composition can be altered. For instance, the deficiency of nitrogen is documented to improve lipid content by accumulation of long-chain fatty acids. Utilization of different media with different composition can considerably change the quality of biomass. Nutrients and water are accounted to contribute up to 50% of the total biomass production cost; therefore, utilization of cheap nutrients source such as wastewater is a promising way to reduce algae production cost (Singh and Das 2014). The pH of culturing media plays one of the most important factors affecting nutrients removal. For instance, the carbon concentrating mechanisms adopted by microalgae considerably depend on the pH, as this parameter determines CO_2 solubility in the culture medium. On the other hand, high pH values result in striping of NH_4^+ and precipitation of PO_4^{3-} out of culture medium.

Open photobioreactors are always susceptible to grazing by herbivorous protozoa and zooplankton which suffer algal productivity quickly in a few days (Park et al. 2011b; Craggs et al. 2013; Carney and Lane 2014). Parasitism by fungi and infection by virus may also considerably reduce the algal pond population within few days and makes changes in algal cell structure, diversity, and succession (Kagami et al. 2007). Therefore, to ensure algal productivity, protection of algae culture from wild predators is very important (Wang and Seibert 2017; Rego et al. 2015).

5.4 Performance Parameters of Photobioreactors

The performance of various kinds of photobioreactors can be evaluated by determining the values of critical parameters as following.

5.4.1 Volumetric Productivity and Areal Productivity

Volumetric productivity of microalgal cultures denoted as P (g/L·d) can be defined as microalgal cell concentration per unit of reactor volume per unit time. It is expressed as:

$$\frac{P = C_f - C_i^{\,i}}{t} \tag{21.20}$$

where C_f and C_i are the final and initial concentration of biomass (dry weight, g/L) measured over period of time t (day) for batch mode. This parameter is used to evaluate performance of column photobioreactors.

Areal productivity denoted as A (g/m^2·d) can be defined as productivity of occupied-land area per unit of time. It is calculated by following equation:

$$\frac{P = C_f - C_i^{\,i}}{S \cdot t \times V} \tag{21.21}$$

where C_f and C_i are the final and initial concentration of biomass (dry weight, g/L) measured over period of time t (day) for batch mode; S is the occupied-land area of photobioreactor; V is the working volume of photobioreactor (L).

5.4.2 Carbon Dioxide Fixation Rate

Carbon dioxide fixation rate can be calculated by the following equation:

$$F = a \cdot P \quad (\mathrm{g/L \cdot d}) \tag{21.22}$$

where a is the amount of carbon dioxide fixed by unit weight of biomass (considered 50% of carbon in the biomass, thus

$$a = 0.5 \times \frac{44}{12} = 1.833 \ \ (\mathrm{g/g\ DCW})$$

5.4.3 Photosynthetic Efficiency (PE)

Photosynthetic efficiency is defined as energy stored in biomass per unit of light energy supplied

$$\mathrm{PE} = Y \frac{\Delta H}{N \cdot hv} \ \ (\%) \tag{21.23}$$

where ΔH is the specific energy content of the biomass; N is the Avogadro number; and hv is the mean photon energy of photosynthetic active radiation (PAR) according to the Planck's law. Y is defined as the ratio between the biomass production rate and the irradiance absorbed by the culture and is expressed as dry cell weight per mole of photons.

$$Y = \frac{\mu \cdot V}{I_0 A a} \tag{21.24}$$

where μ is the specific growth rate (1/day), V is the culture volume (m^3), A is the irradiated surface (m^2), a is the specific biomass absorption coefficient (expressed as m^2/g), and I_0 average light intensity ($\mu E/m^2 \cdot s$).

5.4.4 Hydraulic Retention Time (HRT)

In continuous or semicontinuous cultivation mode, wastewater is retained in the cultivation system for a specific period to reach adequate nutrients removal efficiency. This term is defined as hydraulic retention time (HRT), which is calculated by the following equation (Eq. 21.25):

$$\mathrm{HRT} = \frac{V}{Q} \tag{21.25}$$

where HRT is the hydraulic retention time (day), V is the working volume of cultivation system (m^3), and Q is the influent flowrate (m^3/day).

HRT is a key design parameter for achieving a proper removal efficiency of contaminants from wastewaters. Additionally, HRT is strongly associated with energy consumption of the algae cultivation system. For example, with the system

operated at HRT of over 7 days, the corresponding energy consumption is lower than 2 kWh/m^3, whereas the system with high energy consumption of over 5 kWh/m^3 are obliged to operate at HRT of lower than 4 days. The best scenario ever reported is the system operated at low-depth (<0.1 m) with HRT of 3–5 days, resulting in moderate energy consumption of lower than 2.0 kWh/m^3 (Acien et al. 2016).

5.5 *Different Open Reactor and Closed Reactor Configurations for Microalgae Cultivation in Wastewater*

Cultivation of microalgae in wastewaters can be conducted in either suspended or immobilized-cells cultures. Based on their reactor design, algae cultivation system can be classified as open systems, closed systems, and hybrid system, all of which present how microalgae are exposed to the surrounding environment. To reach effective light penetration, photobioreactors should be designed to have an as high surface-to-volume (S/V) ratio as possible (Posten 2009). This will enhance the photosynthesis efficiency, and that results in high product and biomass productivity of microalgae (Jorquera et al. 2010). Several PBR designs have been developed with different S/V ratio for cultivation of microalgae in wastewater, which are reported in Table 21.2. As shown in Table 21.2, cylindrical, flat plate, or tubular types are popularly adopted designs to maximize light capture. Although solar energy is abundantly available, current light utilization efficiencies are only restricted to 5–6% (Jorquera et al. 2010). Various configurations of PBRs come in various shapes and sizes as depicted in Fig. 21.2. Tubular reactors are either horizontal or vertical columns (cylindrical in shape) which can be divided into bubble column or air-lift reactors for enhanced mixing and mass transfer characteristics (Fig. 21.2a). Cuboidal shapes are flat panel reactors which have large lengths and heights, but narrow width to attain greater light penetration (Fig. 21.2b). Based upon their orientation to sunlight, some researchers also categorize them into vertical or inclined reactors (Molina Grima et al. 1999). Raceways are open to photobioreactors which are configurated with channels, through which the culture is recirculated using paddle wheels (Ugwu et al. 2008; Karemore et al. 2015).

Table 21.2 Surface area-to-volume ratios of various reactor configurations

Reactor design	Illuminated surface area	Volume	Surface area/volume (S/V)
Tubular	πrh (single sided lighting)	$1/3\pi r^2h$	$3/r$
	$2\pi rh$ (dual-sided lighting)	$2/3\pi r^2h$	$6/r$
	$2\pi r_1h_1$* (dual-sided lighting)	$1/3\pi r_2h - 1/3\pi r_1^2h_1$	$(6r_1h_1)/(r_2h\text{-}r_1h_1)$
Flat panel	lh (single sided lighting)	lhw	$1/w$
	$2lh$ (dual sided lighting)		$2/w$
Thin layer	$2lw$	$2lwh$	$1/h$

*Annular lighting is light inserted inside the reactor. r_1 and h_1 are dimension of lighting system

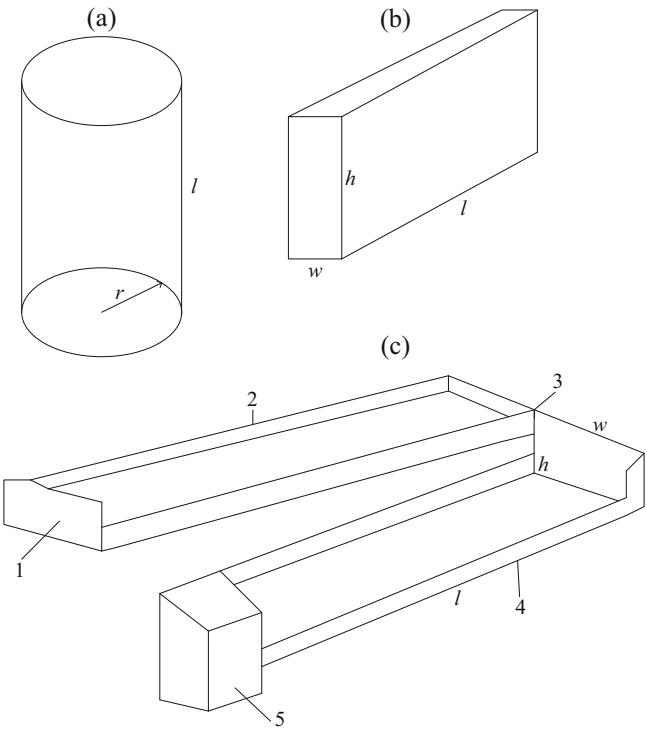

Fig. 21.2 Configuration of several photobioreactor designs: Tubular (**a**), flat panel (**b**), and thin layer (**c**); *h*, height (in thin-layer photobioreactor, *h* is algae culture layer thickness); *l*, length; *r*, radius; *w*, width of reactor. In thin-layer photobioreactor, 1 is inlet module, 2 is upper channel, 3 is flow reversal module, 4 is lower channel, and 5 is retention tank

5.5.1 Open Photobioreactors

Traditionally, raceway pond is a typical open system which has been developed and widely used since 1950s for microalgae production (Ravikumar 2014). The main features of raceway pond are about 30 cm depth and algae culture is mixed and circulated around the raceway track by paddlewheels to ensure all microalgal cells exposed to sufficient sunlight, making photosynthesis process occurred. As the system is cheap in construction and operation, commercial-scale cultivation of microalgae mostly employs open ponds (Habibi et al. 2018; Romero Villegas et al. 2017). The raceway ponds can be installed on non-arable lands near power plants and wastewater treatment plants, helping the system to access to carbon dioxide from flue gas and nutrient supplies. Although utilization of raceway ponds is cost effective, the open systems exposed to surrounding environment get highly contaminated; water is evaporated (particularly in summer time), which leads to suffer culture and reduce productivity. Furthermore, they have low biomass productivity

(0.1–0.5 g/l) when compared to closed photobioreactors (Pulz 2001). For wastewater treatment, raceway ponds or high rate algae ponds (HRAPs) are the first choice for construction. These reactors are operated at culture depths of 20–40 cm (S/V = 5–2.5 (1/m)) and hydraulic retention times of 7–10 days with low energy consumption of 1 W/m^2. Under these operational conditions, irradiance exposed to algal cells is low, resulting in the growth of microalgae, and productivity is low. As previously mentioned, the key to higher yields and lower production costs are largely relied on a low volume culture by decreasing algal layer exposed to light to as low value as technically possible. Thin-layer photobioreactors may appropriately compromise this task since the reactors have higher the surface-to-volume ratio (S/V) (Fig. 21.2c, Table 21.2). The thin-layer photobioreactor was first developed in 1963 and used widely for commercial production of microalgae (Jerez et al. 2016; Přibyl et al. 2015; Doucha and Lívanský 2009; Apel et al. 2017). The main feature of the technology contains a well-mixed microalgal suspension, which follows continuously in a very thin layer (6–8 mm) on inclined lanes, constructed by transparent material and arranged in opposite direction (Lívanský and Doucha 1996; Doucha and Lívanský 2006). The thin-layer photobioreactors are operated at a culture depth from 0.5 to 5 cm (S/V = 200–20 (1/m)) and hydraulic retention time of 3–5 days, with average energy consumption of 10 W/m^2. This special design results making light availability for algal cells are high, resulting in very high microalgae density of up to 40–50 g/L corresponding to areal density of 240–300 g/m^2 (Doucha and Lívanský 2009). For wastewater treatment, biomass productivity of *Scenedesmus* sp. reached up to 42 g/m^2·day (Morales-Amaral et al. 2015a). Moreover, it was reported that nitrogen and phosphorous removal capacities up to 38 mg N/L·day and 3.9 mg P/L·d were obtained on a thin-layer reactor, whereas in the raceway reactor, values achieved below 20 mg N/L·d and 0.4 mg P/L·d (Morales-Amaral et al. 2015a). These value are similar to those previously reported of 47.5 mg N/L·d and 3.8 mg P/L·d using *Muriellopsis* sp., and 27.5 mg N/L·d and 2.7 mg P/L·d using *P. subcapitata* (Morales-Amaral et al. 2015b) or 35 mg N/L·d and 5.7 mg P/L·d. for *N. gaditana* (Sepúlveda et al. 2015). A maximal removal rate of nitrogen from wastewater was reported for *Chlorella* of 8.5 mg N/L·d (Masojídek et al. 2011).

Recently, algal-film photobioreactors, which are regarded as an open system, have been emerging as a potential technology for wastewater treatment using microalgae-bacteria consortia for biomass production (Choudhary et al. 2017; Gao et al. 2015; Kesaano and Sims 2014; Hoh et al. 2016; Genin et al. 2014, 2015; Schnurr et al. 2013). Algae-attached cultures have been developed as commercial name of Algadisk, Rotating Algal Biofilm Reactor (RABR), Rotating Algal Biofilm (RAB), and Algal "disk" with productivity estimated for bench to pilot-scale operation ranging from 0.6 to 50 g/m^2.d on different wastewater sources (municipal and animal wastewater) (Kesaano and Sims 2014). Despite a large potential, the application of algal biofilm-based photobioreactors in wastewater treatment is still limited by the lack of knowledge on system performance, sustainability, reliability, and techno-economic as well as life cycle analysis.

5.5.2 Closed Photobioreactors

Closed photobioreactors typically including flat plate reactors, tubular photobioreactors, and bag system were developed to overcome drawbacks that are associated with open systems, avoiding water evaporation and contamination and increasing photosynthesis efficiency (Acién et al. 2017; Gupta et al. 2015; Borowitzka 1999). Among these design, plate and tubular photobioreactos are well designed to maximize light harvesting (by changing the arrangement of the reactor tubes or panels oriented to the sun) and gas exchange. Despite high biomass productivity, installation and maintenance costs of these reactors are much higher than open ponds, limiting large-scale commercialization capability of the closed systems. The bag photobioreactors use large plastic bags having 0.5 m in diameter coupled with aeration system. This system poses difficulty in maintenance and lack of mixing algae. Closed reactors are suitable for microalgae production applied as high value product for pharmaceutical and food industries.

5.5.3 Hybrid Photobioreactors

Hybrid photobioreactos are developed by the combination of merit from open and closed culturing systems for two-stage cultivation of microalgae (Cai et al. 2013). Principally, the algae inoculum is usually grown in closed photobioreactors to prevent contamination and obtain a healthy feed, which is then used as seeding culture for open ponds. Despite several advantages of these system, large-scale applications is still limited by the cost of the first stage.

5.5.4 Further Analysis of Photobioreactor Performance

The critical parameters governing the adequate design and performance of photobioreactors are first their capacity to satisfy the microalgae to perform biological and biochemical tasks (e.g., photosynthesis, biomass growth, nutrients removal), and second energy requirements to operate the system to reach a designed efficiency. Although in-depth analysis of photobioreactor design has been described (Posten 2009; Ugwu et al. 2008; Karemore et al. 2015; Acién Fernández et al. 2013; Molina Grima et al. 1999; Pulz 2001), there are other opportunities to improve performance of open raceway (Mendoza et al. 2013; Fernández et al. 2016) and tubular phorobioreactors (Gómez-Pérez et al. 2015, 2017) such as optimization of the fluid dynamics to minimize power consumption. To enhance photosynthetic efficiency, the utilization of low-depth culture system is strongly recommended. Moreover, in terms of biological and biochemical aspects, the microalgal consortium and wastewater composition should be characterized to allow the industrial development of microalgae-based wastewater treatment technology.

6 Conclusion

Microalgae consortia-based wastewater treatment process is centered on microalgal metabolism in which inorganic matters degraded by bacteria are assimilated by microalgae. Therefore, system design of photobioreactors should be adequately determined to satisfy microalgal cells for optimal biomass productivity and nutrients removal efficiency. Reactors operated at as high S/V ratio as possible is strongly recommended to meet the task. In terms of biochemical aspect, further research of consortia ecology and the microorganism interactions (microalgal-microalgal, microalgae-bacteria, microalgae-yeasts, and microalgae-fungi) are indeed needed to develop new consortium for industrial use. Furthermore, engineering, system design, as well as algae cultivation conditions should be optimized to achieve high biomass growth (corresponding to high nutrients removal efficiency) and minimized energy consumption and land requirement.

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Chapter 22
Developing Designer Microalgal Consortia: A Suitable Approach to Sustainable Wastewater Treatment

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Abstract Nowadays, large amounts of improperly treated wastes have been discharged into water bodies, resulting in the reduction of water quality and the damage of aquatic ecosystems. One of the most severe issues is eutrophication phenomenon due to the excessive emission of nutrients such as nitrogen and phosphorus. However, most traditional approaches used for nutrient removal have complicated processes, high operation cost, and intensive energy demand. Alternatively, microalgal can provide a potential solution to the problems mentioned above. Microalgal-based technologies are low-cost and sustainable and recycle nutrients into biomass which would be converted to valuable goods. Since the nutrients such as nitrogen and phosphorus in wastewaters are indispensable for microalgal growth, microalgal exhibit superior nutrient removal to other microorganisms. Nevertheless, it is difficult to maintain axenic cultures of microalgal during wastewater treatment processes. Therefore natural and artificial consortia including microalgal consortia or microalgal-bacterial consortia have been utilized in several studies. The application of these consortia in wastewater remediation has many advantages; for example, synergistic relationship between the microorganisms in the consortia can enhance nutrient uptake and resistance to adverse conditions. This chapter reviews wastewater characteristics as nutrient sources for microalgal, formation and construction of microalgal consortia, factors influencing nutrient removal and biomass generation by consortia, the progress of treatment of various wastewaters (including municipal, industrial, and agricultural wastewater), and mechanisms involved in nutrient removal by consortia. Finally, the challenges of microalgal consortia research in bioremediation of wastewaters are also addressed.

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1 Introduction

Large volumes of wastewaters have been generated in the last decades, mainly due to agricultural, municipal, and industrial activities. These wastewaters can lead to serious pollution issues if they are discharged into water bodies without adequate treatment. One of the major issues is that excessive nutrients in wastewaters, mainly nitrogen and phosphorus, may result in eutrophication in surface water such as rivers and lakes. Eutrophication impairs water quality, degrades aquatic ecosystems, and imposes a public health risk on humans (Dodds et al. 2009; Gonçalves et al. 2017). An important demand of wastewater treatment is to reduce the amounts of nutrients to allowable limits before discharge or reuse. Nevertheless, traditional biological treatment technologies have some technical or economic shortcomings involving poor nutrient removal or high-energy consuming (Quijano et al. 2017). For example, the most commonly used anaerobic-anoxic-oxic (A^2O) process contains multistage anaerobic, anoxic, and aerobic reactors with internal recirculation and return activated sludge stream, thus increasing process cost, sophistication, and energy input (Gonçalves et al. 2017).

Microalgal are capable of growing in the wastewater using inorganic nitrogen and phosphorus to produce biomass, whereas bacteria remove nitrogen through anaerobic ammonia oxidation, nitrification, and denitrification and remove phosphorus via biological uptake. Thus wastewater treatment by means of microalgal consortia (microalgal and microalgal-bacterial) has been proposed since 1950s (Oswald et al. 1957). The pioneering process was designed by Oswald named "high rate algal ponds" (HRAPs), which was originally used to improve organics and nutrient removal in municipal wastewater (Oswald 1973). Subsequently, many investigations demonstrated that microalgal-mediated wastewater treatment process could concomitantly produce biofuels as well as high value-added products (Quijano et al. 2017). Accordingly, microalgal can be used to play a dual role in the wastewater treatment and biomass production for diverse applications. Microalgal wastewater treatment presents several advantages over conventional wastewater treatment (Abinandan and Shanthakumar 2015; Gonçalves et al. 2017): (1) O_2 produced during microalgal photosynthesis can reduce energy demand for mechanical aeration; (2) microalgal and bacteria can create a synergy to make the process more efficient; (3) nutrients assimilated by microalgal can be reused; (4) harvested biomass can be used for the generation of biofuels, fertilizers, animal feed, or pharmaceuticals; and (5) oxygenated effluents can be discharged into water bodies. Figure 22.1 provides a general scheme of wastewater treatment and resource recycling using microalgal-bacterial consortia systems.

In the following sections, a comprehensive view of wastewater characteristics as nutrient sources, microalgal consortia formation and construction, factors influencing nutrient removal and biomass generation by consortia, and applications of microalgal consortia for various wastewater treatments is given. Mechanisms involved in nutrient removal by consortia in wastewater bioremediation and major challenges in this process are also addressed. This chapter provides the foundation and motivation for further developing microalgal consortia processes for sustainable wastewater treatment.

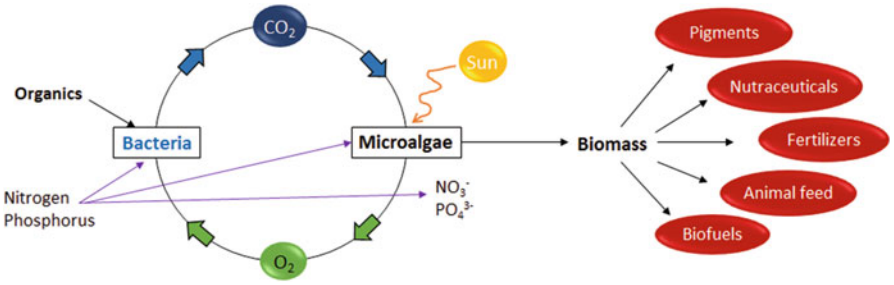

Fig. 22.1 Wastewater treatment by microalgal-bacterial systems and resource recovery. (Adapted from Quijano et al. (2017))

2 Wastewater Characteristics as Nutrient Source

Although many kinds of wastewaters contain carbon, nitrogen, and phosphorus which can be served as main nutrient sources needed for microalgal growth, the treatment performance differs significantly owing to different nutrient compositions. Generally, wastewaters mainly contain oxygen-demanding compounds, organic pollutants, inorganic matters, pathogenic organisms, and sediments (Sonune and Ghate 2004). Table 22.1 exhibits the concentration ranges of nitrogen, phosphorus, and organic carbon from various wastewaters, indicating that the compositions of wastewaters strongly depend on sources. Ammonium is the most common chemical form of nitrogen in the wastewater which can be easily utilized by most algal species and strains. Phosphorus in the wastewater usually exists in the form of inorganic anions such as $H_2PO_4^-$ and HPO_4^{2-}. In this sense, wastewaters or effluents can be used as a cheap source of nutrients for microalgal cultivation.

2.1 Municipal Wastewater

Municipal wastewaters are a mixture consisted of a mass of domestic wastewaters (80–95%) and a small quantity of industrial influents (5–20%) (Guldhe et al. 2017). Moreover, the compositions of municipal wastewaters vary from one location to another, largely dependent on local activities. The representative compositions of municipal wastewater contain organic materials, nutrients such as nitrogen and phosphorus, inorganic compounds, metal ions, and pathogenic microorganisms (Henze and Comeau 2008). Most municipal wastewaters include many nutrients like ammonia, phosphate, and other basic metal ions for microalgal growth. However, they may also contain a series of heavy metal ions including arsenic, cadmium, chromium, copper, lead, etc. Some of these metal ions are micronutrient elements which are necessary for microalgal growth, whereas others are not essential and may be toxic for algal growth when their amounts reach certain levels (Guldhe et al. 2017). Table 22.2 shows the typical characteristics of municipal wastewaters. Totally, municipal wastewater contains less nitrogen and phosphorus compared with agricultural wastewater.

Table 22.1 The major compositions of different types of wastewaters

Wastewater category	Source	Nitrogen (mg L^{-1})	Phosphorus (mg L^{-1})	Carbon (mg L^{-1})
Municipal wastewater	Domestic sewage	25–66 NH_3-N	7–12 PO_4-P	400–500 COD
	Landfill leachate	112–192 NH_3-N	7–9 PO_4-P	3725–4861 COD
	Sewage	1 NO_3-N 23–219 NH_3-N	1–12 PO_4-P	183–380 COD
Agricultural wastewater	Dairy	< 1 NO_3-N 120–350 NH_3-N	35–350 TP	2000–20,213 COD
	Anaerobically digested dairy manure	< 1 NO_3-N 1279–1961 NH_3-N	240 TP	4855–4945 COD
	Piggery	324–656 NH_3-N	117 PO_4-P	1247 TOC
	Anaerobically digested piggery manure	303–495 NH_3-N	n.s.	n.s.
	Potato-processing effluent	54 NO_3-N 12 NH_3-N	48 PO_4-P	745 COD
	Rice effluent	25–95 TN	12–94 PO_4-P	2578–6480 COD
Industrial wastewater	Brewery	2–11 NO_3-N 3–106 NH_3-N	57–326 TP	565–7837 COD
	Carpet mill	0–28 NO_3-N 18–26 NH_3-N	20–35 PO_4-P	1412 COD
	Oil, metal, and chemical	1.9 NO_3-N 1.1 NH_3-N	n.s.	1200 COD
	Starch	49–115 NH_3-N	50–385 TP	2470–15,440 COD
	Wood-based pulp and paper	n.s.	n.s.	1248 COD

Source: Gonçalves et al. (2017)

2.2 *Agricultural Wastewater*

Generally speaking, agriculture is the largest water user as well as a major water polluter. Agricultural wastewater mainly includes animal manure, crop stalks, hulls and leaves, etc. (Guldhe et al. 2017). As the farm scale shifted from small to large, the wastes generated from animal feeding including livestock and poultry have become a primary point source pollution, owing to their spatially intensive organic

Table 22.2 Typical characteristics of municipal wastewaters

Parameters	Values
COD	300–900 mg L^{-1}
BOD	140–350 mg L^{-1}
Total N	30–100 mg L^{-1}
NH_3-N	20–75 mg L^{-1}
NO_3-N + NO_2-N	0.1–0.5 mg L^{-1}
Organic N	10–25 mg L^{-1}
Total Kjeldahl N	30–100 mg L^{-1}
Total P	6–25 mg L^{-1}
Ortho-P	4–15 mg L^{-1}
Organic P	2–10 mg L^{-1}
TSS	25–600 mg L^{-1}
VSS	200–480 mg L^{-1}
pH	7–8
Alkalinity	1–7 Eqv m^{-3}
Aluminum (Al)	350–1000 μg L^{-1}
Cadmium (Cd)	1–4 μg L^{-1}
Chromium (Cr)	10–25 μg L^{-1}
Copper (Cu)	30–70 μg L^{-1}
Lead (Pb)	25–80 μg L^{-1}
Mercury (Hg)	1–3 μg L^{-1}

Source: (Guldhe et al. 2017)

matter and nutrients. Animal wastewater is often rich in nutrients such as nitrogen and phosphorus (Table 22.1). Usually, ammonium occupies almost half of the total nitrogen in animal wastewater, while organic nitrogen accounts for the other. In spite of this, the concentrations and forms of nutrients in agricultural wastewater are still dependent on wastewater sources. Many factors such as animal diet, age, productivity, management, and site would strongly influence the nutrient content in animal wastes (Cai et al. 2013). It is noted that the N/P ratio is usually 2–8 for dairy, piggery, and cattle farm wastewater (Cai et al. 2013). In addition, agricultural wastewater may also contain antibiotics, herbicides, fungicides, and insecticides, which would potentially inhibit microalgal growth.

2.3 Industrial Wastewater

Different industries or sectors may produce different pollutants, which generate various types of industrial wastewater. Generally, industrial wastewater varies depending on the source activities more significantly than agricultural wastewater (Chiu et al. 2015). Industrial wastewater from diverse sources may contain high levels of easily biodegradable and refractory organics, inorganic compounds, and even potential inhibitors (Table 22.3). Moreover, most industrial wastewaters

Table 22.3 Wastewater characteristics for different types of industrial wastewater

Industrial wastewater streams	COD (mg L^{-1})	BOD (mg L^{-1})	TOC	NH_3-N (mg L^{-1})	Total N (mg L^{-1})	Total P (mg L^{-1})	TSS (mg L^{-1})	pH
Steel industry	150–6000	–	–	10–300	7–8	–	2500–500	7–8
Pharmaceutical and chemical industry	240–35,600	800–114,000	–	< 0.2	5.25–7500	8.9–4200	0.06–1679	<1–6.7
Textile industry	728–6000	250–700	90.6–108	4.8–100	21–57	0.83–120	16–46	7.8–10.4
Mining industry	–	–	27.3	–	0.2	0.23	–	4.2
Olive mill	–	–	26,460–32,680	–	410–1020	30–1000	41,900–54,760	4.9–5.1
Food processing industry	440–3500	132–16,000	–	–	5.7–9.5	4–57.3	2.43–13,300	6.35–7
Tannery industry	16,000	5000	–	450	273	21	–	–
Carpet industry	106–1412	2–487	–	0.57–25.85	–	3.47–13.83	4–268	6.5–8.0
Paper pulp industry	1120–1160	200–210	–	–	–	–	80–90	5.5–6.8
Milk and dairy industry	10251.2	4840.6	–	–	663	153.6	5802.6	8.34
Sugar industry	1360–2000	300–2200	–	–	–	–	220–790	6.5–8.8

Source: Guldhe et al. (2017)

contain many heavy metals and very few nutrients (N and P) (Ahluwalia and Goyal 2007). Therefore, it is considered that industrial wastewaters are unsuitable for microalgal growth due to their unbalanced nutrient constituents and toxic contaminants. However, there are still several reports demonstrating the potential of microalgal cultivation in industrial wastewaters for nutrients and heavy metal removal as well as biomass production, such as carpet mill effluent (Chinnasamy et al. 2010). Additionally, it is worthy to mention that food wastewater, as another kind of industrial wastewater, has been reported to cultivate microalgal effectively (Ji et al. 2015). Normally, this kind of wastewater such as molasses wastewater and olive oil wastewater is rich in nutrients and organic carbon.

In summary, in order to exploit microalgal more effectively in wastewater treatment, the characteristics of the wastewater should be evaluated before treated. N/P ratio of the wastewater, as one of the most important indicators, largely affects microalgal biomass production and nutrients uptake. According to the universal microalgal composition, N/P molar ratios less than 5:1 would result in nitrogen limitation, while N/P molar ratios more than 30:1 would lead to phosphorus deficiency (Gonçalves et al. 2017).

3 Formation and Construction of Consortia

Although microalgal show superior capability of removing nutrients from various wastewaters, it is difficult to sustain monocultures of microalgal during wastewater treatment processes. On the other hand, several studies have indicated the advantages of mixed consortia over monocultures in wastewater treatment (Table 22.4). In comparison with monocultures, mixed consortia which consist of many microalgal species and/or bacteria are deemed to be more stable especially during large-scale cultivation (Chen et al. 2015). Application of these consortia with varying metabolic potential may develop a robust system, thereby enhancing the resistance to adverse environments and invasive species (Gonçalves et al. 2017). The mixed consortia may form spontaneously in nature or be engineered artificially. Compared to naturally formed consortia, artificial consortia can increase the abundance of the specific species with high growth rates and nutrient removal efficiencies. However, it should be taken into account that if the specific species are adaptable to the environment and compatible to the wastewater. During outdoor cultivation, environment varies very often, and wastewater endogenous microorganisms may affect artificial consortium. Generally, it is preferred to choose robust species with a wide tolerance to environmental conditions (Liu et al. 2017).

So far, the most commonly used mixed consortia for wastewater treatment can be divided into two categories, one is microalgal consortia, which are merely consisted of photosynthetic microorganisms (eukaryotic and/or prokaryotic), and the other is microalgal-bacterial consortia, which are consisted of photosynthetic microorganisms and bacteria. Table 22.5 summarized the main characteristics of both consortia.

Table 22.4 Advantages and disadvantages of using mixed consortia in wastewater treatment

Advantages	Disadvantages
Robust systems (able to resist to adverse environments and invasive species)	Wide variety of possible microalgal combinations
Broad specificity to different nutrients (different microorganisms have different nutrients requirements)	Difficulties in the consortia construction (microbial selection, ratio between microorganisms, etc.)
Cooperative interactions can increase removal efficiencies	Difficulties in maintaining the consortia stable in longer processes and in open systems
Can be instead of secondary treatment regarding microalgal-bacterial consortia, reducing the aeration costs and CO_2 emissions	

Source: Gonçalves et al. (2017)

Table 22.5 Main characteristics of microalgal consortia and microalgal-bacterial consortia

Microalgal consortia	Microalgal-bacterial consortia
Established interactions poorly documented	Interactions well reported
Cooperative and competitive interactions can occur	Cooperative and competitive interactions can occur
Can be applied in the tertiary treatment stage of wastewater treatment	Nutrients exchange is the basis for cooperative interactions
Improve effective removals of nitrogen, phosphorus, and other elements, such as heavy metals	Can be applied in both secondary and tertiary treatment stages of wastewater treatment
	Improve effective removals of nitrogen, phosphorus, and organics, reducing the aeration in the secondary treatment

Source: Gonçalves et al. (2017)

3.1 Microalgal Consortia

In wastewater treatment processes, interactions between microalgal in the consortia have several advantages. First, they can improve the overall nutrients uptake as different microalgal have different nutrient requirements. Secondly, they may resist to contaminants and invasive predators due to the induction of allelochemicals. Thirdly, the combination of unicellular microalgal species with filamentous ones can improve settleability, which benefits for harvesting algae. Additionally, the use of microalgal consortia can ensure the sustainability and stability of the treatment process since the loss of some microalgal species can be compensated by the other species in the consortia (Gonçalves et al. 2017).

Several researchers have studied the use of both natural and artificial microalgal consortia in wastewater treatment. For instance, the potential of using microalgal consortia (*Chlorella* sp., *Scenedesmus* sp., and *C. zofingiensis*) to treat dairy wastewater has been evaluated by Qin et al. (2016, 2018). They noted a much higher COD removal (57.01–62.86%) and phosphorus removal (91.16–95.96%) by microalgal consortia than the monoculture of *Chlorella* sp. In addition, the

lipids extracted from microalgal consortia were considered to be more suitable for biodiesel production. In order to obtain highly settleable microalgal consortia, Renuka et al. (2013) have analyzed the potential of a selected microalgal consortium, native filamentous microalgal strains, and native unicellular microalgal strains for the treatment of primary treated wastewater. They found the removal efficiencies of NO_3-N, NH_4-N, and PO_4-P were 81.5–83.3%, 100%, and 94.9–97.8%, respectively, in all studied consortia.

3.2 Microalgal-Bacterial Consortia

Microalgal and bacteria are well-known to form consortia in the nature. The possible interactions between these microorganisms in microalgal-bacterial consortia are summarized in Table 22.6. Eight bacterial strains were isolated from a long-term laboratory culture of *Chlorella ellipsoidea*, and it was found that each bacterial strain is able to promote microalgal growth to various extents (Park et al. 2008). Generally, microalgal are able to produce oxygen by photosynthesis, while bacteria can release CO_2 by respiration. Oxygen and CO_2 can be exchanged between microalgal and bacteria, as microalgal can capture CO_2 for photosynthesis and bacteria need oxygen as an electron acceptor for their metabolism (Mujtaba and Lee 2017). Additionally, bacteria are able to excrete growth-promoting factors, such as vitamins (e.g., biotin, thiamine, and cobalamin) and siderophore (important chelating agents for microalgal growth under iron deficiency) (Gonçalves et al. 2017). Also, microalgal can act as a habitat for bacteria, resistant to unfavorable environmental conditions, and improve bacterial growth through releasing some extracellular metabolites (Unnithan et al. 2014). In the co-cultivation system, it has been indicated that bacteria might secrete extracellular polymeric substances (EPS) which promote bacterial aggregation with microalgal (Zhou et al. 2014). The sizes of the resulting bio-flocs range from 100 to 5000 μm, which can increase the biomass settleability and reduce harvesting cost (Quijano et al. 2017).

The formation of microalgal-bacterial consortia is a dynamic process and can be divided into four stages: stage 1, microalgal are adhered onto the surface of flocculated sludge due to EPS bridging and large superficial area of sludge flocs; stage 2, nascent bacteria are adhered onto microalgal surface due to the phycosphere; stage 3, the microalgal-bacterial consortia continue to grow; stage 4, dynamic balance is formed between the attachment and detachment of the algal and bacterial biomass (Wang et al. 2016).

In microalgal-bacterial consortia systems, nitrogen removal can be attained through biomass assimilation and nitrification/denitrification process, depending on the wastewater characteristics and reactor operating regime. Su et al. (2011) found that biomass assimilation was the main nitrogen removal mechanism, accounting for 45% of total inlet nitrogen in batch wastewater treatment with a microalgal-bacterial culture. González-Fernández et al. (2011) compared nitrogen transformations by microalgal-bacteria consortia treating fresh swine slurry with

Table 22.6 The possible interactions between microalgae and bacteria

	Benefits	Drawbacks
Algae	CO_2 release from bacterial metabolism	Algicidal effects of some bacteria
	Stimulative effects and essential nutrients from bacterial metabolism	
	Enhanced flocculation by associated bacteria	
Bacteria	Oxygenation from algae	Increase in pH due to associated algal metabolism
	Algal organic matter as a carbon source	Increase in temperature due to associated algal metabolism
		Antibacterial effects from some algae

Source: Wang et al. (2016)

anaerobically digested slurry in open ponds and found that substrate source significantly affected nitrogen transformation. Nitrification followed by biomass uptake was the major nitrogen transformation in anaerobically digested slurry fed ponds, while denitrification was the main nitrogen transformation in fresh slurry fed ponds under real conditions. A photo-sequencing batch reactor (PSBR) was adopted for shortcut nitrogen removal using microalgal-bacterial consortia system, where O_2 generated by microalgal stimulated ammonium-oxidizing bacteria (AOB) during light periods, and dissolved oxygen (DO) was promptly reduced by bacterial metabolism and algal respiration to facilitate denitrification during the dark period (Wang et al. 2015).

Currently, there are three main microalgal-bacterial consortia in use: (1) algae plus wastewater, (2) algae plus activated sludge, and (3) co-culture of algae and selected bacteria. Generally, bacterial community structure in the microalgal-bacterial consortia can be strongly influenced by algal species and phycosphere. Co-cultures of bacteria and microalgal have been evaluated including both naturally formed and artificially engineered consortia. For natural consortia, *Brevundimonas* and *Sphingomonas* have been reported to be commonly occurring bacterial genus (Tate et al. 2013). For artificial symbioses, the use of plant growth-promoting bacteria *Azospirillum* and *Bacillus* as well as organic-degrading bacterium *Pseudomonas* has been studied (de-Bashan et al. 2002b; Liang et al. 2013; Mujtaba et al. 2015).

4 Factors Influencing Nutrient Removal and Biomass Generation by Consortia

Compared to conventional mechanical treatment facilities, the microalgal treatment is more cost-effective in construction and operation (DOE 2010). Microalgal culture has demonstrated the viability to removal of aromatics, nutrients, and enhance algal biomass production (Chavan and Mukherji 2010; Madadi et al. 2016; Hodges et al. 2017). Compared to monoculture of microalgal, a synergistic algal-bacterial system is

more economic, and the synergistic effect of the two microorganisms enhances the treatment efficiency for total nitrogen (TN), total phosphate (TP), and organic pollutants in the wastewater. Environmental conditions and biotic interactions are the main factors influencing nutrient removal and biomass generation by the consortia.

4.1 Environmental Conditions

4.1.1 Light Intensity and Light/Dark Cycle

Light as an environmental factor not only affects the photosynthesis and metabolic pathway of algae but also affects the economic efficiency of algae growth process. Light intensity and photoperiod are the two key factors in determining the microalgal growth rate.

Regarding light intensity, large quantities of algal cell require higher light intensity to overcome the self-shading effect in culture (Franco-Morgado et al. 2017). According to Thawechai et al. (2016), when the light intensity is lower than the light saturation of microalgal, the algal cell does not grow well. Lipid accumulation and pigment productivity increased under strong light intensity (Gouveia et al. 2014). According to He et al. (2015), different light intensities affect the carbon partition between proteins, carbohydrates, and lipids; the higher light intensity stimulates lipid accumulation. Different light intensity also affects the quality of biodiesel produced by microalgal (Krzemińska et al. 2015). With the increase of irradiance, the C16–18 increased from 76.97% to 92.24%, and the C18:3 content decreased from 12% to 7.44%, meeting the requirements of biodiesel.

Regarding light/dark cycle, during the illuminated period, the photosynthesis is responsible for the net oxygen production in algal cell. However, in the dark period, the microalgal and related bacteria consumed DO by respiration (Franco-Morgado et al. 2017). Sriram and Seenivasan (2015) reported that the 16 h/8 h light/dark cycles and the light intensity of 16,000 Lux were the optimal conditions for maximum carbohydrate accumulation in *Desmodesmus* sp. The maximum biomass (1.033 g/L) and lipids (22.5%) were achieved at 8000–32000 Lux light intensity and 16 h/8 h light/dark cycles. The removal rate of organic carbon under the light/dark cycle is higher than that under continuous lighting, but the effect of nitrate removal is opposite (Lee and Lee 2001).

4.1.2 Temperature

The temperature factor could affect the biochemical reactions and eventually may affect the photosynthetic CO_2 assimilation (Bohutskyi et al. 2016). *Chlorella* cultured at 15 °C are more conducive to the accumulation of saturated fatty acids (SFA), at 20 °C are more conducive to the accumulation of monounsaturated fatty acids (MUFA), while at 25 °C are more conducive to polyunsaturated fatty acid

accumulation (PUFA) and C14-C18 components (Zhao 2016). Some species of microalgal can cultivate at high temperatures, whereas other species can tolerate at low temperatures. Generally, optimum growth temperature 18–25 °C is the most favorable temperature for algal cultivation (Gonzalez-Fernandez et al. 2016). The nitrogen removal in the wastewater, including the air stripping and the nitrification process, is also strongly influenced by temperature (Jämsä et al. 2017).

4.1.3 pH

In the microalgal cultivation, pH rises due to the photosynthetic CO_2 assimilation (Tan et al. 2016). The viability of alga grown on alkaline pH environment increases its potential for large-scale outdoors by inhibiting bacteria or protozoa contaminants. The activation of acetyl-CoA carboxylases (ACCs) is pH-dependent; therefore, the pH plays an important role in influencing the lipid accumulation process in the algae (Franco-Morgado et al. 2017). pH has different effects on biomass production and lipid accumulation. The biomass and lipid concentrations were highest at pH 8 and 6, respectively (Mandotra et al. 2016). When pH is lower than 8, the binding ability of the phosphate to the metal element is strong, and when the pH is high, the OH^- is exchanged with PO_4^{3-} and the phosphorus is released into water. The release of phosphorus promotes the growth of algae (Christer and Lars-Anders 2005).

4.1.4 Nutrients

Wastewater contains large amount of nutrients. Nitrogen and phosphate in wastewaters are the major causes of eutrophication and have harmful effects on freshwater. In general, the wastewater from the pig industry has a higher N and P content than municipal wastewater. The soluble COD content is higher in wastewater from brewery, food, and agricultural products processing (Gonçalves et al. 2017). The high concentration of COD has synergistic effect on algal growth if N and P are presented in sufficient amount (Phang et al. 2000; Cho et al. 2011; Min et al. 2011). Absorption of carbon, nitrogen, and phosphorus by rapidly growing algae is performed at an atomic ratio of 106:16:1 (Redfield ratio). If the ratio of N/P in wastewater is higher than the Redfield ratio (N: P = 16), the algae are most likely to be limited by phosphorus, while if less than the Redfield ratio (N: P = 16), they may be nitrogen limitation (Christer and Lars-Anders 2005).

4.2 Biotic Interactions Within the Consortium

Researchers have studied the effect changes of microalgal on bacterial community structure, each microalgal has themselves consortium formation, and the relationship is unique between them. However, the mechanisms for the formation of microalgal

Table 22.7 Microalgae and cyanobacteria-bacteria consortia formed on the common substrates (Liu et al. 2017)

Substrate	Formation	Dominant microorganisms
PVC pipe	Natural	*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteria*
Fiberglass		Achrochaete, amphora, Berkeleya, cyanobacteria, Gloeothece, Grammatophora, Lyngbya, Licmophora, Melosira, Navicula, Uliva, Ulothrix
PVC filler		Cyanobacteria, Cladophora, Oedogonium, Spyrogyra, Vaucheria, Bacteroidetes, Planctomycetes, Proteobacteria
PVC sheet		Filamentous green algae, Chroococcus, Nitzschia, Phormidium, Pseudanabaena
Nylon netting		*Rhizoclonium hieroglyphicum*, *Microspora willeana*, *Ulothrix zonata*, *Rhizoclonium hieroglyphicum*, *Oedogonium sp.*
PE liner		Cladophora, Cyclotella, Navicula, Nitzschia, Oscillatoria, Spyrogyra, Ulothrix
Plastic liner	Artificial	Filamentous cyanobacteria, Chlamydomonas, Desmodesmus, Stigeoclonium
PVC plate		Filamentous cyanobacteria, *Bacteroidia*, *Betaproteobacteria*, *Flavobacteria*, *Gammaproteobacteria*

consortium and nutrient removal are still not clear. The wastewater treatment using microalgal or cyanobacteria-bacteria consortia has been widely studied (Liu et al. 2017). A large number of researchers have made analysis of the microflora in microalgal community, some of which was formed by artificial and others were microalgal consortium in nature (Table 22.7). Microalgal always coexist with other microbes in nature, and there are an intricate relationship between them (Fig. 22.2). As the primary producer of the aquatic environment, algae converts carbon dioxide into organic matter through photosynthesis, providing nutrients for heterotrophic organisms. Microalgal and bacteria have complex symbiotic relationships, and O_2/CO_2 exchange is one of the well-known interactive relationships. Microalgal can generate oxygen for aerobic bacteria through photosynthesis, and bacteria release carbon dioxide, which in turn would be used by microalgal (Subashchandrabose et al. 2011). People use this kind of symbiosis to deal with organic pollutants, effectively remove organics pollutants, but also greatly reduce the cost of consumption.

Zhang et al. (2016) summed up the relationship between bacteria and microalgal, including evolutionary relationships, nutritional dependence, metabolic complementarity, and collaborative biosynthesis. Ramanan et al. (2016) elaborated the interactions between bacteria and microalgal and discussed the various environmental and technological applications utilizing these interactions from the perspective of evolution and ecology. Some bacteria not only enhance algal growth rate but also help in nutrient removal (Renuka et al. 2013; Qin et al. 2016) and the biomass harvesting (Lee et al. 2013; Kim et al. 2011), both essential processes in algal biotechnology. Previous studies have shown that there are symbiotic relationships between bacteria

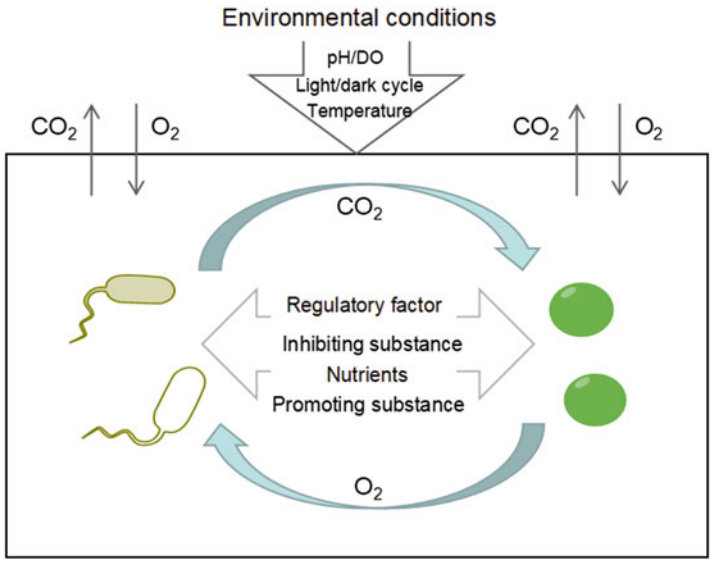

Fig. 22.2 Possible relationship between bacteria and algae in an inter-algal environment

and algae in host coral. Microalgal provide oxygen, and bacteria can provide symbiont carbon source by decomposing organic matter (Hu et al. 2013). A study pointed that *Rhizobium* sp. provides nitrogen source for *Chlorella vulgaris*, and *Chlorella vulgaris* provides carbon source for *Rhizobium* sp. in the symbiosis of the algae (*Chlorella vulgaris*) with the *Rhizobium* sp. (Kim et al. 2014). N_2-fixing cyanobacterial symbionts provide N for the diatoms; furthermore the N_2 fixation and the symbiotic growth rates of *Richelia* and *Calothrix* symbionts were higher compared with the rates estimated for the cells living freely (Foster et al. 2011). In addition C conversion efficiency and the algae growth and content of lipid, the diversity of fatty acids could be enhanced, which is beneficial to the production of biofuels, when the bacterial communities were included (Vasseur et al. 2012; de-Bashan et al. 2002b).

As Fig. 22.3 shows, the assimilation, ammonia oxidation, nitrite oxidation (nitrification), and denitrification may occur in the interaction of microalgal-bacteria consortium. During the photoautotrophic and heterotrophic consumption, the assimilation of microalgal is the major inorganic nitrogen removal pathway (Gonçalves et al. 2017). Except for assimilation, the ammonia volatilization, nitrite oxidation (nitrification), and denitrification are also the key process for inorganic nitrogen removal (Basílico et al. 2016; Daims et al. 2015). Apart from N, P plays an irreplaceable role in the metabolic function of microalgal consortium, in particular the inorganic forms, which can be phosphorylated into the organic compounds such as DNA, RNA, and lipids. In addition to the absorption and utilization of essential phosphorus for the microbial growth, some microalgal (e.g., *Cladophora*) and bacteria (e.g., *Acinetobacter*) can absorb large amounts of phosphorus and store it

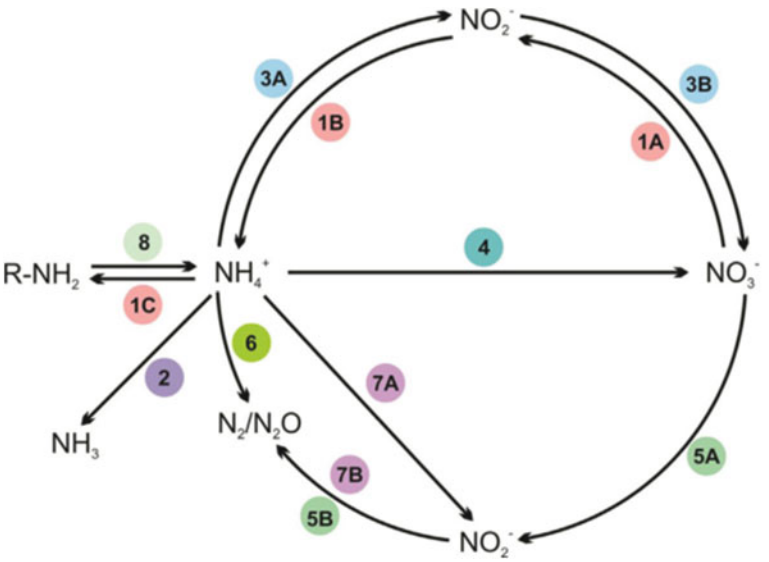

Fig. 22.3 Nitrogen removal pathways by the microalgae-bacteria consortium: (1) inorganic nitrogen assimilation by microalgae (1A nitrate reductase, 1B nitrite reductase, 1C glutamine synthetase); (2) ammonia volatilization caused by pH increase; (3) ammonia nitrification (3A, oxidation of ammonia to nitrite; 3B, oxidation of nitrite to nitrate); (4) nitrification by a single microorganism; (5) denitrification (5A, reduction of nitrate to nitrite; 5B, reduction of nitrite to N_2); (6) ammonium oxidation; (7) shortcut denitrification in the nitrite way (7A, oxidation of ammonia to nitrite; 7B, reduction of nitrite to N_2); (8) organic nitrogen mineralization. (Source from de-Bashan et al. (2002a))

as polyphosphate in cells (Schmidt et al. 2016; Higgins et al. 2008). Also, through precipitation with Ca and Mg at high pH, P can be removed from wastewater (Lu et al. 2016). These complex mechanisms may be related to the growth-promoting molecules secreted by certain bacteria. Overall, the diversified N and P removal mechanisms of microalgal-bacteria consortium make them an advanced platform for wastewater phosphorus removal.

5 Treatment of Different Wastewaters

Wastewater is defined as disposable water or liquids containing waste as a result of agricultural, urbanization, domestic, and industrial practices. Mostly wastewater can contain a huge amount of organic pollutants, oxygen-demanding wastes, inorganic compounds, pathogenic organisms, sediments, and nutrients, e.g., nitrogen and phosphorus (García et al. 2017). Every day human wastes of around two million tons are drained out in water bodies (Cuellar-Bermudez et al. 2017). More than 70% of wastewater is treated in developed countries, while developing countries process

28–38%, and undeveloped countries treat around 8% of whole wastewater (Sato et al. 2013). Nowadays in developed countries, physical and aerobic biological technologies are being used to treat wastewater, but there are more strict regulations regarding environment which are forcing current facilities to move to advanced level. Moreover greenhouse gas emission and high costs encourage others to find more sustainable alternatives (Li et al. 2015). Nitrogen and phosphorus can be effectively uptaken by microalgal from wastewaters. It is reported that microalgal eliminate a large nitrogen and phosphorus with elimination efficiencies (80–100%) from wastewaters of various sources, e.g., industrial, municipal, and agricultural (Phang et al. 2000). The degradation processes can be effectively performed by using microalgal consortia; otherwise these complicated processes would be difficult to complete if only monocultures are used. Moreover, through the application of these consortia, a robust system may develop a system which is able to resist invasion by other species and environmental fluctuations (Subashchandrabose et al. 2011). In many recent studies, efficiency of microalgal consortia (microalgal-bacterial) in several usages, including eliminating nutrients and production of biomass, has been reported (Rawat et al. 2011). The usage of polycultures for elimination of nutrients can be very effective because it is a combination of micro-organisms with various metabolic activities and having different environmental conditions resulted to develop a robust biological system that can operate under multiple nutrient loads and environmental conditions (Boonma et al. 2015). Moreover, higher nutrient uptake rates can be resulted by cooperative interactions formed among the microorganisms synthesizing the consortia (Johnson and Admassu 2013).

5.1 Municipal Wastewater

For a primary treated municipal wastewater, a consortium formed by *Chlorella* sp. and *Scenedesmus* sp. was found very effective for the removal of nitrogen and phosphorus (Koreivienė et al. 2014). It is found that efficiency of total nitrogen (TN) elimination is 88.6–96.4% and for total phosphorus (TP) removal is 99.7–99.9%. It is expected that nutrient uptake rates are increased by increasing growth of microalgal. As studied by de-Bashan et al. (2004), in mixed culture containing *Azospirillum brasilense*, *C. vulgaris*, and *C. sorokiniana* in alginate form, not only microalgal growth was increased, but removal of nitrogen and phosphorus was also enhanced when a municipal wastewater was used as culture medium. These systems enhance existing wastewater treatment processes and were very effective in nutrient removal because (de Godos et al. 2009) (i) the expenses can be notably decreased to oxygenate activated sludge tanks (ii) since the bacteria discharge CO_2 which is used by microalgal to form organic matter so the greenhouse effects related to plants of wastewater treatment are assumed insignificant (Karya et al. 2013).

It is studied that ammonium ions are nitrified by bacteria in wastewater ranging from 81% to 85% besides uptaken by algae in photobioreactors. Additionally, the uptake of phosphorus was influenced by nitrogen availability in the system

(Beuckels et al. 2015); it shows that well-maintained algal-bacterial consortium can eliminate nutrients (N and P) and organic contaminants. Phosphorus is also eliminated by biological process. The uptaken phosphorus is used in the formation of nucleotides, nucleic acids, and phospholipids by microalgal (Miyachi et al. 1964). There is another biological system used by bacterial cells which is extra absorbance of phosphorus (Powell et al. 2008). It is studied that efficiency of energy in BOD elimination from domestic wastewater is enhanced by microalgal because microalgal provide O_2 to the heterotrophic aerobic bacteria (Munoz and Guieysse 2006).

5.2 *Industrial Wastewater*

Phenols and their derivatives are potentially harmful for organisms (plants and microorganisms) even though these are at very minute amount, so they are counted as important pollutants. Advanced oxidation processes (AOPs) are proved very useful techniques for degrading organic compounds that are based on releasing of reactive species like sulfate radicals and hydroxyl (Liotta et al. 2009). To degrade phenol, heterogeneous photocatalysis is applied (Das et al. 2005). Olive washing wastewater (OWW) contains high amounts of phenolic compounds which is biologically treated using a stable microbial consortium formed by bacteria (*Pantoea agglomerans* and *Raoultella terrigena*) and microalgal (*Chlorella vulgaris* and *Scenedesmus obliquus*). This study showed that several bioremediation mechanisms could be enhanced by phototrophic contribution (like eukaryotic microalgal) in combination with heterotrophic bacteria which is regarded a recent technique to olive wash water (OWW) biotreatment. The consortium utilized in it may be a promising bioremediation means, but in literature this approach has not been elaborated (Maza-Márquez et al. 2014).

It is described that *Scenedesmus* sp., *Ankistrodesmus* sp., and *Chlorella* sp. have been successfully utilized for the bioremediation of the wastewater of paper industry and olive oil mil (Pinto et al. 2013). In mechanisms of wastewater treatment, along with basic systems by which components of wastewater degraded or adsorbed. Organic compounds having N are degraded well by microalgal-bacteria consortia than with bacteria alone, because microalgal assimilated released NH_4^+ (Risgaard-Petersen et al. 2004). Riaño et al. (2011) reported that for the treatment of fish processing wastewater, a microalgal-bacteria symbiosis was applied; it increased elimination of COD (chemical oxygen demand) up to 71% of the initial quantity. In the elimination of uranium in mining wastewater, the change of U when it passed through a wetland was recognized, and higher number of both anaerobic and aerobic microbial consortia in the less redox sediments and the existence of uraninite were reported. It is showed that some biosorption by the microbial consortia was an effective tool of U elimination (Groudev et al. 1999).

Borde et al. (2003) first studied the treatment of benzene containing wastewater by utilizing algal-bacterial consortium, but results showed the pollutants were not

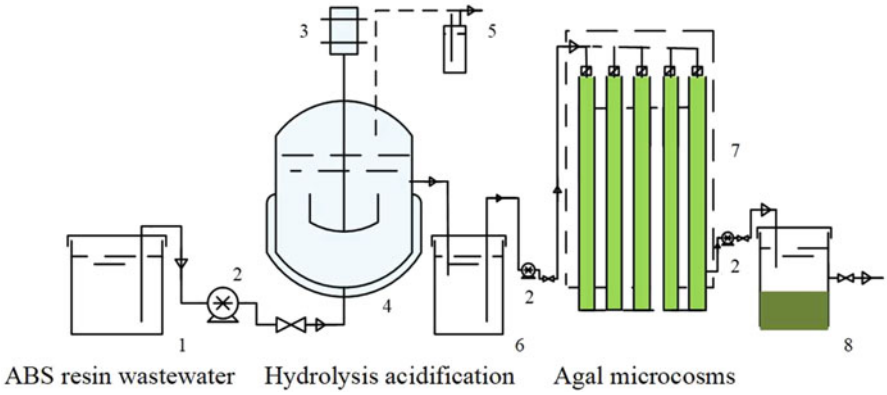

Fig. 22.4 Diagram of two-stage coupling system for benzenes petrochemical wastewater's advanced treatment based on hydrolysis acidification coupled with algal-bacterial microcosms (1, wastewater tank; 2, pump; 3, electric motor; 4, hydrolysis acidification reactor; 5, biogas; 6, effluent tank; 7, algal photobioreactor; 8, flocculation collection tank) (Huo et al. 2018)

completely removed. Later, Abed and Köster (2005) treat petrochemical wastewater containing dibenzothiophene and phenanthrene by using algal-bacterial consortium; this process took 20–30 days to be completed. So, the algae microcosm system is less than ideal to treat the petrochemical wastewater having benzene. Pollutants are toxic and very complicated that make the algal treatment even more difficult. Huo et al. (2018) show a successful illustration of a novel two-stage process coupling hydrolysis acidification with algae microcosm for treatment of acrylonitrile-butadiene-styrene (ABS) resin manufacturing wastewater (Fig. 22.4). After hydrolysis acidification, the wastewater showed less toxicity and increased biodegradability. Coupling with the algae microcosm treatment system, the COD, phosphate and nitrogen, and other organic compounds in the petrochemical wastewater were effectively cleaned. This two-stage coupling system achieved cleaning of the benzene-containing petrochemical wastewater while obtaining microalgal biomass resources at less cost.

5.3 Agricultural Wastewater

It is studied that bacteria produce substances like jasmonic acid, brassinosteroids, polyamines, cytokinins, auxins, and abscisic acid which have potential to regulate metabolism and growth in several species of microalgal such as *Dunaliella* sp. and *Chlorella* (Kouzuma and Watanabe 2015). The microalgal consortium idea can be represented by N_2-fixing bacteria like microalgal and *Azospirillum*. Studies showed that *Azospirillum brasilense* produce indole-3-acetic acid which promotes NH_4^+ in *C. vulgaris* cells (Meza et al. 2015). Addition of exogenic N in wastewater can removed by getting benefits of the cooperative relationship of the N2-fixing bacteria

Azospirillum. So, consortia or mixed populations can make the rate of removal higher which is negligible or less by individual species (Brenner et al. 2008). In the study of wastewater having organic P, organophosphorus pesticides are in higher concentrations up to 50 μg L^{-1} in agricultural effluents (Hultberg et al. 2016). Organic P elimination from pesticides is studied by Ibrahim et al. (2014). In the study of wastewater, mixotrophic metabolism of bacteria and microalgal can eliminate organic pollutants through process of using organic pollutants and/or by biodegradation (Markou et al. 2012). No doubt, magnificent results were obtained by cultivating filamentous algae in animal farm effluents and domestic and nutrient-contaminated surface water (Mulbry et al. 2008).

Moreover, algal biomass has various harmful heavy metals like cadmium, lead, copper, zinc, nickel chromium, and arsenic. These metals can incorporate into soil by atmospheric deposition; by applying animal manure, metal-contaminated wastewater, and compost and inorganic fertilizers; and by being washed from agricultural fields during rainfall (Nicholson et al. 2003).

6 Mechanisms for Removing Nutrients by Consortia

Many reports indicated that the use of microalgal consortia for wastewater treatment has a good effect on biomass production, nutrient cycling, organic pollutants, heavy metals, and bioremediation of many other pollutants (Manzoor et al. 2016; Renuka et al. 2013). It is important to understand the removal mechanism of nutrients in wastewater by microalgal consortia to treat wastewater (Gonçalves et al. 2017). This section mainly reviews the mechanisms of nutrient removal by microalgal consortia.

6.1 N and P Removed by Consortia

The main pollutants in water are organic and inorganic nutrients. Bacteria are the major decomposers of organic matter, and algae are the main absorbers of nutrients in water bodies. People use this symbiotic action of algae-bacteria to treat pollutants in wastewater, which can effectively reduce pollutant emissions and greatly reduce cost. The basic mechanism for purifying water quality by microalgal consortia is that microalgal supply oxygen to water bodies through photosynthesis, increase dissolved oxygen in water bodies, and allow aerobic bacteria to continuously degrade organic matter; the CO_2 produced by aerobic bacteria in the degradation of organic matter can be reused by algae for photosynthesis; the system formed by this cycle is also called "algal-bacterial symbiosis" (Mu et al. 2005; Natrah et al. 2014).

The consortia (microalgal and bacteria) remove N, P nutrients in wastewater mainly through three aspects (Xing et al. 2009; Wang et al. 2018): oxidative degradation by bacteria, assimilation by microalgal, and changes in pH of symbiotic

environment. Aerobic bacteria use O_2 produced by microalgal to degrade organic matter in wastewater to produce CO_2 and aminate nitrogenous organics, followed by nitration to produce ammonia nitrogen, nitrite, and nitrate. At the same time, in this process, phosphorus-containing organic matter can be degraded to orthophosphate for the growth and utilization of microalgal. Microalgal use the CO_2 produced by bacteria for photosynthesis and assimilation of N, P, and other nutrients.

Both organic nitrogen and inorganic nitrogen can be utilized by microalgal. The most common forms of inorganic nitrogen include nitrates, nitrites, nitrogen, ammonium, hydrogen nitrate, ammonia, and nitrogen oxides (Barsanti and Gualtieri 2006; Gonçalves et al. 2017). Some species of blue-green algae can fix N2 and convert it to ammonia nitrogen, which can be used to synthesize amino acids and proteins (Cai et al. 2013; Barsanti and Gualtieri 2006; Gonçalves et al. 2017). The research of Vílchez et al. (1997) showed that the process of inorganic nitrogen absorption and utilization by microalgal was divided into three steps. In the first step, microalgal absorbed nitrate, nitrite, and ammonia nitrogen into cells with the help of specific enzymes; after that, under the catalytic action of related enzymes, microalgal used ATP to reduce nitrates and nitrites to ammonium; finally, the reduced ammonium was incorporated into the carbon skeleton by microalgal. Since the utilization of NH4-N by microalgal does not require reduction, it is believed that microalgal will preferentially utilize this form of nitrogen source (Gonçalves et al. 2017). Therefore, microalgal preferentially utilize ammonia nitrogen in the absorption and utilization of nitrogen sources compared to nitrates and nitrites (Wang et al. 2018).

Phosphorus is an essential element of nucleic acids, cell membranes, and energy stores. It is also a constant element necessary for the growth of microalgal. Microalgal usually preferentially absorb two forms of inorganic ions of HPO_4^{2-} and $H_2PO_4^-$. PO4-P is assimilated by microalgal mainly through three ways, namely, oxidative phosphorylation, photophosphorylation, and phosphorylation at the substrate level (Gonçalves et al. 2017). Phosphorus absorbed by microalgal can be synthesized nucleic acids or energy transfer substances, etc.

Stumn and Morgan (1996) proposed that the algae formula was approximately $C_{106}H_{263}O_{110}N_{16}P$. During the growth of microalgal, CO_2 is used as the carbon source, and inorganic ions (such as NH4, NO_3^-, NO_2^-, $H_2PO_4^-$ etc.) and organic substances (such as urea) in wastewater are used in photosynthesis. The N, P, and other elements are added into the carbon skeleton of the algae cells. At the same time, microalgal release O_2 into the water, increasing the dissolved oxygen (DO) content in the water, which is good for the growth of other organisms (Xing et al. 2009). The response of microalgal to assimilation of NH_4^+ is as follows:

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2$$

Since the microalgal use CO_2 as a carbon source for photosynthesis, the CO_2 content in the wastewater is reduced, and the pH value is increased. That will lead to enhance ammonia nitrogen volatilization, and phosphate and calcium ions form calcium phosphate precipitates at high pH, thereby achieving effective removal of phosphorus and nitrogen (Su et al. 2012; Aslan and Kapdan 2006; Cai et al. 2013).

6.2 Difficult-to-Degrade Organics Removed by Consortia

The biodegradable organics have high toxicity and complex components. Such contaminants include organic cyanide, organic pesticides, organic dyes, antibiotics, etc. General biological treatment methods are difficult to degrade such organic substances, and the presence of such pollutants can have toxic effects on microorganisms, inhibit microorganisms' growth, and affect sewage treatment. Many microalgal can effectively enrich and degrade a variety of refractory organic compounds, such as antibiotics, organochlorines, pesticides, azo dyes, etc. Some microalgal have good tolerance to high concentrations of refractory organics (Xiong et al. 2016). By combining the characteristics of bacteria and microalgal to construct a symbiotic system of bacteria and algae, it can degrade refractory organic matter and effectively remove those pollutants. When microalgal-bacterial consortia is used to treat difficult-to-degrade organics such as antibiotics, it is mainly through the action of bacteria and algae. Bacteria mainly remove antibiotics through co-metabolism of organisms, but the amount of adsorption, volatilization, hydrolysis, and mineralization is relatively small. Some studies have found that nitrification sludge and *Nitrosomonas europaea* can effectively degrade antibiotics such as pollutants through co-metabolism degradation (Yin et al. 2014). Antibiotics have a direct effect on the algae itself and may both inhibit the growth of algae and may also have toxic stimulatory effects at specific concentrations. Further activation of proteases regulates synthesis and induces gene expression (Ma et al. 2012; Yin et al. 2014). At low concentrations, antibiotics can be removed by absorption and biodegradation by microalgal; however, antibiotics may inhibit the growth of microalgal at high concentrations (Pan et al. 2008; Bai and Acharya 2016). The tolerance of microalgal to antibiotics is much higher than that of bacterial microbes (Bai and Acharya 2016). When microalgal-bacterial consortia is used to treat difficult-to-degrade organics of antibiotics, the symbiotic relationship between bacteria and algae can enhance the activity of the bacteria and increase the tolerance of the system to antibiotics.

6.3 Heavy Metal Ions Removed by Consortia

Microalgal consortia remove heavy metal contaminants primarily through biosorption and bioconcentration processes. At low concentrations, microalgal consortia remove heavy metal ions from water in two stages (Zhi et al. 2011):

(i) Bio-adsorption: Metal ions attach to the surface of microbial cells through complexation, coordination, ion exchange, etc. the reaction time of this process is short, and there is no need to provide energy.
(ii) Bio-enrichment: Metal ions bind to specific enzymes on the surface of microbial cells and are then transported into the cells. This process has a long reaction time and is irreversible, which is related to metabolic activity.

Microalgal consortia can effectively remove heavy metals, mainly because microalgal have higher tolerance to heavy metal ions than bacterial microbes, which can improve the removal effect of consortia on heavy metal ions (Calabrese 1999; Davis et al. 2003). The mechanism of adsorption of heavy metal ions by microalgal is mainly ion exchange mechanism and complexation mechanism. The cations can undergo complexation or chelation reactions with molecules or anions (base pairs) with free electron pairs. Complexes are formed by complexing metal cations in wastewater with negatively charged functional groups in proteins, lipids, and polysaccharides in cells, such as -$CONH_2$, R-SH, -NH_2, -COOH, imidazole, sulfur ether, sulfate, phosphate, etc. The main ion exchange effect is that the metal cations contained in the wastewater will displace the protons on the algal cell wall, and other metal ions will act on the cell wall surface through the electrostatic attraction between the ions or through the coordination bond (Davis et al. 2003; Li et al. 2016). Davis et al. (2003) showed that the ion exchange mechanism is the most effective process to reflect the bio-adsorption of heavy metal ions by algae cells. Alginate and sulfate in polysaccharides have also been found to have significant ion exchange capacity.

7 Challenges of Microalgal Consortia Research in Bioremediation of Wastewaters

Microalgal and microalgal-bacterial consortia have broad application prospects in wastewater treatment and recycling. However, in the research field of wastewater treatment using microalgal consortia, further research is still needed in many aspects such as metabolic mechanism, mass transfer process, process kinetics simulation, and reactor design for removing pollutants.

1. Microalgal consortia have certain advantages in the removal of contaminants. However, the interfacial forces and binding conditions of the bacteria and algae binding in the microalgal environment are still unclear; the contribution of bacteria and microalgal to contaminant removal has not yet been explored. Therefore, the mechanism of microalgal consortia removal of contaminants can be further studied by linking microscopic mechanism studies with pollutant removal characteristics.
2. There are also problems such as biofilm shedding in microalgal-bacterial consortia. The formation and maintenance of microenvironment in consortia, symbiotic mass transfer, decay balance of algal growth, metabolic mechanism, etc. need to be further studied (Li and Xie 2006).
3. The microalgal consortia not only removes pollutants from the water but also fixes CO_2 in the gas. In order to achieve synchronous treatment of wastewater and waste gas, further efforts are still needed to rationally design photobioreactors.
4. In microalgal consortia, the relationship between microalgal and bacteria should be the presence of bacteria is conducive to algae growth, can enhance algae

tolerance to biological and abiotic stresses, and secrete antibacterial substances and hydrolytic enzymes to eliminate harmful organisms, thus improving the biomass production of algae; algae can provide useful nutrients for the growth of bacteria. However, the mechanism of this effect is not yet clear. In the future, there is still a lot of work to be done, which can focus on the following points:

(a) Phylogenetic analysis of microbial community structure of microalgal consortia. The bacteria that act on microalgal are specific. The conclusion can be drawn by analyzing the structure and biochemical analysis of microalgal-bacterial populations in many different types of microalgal consortia (Kim et al. 2014).
(b) Exploration of chemical signaling substances and mechanisms of interaction between bacteria and microalgal in microalgal consortia through metabolome and transcriptome analysis. The relationship between microalgal and bacteria is not just direct physical contact for material exchange, but it may also be linked together by some medium.

5. Although the use of microalgal consortia to treat wastewater can achieve good results, there are still some problems, such as: (1) microalgal do not have strong tolerance to livestock and poultry wastewater treatment efficiency is not high; (2) industrial reactor device is not perfect; (3) the concentration of microalgal cells is low, and high cost of later separation of microalgal (Christenson and Sims 2011). Therefore, microalgal immobilization culture technology may be the focus of future research. In particular, new immobilization techniques such as bio-immobilization should be thoroughly studied to make the microalgal consortia system play an effective role in dealing with pollution, thus contributing to the widely used aquaculture wastewater treatment (Zhang et al. 2017). In addition, the treatment of aquaculture wastewater and microalgal resources by microalgal consortia is sustainable and will have great potential for development.
6. So far, most studies on the use of microalgal consortia to remove nutrients from wastewater have been conducted under small laboratory conditions. Although some studies have shown that microalgal and microalgal bacterial consortia can effectively remove nutrients from different wastewaters, further research in this area is needed to optimize culture parameters in large scale (Gonçalves et al. 2017).

8 Conclusion

Although there have been a lot of research reports on the treatment of wastewater by microalgal cultivation, there is insufficient understanding of the treatment of wastewater by using microalgal consortia, which is mainly caused by too many possible combinations available (Gonçalves et al. 2017). This chapter introduced a suitable approach to sustainable wastewater treatment – microalgal consortia, including the microalgal consortia formation and construction, factors influencing nutrient removal and biomass generation by consortia, microalgal-mediated treatment of different wastewaters (including municipal, industrial, and agricultural wastewater),

mechanisms for removing nutrients by consortia, and the challenges of microalgal consortia research in bioremediation of wastewaters. In the microalgal consortia system (microalgal and microalgal-bacteria consortia), the relationship between algae and bacteria may be more complicated than the relationship between different species of microalgal. Compared with the single microalgal system and single bacterial system, microalgal-bacterial consortia exhibit stronger contaminant tolerance. Therefore, the use of microalgal-bacterial consortia to treat some types of wastewater (such as high N, P wastewater, heavy metal ion wastewater, antibiotic wastewater, etc.) has certain advantages. However, it is still necessary to reasonably select the species of microalgal and bacteria based on the characteristics of the wastewater.

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Chapter 23
Biosorption of Heavy Metals and Dyes from Industrial Effluents by Microalgae

Wan-Loy Chu and Siew-Moi Phang

Abstract Discharge of industrial effluents containing heavy metals and dyes is of concern as the pollutants may adversely affect the environment by contaminating surface- and groundwater resources. Heavy metals and dyes are very persistent and may pose a threat to various organisms and human health. Physicochemical methods such as chemical precipitation and adsorption are commonly used to remove heavy metals, while flocculation, flotation, membrane filtration and activated carbon are used to treat dye wastewater. However, these conventional technologies are costly and may not be fully effective in removing heavy metals and dyes. Biosorption refers to the ability of living or dead biomass to sequestrate pollutants such as heavy metals and dyes through passive binding. The biosorption capacity of microalgae is due to their high surface-to-volume ratio and high binding affinity. The mechanisms involved in biosorption include ion exchange, complexation, precipitation and physical adsorption. Functional sites on the cell wall of microalgae involved in biosorption include carboxyl, imidazole, sulfhydryl, amino, phosphate and sulphate moieties. Despite the extensive research in this area, there has been limited success in commercializing the technologies using algal biosorbents. Detailed economic and market analyses are required to assess the feasibility of the technologies. Integration of wastewater treatment and biofuel production with heavy metal and dye removal using biosorption process would be an attractive approach. Apart from treating the wastewater, the microalgae can be harvested for biofuel production, and the residual biomass can be used for biosorption of heavy metals and dyes.

Keywords Biosorption · Heavy metals · Dyes · Microalgae · Bioremediation

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1 Introduction

Anthropogenic activities and industrialization have generated large quantities of aqueous effluents containing toxic metals as well as various organic pollutants including dyes. Industrial wastewaters containing heavy metals and dyes are of concern as they can cause detrimental effects on ecosystems and can be hazardous to human health (Bilal et al. 2018; Vikrant et al. 2018).

Effluents that contain high amounts of heavy metals include those from sources such as mining, plastic, fertilizer, textile, dyeing, paper and paint industries (Abbas et al. 2014). Contamination of drinking water resources due to discharge of industrial effluents laden with heavy metals is a great concern (Azimi et al. 2017). Furthermore, most of the heavy metal ions are toxic to living organisms, and thus, efficient removal of the toxicants before discharge is crucial (Dhankhar and Hooda 2011).

Effluents originating from textile, wool and tanning industries are a major source of dyes that pollute the aquatic ecosystems (Vikrant et al. 2018; Ghosh et al. 2016). There are over 100,000 types of commercially available dyes and pigments, with annual production of over 7×10^5 tons worldwide (Robinson et al. 2001). In the textile industry, up to 200,000 tons of these dyes are lost in the effluents due to inefficiency in the dyeing process (Ogugbue and Sawidis 2011). Contamination of water bodies by synthetic dyes is regarded as a global environmental problem that poses a threat to aquatic ecosystems as well as human health. Synthetic dyes can adversely affect growth and metabolic activities, especially the photosynthetic activity of microalgae, which play an important role as primary producers in the food chain. For instance, exposure to Congo red reduced the growth rate and adversely the photosynthetic efficiency of *Chlorella vulgaris* (Hernandez-Zamora et al. 2014).

Microalgae have been used in bioremediation of agroindustrial wastewaters, including the removal of pollutants such as heavy metals and dyes (Phang et al. 2015). *Chlorella vulgaris* grown in high rate algae ponds (HRAP) has been shown to have potential application for bioremediation of textile wastewater, especially in colour removal (Lim et al. 2010). Microalgae are known to have enormous potential for bioremediation of heavy metals due to their ability to concentrate metal ions (Suresh Kumar et al. 2015). One of the major processes involved in removing heavy metals and dyes by microalgae is through biosorption (Bilal et al. 2018). Algal biomass has high binding affinity for metals and dyes as the algal cell wall and exopolysaccharides contain diverse functional groups (Romera et al. 2006; Mohan et al. 2008; Maurya et al. 2014; Kumar et al. 2016).

The primary aim of this chapter is to review the current status of research on biosorption of metals and dyes using both living and non-living biomass of microalgae. This review will also highlight the potential applications of microalgae as biosorbents for bioremediation of industrial effluents containing metals and dyes.

2 Sources of Heavy Metal and Dye Pollutants

The major sources of wastewaters containing heavy metals are the electroplating, mining, tanning, metal finishing, electronic circuit, steel, aluminium and textile industries (O'Connell et al. 2008; Noreen et al. 2017). Other anthropogenic sources of heavy metals include landfill leachate, fertilizers and manure sewage sludge. Typical toxic metals found in industrial effluents include cadmium (Cd), copper (Cu), nickel (Ni), chromium (Cr), cobalt (Co), zinc (Zn) and lead (Pb). Effluents arising from electroplating industries are complex as they contain a variety of metal ions with counter anions, surfactants and organic/inorganic additives (Islamoglu et al. 2006). Such effluents contain high amounts of Cd (28,656 mg/L), with medium amounts of Zn, Cu and iron (Fe) and small amounts of Ni, Co and manganese (Mn). In addition, the total Cr in wastewater from chrome plating industries may range from 0.1 to 0.9 g/L in a diluted rinse discharge stream to 78–286 g/L in an electrolytic bath stream (Agrawal et al. 2006). In comparison, the raw effluent from copper smelting and refinery contain elevated levels of Cd (3810 μg/kg) and mercury (Hg; 760 μg/kg) (Chojnacka et al. 2004).

The concentrations of Cr from tanning operations may range from 0.5 to 4.0 g/L. High concentrations of arsenic (As; 54 mg/L), Fe (5.8 g/L) and Zn (1.6 g/L) along with SO_4^{2-} (17.7 g/L) have been reported in raw mining water from a former ore mining activity on the Kank Hill, Czech Republic (Doušová et al. 2005). In comparison, textile effluents contain high amounts of heavy metals such as Pb (0.13–0.25 mg/L) and Cd (0.10–0.20 mg/L) (Noreen et al. 2017). Animal husbandry, especially pig farming, is another source of heavy metal pollution, as pig manure may contain high levels of metals such as Cu (151.11 mg/kg) and Zn (538.29 mg/g) (Feng et al. 2018). The metals may cause a wide range of detrimental human health effects, including toxic effects on the nervous and reproductive systems (Pb), cardiovascular system (As) and respiratory and gastrointestinal systems (Cd) and carcinogenic effect (Cr) (Bilal et al. 2018).

There are many types of dyes, which can be broadly categorized into non-ionic (disperse dyes), anionic (direct, acid and reactive dyes) and cationic forms (basic dyes) (Vikrant et al. 2018). Azo dyes are the most common dye stuff used in the textile industry; the chromophoric azo groups (-N=N-) present in these dyes can undergo reductive cleavage resulting in the formation of highly toxic aromatic amines (Bruschweiler and Merlot 2017). The major sources of effluents containing dyes are from industries such as textile, paper, plastics and dyestuffs (Crini and Badot 2008; Vikrant et al. 2018). Such industries consume substantial amounts of water and use significant amounts of chemicals during manufacturing and dyes to colour their products. As a result, a considerable amount of coloured wastewater is generated. For instance, the apparent colour of textile wastewater may range from 169.67 to 1937.33 PtCo unit, with high levels of metals such as Pb, Cd, Zn and Mn (Lim et al. 2010). The effluents, if discharged untreated, are a major source of aquatic

pollution. The pollutants are not only toxic but also cause adverse impact on the aesthetic value of receiving water bodies. For instance, pulp and paper mills generate coloured effluents with high chemical oxygen demand (COD), biological oxygen demand (BOD) and suspended solid (mainly fibres) and contain toxic chemicals such as chlorinated phenolic compounds (Pokhrel and Viraraghavan 2004).

Efficient treatment is required before coloured wastewater is discharged as the presence of very small amounts of dyes in water is highly visible and undesirable. Wastewater containing dyes is not easy to treat as the chemicals consist of recalcitrant molecules, particularly azo dyes, that are resistant to aerobic digestion and stable to oxidizing agents (Fomina and Gadd 2014). Effluents containing metal complex dyes from textile and leather industries not only contaminate water bodies with synthetic dyes but also heavy metals such as Cr, Co, Cu and Ni (Ghosh et al. 2016).

3 Technologies for Removal of Heavy Metals and Dyes

Efficient treatment of effluents containing metals is required before discharge of the wastewater to the environment. Conventional technologies used to remove heavy metals from aqueous solution include chemical precipitation, lime (calcium hydroxide) coagulation, solvent extraction, membrane filtration, reverse osmosis, ion exchange and adsorption (O'Connell et al. 2008; Kurniawan et al. 2006; Azimi et al. 2017). Chemical precipitation is most widely used for removal of heavy metals from electroplating wastewater; however, it is non-selective and produce large quantities of sludge (Kurniawan et al. 2006; O'Connell et al. 2008). Using this method, metals are precipitated by the addition of lime (calcium hydroxide), sulphide and caustic soda. Additional methods such as chemical extraction, bioleaching process, electrokinetic process and supercritical extraction need to be applied to remove the heavy metals from the sludge before disposal (Babel and del Mundo Dacera 2006).

Ion exchange is the second most widely used technique for metal removal from industrial effluents (O'Connell et al. 2008). In this technique, there is a reversible exchange of ions between the solid and liquid phase. A column of resin is used to remove ions from an electrolytic solution and releases other ions of similar charge in a chemically equivalent amount. Another widely used method is adsorption, which involves the physical adherence or binding of ions or molecules onto two-dimensional surface (Fomina and Gadd 2014). The common adsorbents used include activated carbon, carbon nanotubes and sawdust (Azimi et al. 2017). An advantage of the process is that the adsorbents can be regenerated by desorption. The disadvantages of the above-mentioned physicochemical techniques include the large amount of sludge generated, high chemical consumption and high operation costs (O'Connell et al. 2008).

Biosorption is an attractive method for removal of metal ions as it is comparable to the well-established ion-exchange resin-based treatment method (Volesky 2007). According to Gadd (2009), biosorption can be simply defined as *the removal of*

substances from solution by biological material. Biosorption involves physicochemical and metabolically independent process that is based on a variety of mechanisms including absorption, adsorption, ion exchange, surface complexation and precipitation (Fomina and Gadd 2014). The biological material used may include both living and dead microorganisms and their components. The biosorption process involves rapid and reversible binding of ions onto functional groups that are present on the surface of biomass (Michalak et al. 2013). Such process is independent of cellular metabolism, in contrast with bioaccumulation, which requires metabolic activity of living organisms (Davis et al. 2003).

The target sorbate removed from aqueous solution using biosoprtion include metals, dyes, fluoride, phthalates and pharmaceuticals. In the context of heavy metal removal, biosorption involves passive mechanisms of metal binding that are not driven by metabolism (Volesky 2007) compared to bioaccumulation, which involves active uptake of metals by living biomass (Chojnacka 2010). Metal removal by living biomass of microalgae may involve both adsorption and active uptake (Cheng et al. 2016; Kumar et al. 2016). For instance, adsorption kinetics of six metal ions (Al, Zn, Hg, Pb, Cu and Cd) on living cells of six species of microalgae showed that removal of the metals was through a combination of adsorption and accumulation (Schmitt et al. 2001).

Various physicochemical methods are used for the treatment of dyes, including oxidative remediation, adsorption, coagulation, membrane separation and ion exchange (Vikrant et al. 2018). Some of the shortcomings associated with such technologies include high operating/energy costs, generation of large amounts of sludge and production of damaging byproducts. Bioremediation is an attractive approach in the treatment of dye wastewater. For such purpose, the use of a consortium of microalgae, bacteria and fungi is more efficient than pure cultures for effective decolonization process (Forgacs et al. 2004). The individual strains may attack at different positions of the molecules or utilize the breakdown products from other strains. The use of low-cost and efficient solid materials as biosorbents for removing synthetic dyes from water and wastewater is regarded as a simple and economical method (Forgacs et al. 2004).

4 Use of Microalgae as Biosorbents for Removal of Heavy Metals and Dyes

According to the statistical review by Romera et al. (2006), algae have been less used as biosorbents compared to other kinds of biomass, especially fungi and bacteria. However, the interest in this field has increased significantly, as inferred from the publications produced in recent years. Amongst algae, the brown seaweeds, especially *Sargassum*, have been shown to be good biosorbents for heavy metals, which could be due to their abundant cell wall polysaccharides and extracellular polymers (Romera et al. 2006; Brinza et al. 2007; Wang and Chen 2009). The analysis by

Romera et al. (2006) showed that algal biomass has the highest sorption affinity for Pb, followed by Cd, Cu, Ni and Zn.

The use of algae as biosorbents has several advantages, including (1) diverse multifunctional groups on their surface, (2) relatively small and uniform distribution of binding sites on the surface, (3) less preparatory steps required, (4) less usage of harsh chemicals, (5) the algal biomass that can be easily produced and recycled and (6) good retention capacity (Bilal et al. 2018). It is advantageous to use microalgae instead of seaweeds as biosorbents as the former can be grown on a large scale to generate sufficient biomass. Furthermore, mass culture of microalgae can be integrated with other applications, especially for bioremediation of wastewater and production of biofuels (Chu 2017).

4.1 Biosorption of Heavy Metals

The potential of microalgae as a tool for remediation of heavy metals in industrial effluents has been reviewed by many authors (e.g. Mehta and Gaur 2005; Suresh Kumar et al. 2015). There are also critical reviews focusing on the use of microalgal biomass for removing heavy metals by biosorption (Vijayaraghavan and Balasubramanian 2015; Kumar et al. 2016; Bilal et al. 2018). The removal efficiencies of heavy metals by microalgae vary greatly with species, ranging from 0.17 to 1055 mg/g biomass, as compiled in an extensive review by Suresh Kumar et al. (2015). Various microalgae and cyanobacteria including green algae such as *Chlorella*, *Chlamydomonas* and *Desmodesmus* and diatoms such as *Phaeodactylum*, *Cyclotella* and *Aulosira* as well as cyanobacteria such as *Spirulina*, *Oscillatoria* and *Phormidium* have been assessed in terms of their capability to remove metals. A summary of various studies on the use of microalgae for biosorption of heavy metals is presented in Table 23.1.

Most studies related to metal sorption focussed on Cu(II), with *Chlorella vulgaris* being the most commonly used microalga, tested in various forms, ranging from non-living, living, free to immobilized cells (Suresh Kumar et al. 2015). Wilke et al. (2006) examined the biosorption abilities of 37 strains of algae in removing Cd(II), Pb(II), Ni(II) and Zn(II) from aqueous solution. The study showed that the cyanobacterium *Lyngbya taylorii* exhibited high uptake capacities for the metals, with the order of selective sorption as follows: Pb >> Ni > Cd > Zn. Recently, Dirbaz and Roosta (2018) assessed the biosorption capacities of four microalgae in removing Cd (II) and found that *Parachlorella* sp. showed the highest metal uptake (96.2 mg/g biomass). There have also been studies on biosorption of toxic elements, particularly radionuclides, by microalgae (Naya et al. 2003; Bilal et al. 2018). While there have been many reports on biosorption of heavy metals, most studies were based on defined media or synthetic metal solution rather than industrial effluents (Table 23.1).

Bakatula et al. (2014) reported the high sorption efficiency of Cu, Co, Cr, Fe, Hg, Ni, Zn and uranium (U) in single- and multi-ion solutions by the filamentous green

Table 23.1 Summary of the studies on the use of microalgae for biosorption of heavy metals

Microalgae	Heavy metals	Metal concentrations tested	Biomass used	Test system	Findings	References
Chlamydomonas reinhardtii	Hg(II), Cg(II) and Pb(II)	100 mg/L	Cells harvested from logarithmic phase cultures	Algal biomass (800 mg/L) transferred into metal ions in NaCl solution (25 mL), agitated magnetically	Biosorption capacity	Tuzun et al. (2005)
					Hg(II) – 72.2 mg/g	
					Cd(II) – 42.6 mg/g	
					Pb(II) – 96.3 mg/g;	
					Optimum pH for maximum adsorption	
					Hg(II) and Cd (II) – pH 6.0	
					Pb(II) – pH 5.0	
					Followed Freundlich biosorption model	
Chlorella sp.	Cd (II)	10 mg/L	Algae immobilized in water hyacinth-derived pellets	Conical flasks	Maximal removal efficiency: 92.45% by the water hyacinth leaf biochar pellet immobilized with algal cells	Shen et al. (2018)
Chlorella minutissima	Cd, Cu, Mn and Zn	Cd: 0.2–0.6 mM	Dead (lyophilized) biomass	150 mL conical flask containing 50 mL metal solution	Maximum adsorption capacity	Yang et al. (2015)
		Cu: 0.2–1 mM				
		Mn: 2–6 mM			Cd – 35.36 mg/g	
		Zn: 2–6 mM			Cu – 3.28 mg/g	

(continued)

Table 23.1 (continued)

Microalgae	Heavy metals	Metal concentrations tested	Biomass used	Test system	Findings	References
					Mn –21.19 mg/g	
					Zn –33.71 mg/g	
Chlorella vulgaris	Cd (II)	100 mg/L	Live and dead biomass	500 mL conical flasks (with 200 mL metal solution)	Maximum adsorption capacity	Cheng et al. (2016)
					Live biomass – 16.34 mg/g	
					Dead biomass – 16.65 mg/g	
Chlorella vulgaris	Fe(II)	30–300 ppm	Dried biomass (freely suspended cells and immobilized in Ca-alginate)	Flasks containing 100 mL defined medium spiked with metals or palm oil mill effluent (POME)	Biosorption capacity (defined medium)	Ahmad et al. (2018)
	Mn(II)					
	Zn(II)				Suspended cells	
					Fe(II) – 74.54 mg/g	
					Zn(II) – 69.19 mg/g	
					Mn(II) – 65.14 mg/g	
					Immobilized cells	
					Fe(II) – 128.83 mg/g	
					Zn(II) – 115.90 mg/g	
					Mn(II) – 105.29 mg/g	

					Biosorption capacity (palm oil mill effluent, POME)	
					Suspended cells	
					Fe(II) – 15.23 mg/g	
					Zn(II) – 11.03 mg/g	
					Mn(II) – 9.43 mg/g	
					Immobilized cells	
					Fe(II) – 25.76 mg/g	
					Zn(II) – 21.76 mg/g	
					Mn(II) – 18.74 mg/g	
Desmodesmus multivariabilis; *Scenedesmus acuminutus*; *Chloroidium saccharophilum*; *Stichococcus bacillaris*	La	15–150 mg/L	Dead biomass	250 mL conical flasks	*Desmodesmus multivariabilis* – most efficient at adsorbing La	Birungi and Chirwa (2014)
					Maximal sorption capacity (q_{max}) = 100 mg/g	
					Affinity = 4.55 L/g	
Gelatinous colonies of cyanobacteria from paddy fields (*Cyanothece* spp., *Leptolyngbya* spp.	Cu(II), Cd (II) and Pb (II)	Cu(II) and Cd (II): 1, 2, 5, 10, 20, 30, 40 and 50 mg/L	Dried biomass	Not described	Maximum adsorption capacities (mg/g biomass)	Tran et al. (2016)
					Cu (II): 27.78	
					Cd (II): 28.57	

(continued)

Table 23.1 (continued)

Microalgae	Heavy metals	Metal concentrations tested	Biomass used	Test system	Findings	References
and *Phormidium* spp.)		Pb(II): 10, 20, 30, 40, 50, 80 and 100 mg/L			Pb (II): 76.92 Maximum desorption using 0.1 M HNO_3: >90%	
Oedogonium sp.	Cu, Co, Cr, Fe, Hg, Ni, Zn and U in single and multi-ion solutions	50–500 mg/L	Filamentous algal biomass collected from the dam receiving gold mine effluents	250 mL screw top polypropylene flasks	Single-ion solutions Maximal adsorption capacity at pH 2–5 for Cr, Cu, Ni and Zn Multi-ion solutions: adsorption capacity for the metals was constant over the pH 2–7 (except for Ni and U)	Bakatula et al. (2014)
Oedogonium sp. *Nostoc* sp.	Pb(II)	0.1–0.8 g/L	Freshly collected from ponds, ditches, etc.; dried before use	Not described	Maximum Pb (II) biosorption capacity *Oedogonium* sp. 145.0 mg/g *Nostoc* sp. 93.5 mg/g	Gupta and Rastogi (2008b)
Parachlorella sp.	Cd(II)	18–180 mg/L	Biomass from cultured microalgae	Equilibrium study: 0.02 g biomass in 20 mL Cd(II) solution Kinetic study: 1 g/L, 5 L shaking bioreactor at 1 rpm	Maximum uptake: 96.2 mg/g Equilibrium: Langmuir model Kinetics – pseudo-first order	Dirbaz and Roosta (2018)

Phaeodactylum tricornutum	Cd (II)	Cd (II): 1, 5, 10, 25, 50, 75 and 100 mg/L	Live cells	Glass (Pyrex) bottles with 500 mL seawater	Biosorption capacity 67.1 mg/g after 96 h with ~40% of this capacity in the intracellular fraction	Torres et al. (2014)
Scenedesmus obliquus	Cd (II)	Influent conc.: 2.5–7.5 mg/L Flow rate: 15 ml/min	Living cells immobilized in loofa sponge	Fixed-bed column	Adsorption capacity of 38.4 mg (breakthrough time at 15.5 h) was achieved at a flow rate of 5 ml/min with an influent concentration of 7.5 mg Cd/L	Chen et al. (2014)
Scenedesmus obliquus CNW-N	Cd(II)	25–200 mg/L	Algae grown in photobioreactor aerated with 2.5% CO_2; biomass harvested by centrifugation and concentrated by lyophilization	0.8 g dry weight/L suspended in 250 mL metal solution (flasks?)	Maximum biosorption capacity: 68.6 mg/g Optimum biosorption capacity: pH 6.0 Optimal temp: 30 °C Adsorption equilibrium Langmuir model Kinetics: pseudo-second order	Chen et al. (2012)
Scenedesmus quadricauda *Neochloris pseudoalveolaris*	Co(II), Cr(III), Pb (II), Cd(II), Ni (II) and Mn(II)	5–40 mg/L	Living cultures	50 mL polyethylene centrifuge tubes (10 mL metal ion solution)	Biosorption capacity Co(II) – 2.14–52.48 mg/g Cr(III) – 1.98–81.98 mg/g Pb(II) – 8.05–4.26 mg/g Cd(II) – 7.81–24.96 mg/g	Kızılkaya et al. (2012)

(continued)

Table 23.1 (continued)

Microalgae	Heavy metals	Metal concentrations tested	Biomass used	Test system	Findings	References
					Ni(II) – 2.17–55.71 mg/g	
					Mn(II) – 3.54–75.20 mg/g	
Spirogyra sp.	Pb(II)	100 and 200 mg/L	Collected from a pond, sun dried and then oven dried at 70 °C for 24 h	250 mL flask with 100 mL metal solution	Maximum adsorption capacity – 140 mg/g biomass at pH 5.0 in 100 min with 200 mg/L of initial concentration	Gupta and Rastogi (2008a)
					Equilibrium – Langmuir isotherm	
					Kinetics – pseudo-second order	
					Endothermic	
Spirulina platensis	Cr(III)	0–156.3 mg/L	Freshly harvested biomass	Conical flasks (50 mL metal solution)	Cr(III) uptake followed Langmuir isotherm model	Li et al. (2006)
					pH – the most important factor that influences metal uptake; more efficient at higher pH (i.e. pH 6)	

Spirulina platensis	Pb (II)	20–200 mg/L	Dead biomass	Conical flasks (100 mL metal solution)	>90% removal	Al-Homaidan et al. (2016)
					Optimal conditions for maximal adsorption	
					pH 3, 2 g adsorbent dose, 26 °C, 100 mg/L Pb initial conc., 60 min contact time	
Spirulina sp.	Effluent from copper smelter and refinery, with elevated levels of Hg and Cd	Cd – 3810 ug/kg	Cells lyophilizate	Cells lyopilizate (0.25 g) added to 1 L of effluent in aerated stirred tank reactor	Metal concentration factor: 80–4250	Chojnacka et al. (2004)
		Hg – 760 ug/kg			Bioaccumulation capacity	
					Cd – 463 ug/g biomass	
					Hg – 1340 ug/g biomass	
Ulothrix zonata	Cu(II)	5–50 mg/L	Algae collected from irrigated water channels; dried at 100 °C for 5–6 h	250 mL flask	Rapid removal of Cu (II) in the first 20 min	Nuhoglu et al. (2002)
				Algal suspension (10 g/L)	Optimum pH for Cu (II) removal – 4.5	

alga *Oedogonium* sp., collected from a site receiving gold mine wastewater. The adsorption equilibrium fitted the Freundlich model. The test algal biomass could be repeatedly used for three cycles of adsorption/desorption. In another study, Birungi and Chirwa (2014) investigated the adsorption and desorption kinetics of lanthanum (La) on four freshwater microalgae. Amongst the species tested, *Desmodesmus multivariabilis* was found to be most efficient at adsorbing La (100 mg/g) and was the best desorbent, with metal recovery of higher than 99%.

The affinity of microalgae in removing metals by biosorption varies with the type of metals. For instance, the affinity of *Chlamydomonas reinhardtii* for selected metals can be ranked in the following order: Pb(II) > Hg(II) > Cd(II) (Tuzun et al. 2005). The same order of biosorption capacities was observed when the biosorbent was tested in multi-metal system consisting of the three metals. This could be attributed to competitive binding amongst the metal ions for binding sites on the algal surface. Rajfur et al. (2012) compared the biosorption capacities of the biomass of *Spirogyra* sp. in removing Cu(II) in static and dynamic system. In the static system, the algal biomass was placed in a perforated container immersed in a fixed volume of $CuSO_4$ solution, while in the dynamic system, there was continuous flow of the metal solution. The sorption parameters were influenced by the ratio of algal biomass to the volume of metal solution in the static system but not in the dynamic system.

Monteiro et al. (2009) compared the capacities of two strains of *Desmodesmus pleiomorphus*, one isolated from a polluted site and another from culture collection, in removing Cd(II). The Cd(II) removal capacities of live cells of the wild and culture collection strains were comparable, with maximal Cd(II) removal of 61.2 and 76.4 mg/g biomass, respectively. The metal ions were mainly removed by adsorption onto cell surface of the microalgae, with an initial fast uptake, reaching maximum removal after 1 day exposure. Doshi et al. (2006) assessed the efficiency of bloom algae, consisting predominantly of *Chlorella* sp., in removing Cd(II) and Ni(II) and methyl orange (azo dye) by biosorption. The study found that the algal biomass was more efficient in removing metal ions compared to the dye. In addition, Nuhoglu et al. (2002) reported the potential of using dried biomass of *Ulothrix zonata* collected from the wild as a low-cost biosorbent for removal of Cu(II). In another study, dried biomass of the filamentous alga, *Spirogyra*, was reported to be an efficient biosorbent for Pb(II) from aqueous solution (Gupta and Rastogi 2008a). Nayak et al. (2003) assessed the biosorption capacities of living cells of three genera of algae in removing heavy metals and toxic radionuclides. Amongst the algae, *Spirulina* showed the highest radionuclide accumulation at pH 8, while *Oedogonium* adsorbed highest amount of Hg at acidic pH.

Various pretreatments may be employed to enhance metal sorption capacity by modifying cell wall properties of the microalgae (Mehta and Gaur 2005). Physical treatments that can be used include heating/boiling, freezing/thawing, drying and lyophilization, while chemical treatments may include washing the biomass with detergents, cross-linking with organic solvents and acid and alkali treatments (Wang and Chen 2009). For instance, *Oedogonium hatei* biomass treated with 0.1 M HCl was found to be an efficient biosorbent for Ni(II) ions from aqueous solution (Gupta et al. 2010). Similarly, pretreatment with 0.1 mM HCl enhanced the sorption

capacity of biomass of *Chlorella vulgaris* in removing metals from single and binary metal solutions (Mehta et al. 2002b). It was suggested that acid pretreatment released the adsorbed cations, including metal ions from the algal biomass, freeing the sites for metal binding. Pretreatment with $CaCl_2$ is another method used for the activation of algal biomass for biosorption (Mehta and Gaur 2005). For instance, pretreatment with $CaCl_2$ enhanced Pb(II) sorption capacity of *Spirulina maxima* biomass by 84–92% (Gong et al. 2005).

Immobilized algal cells have also been used in removing metals and have been shown to be more efficient than free cells. Recently, Shen et al. (2018) reported the high efficiency of Cd (II) removal using *Chlorella* sp. immobilized in water hyacinth-derived pellets. A maximum bioaccumulation capacity of 13.81 mg/g of the complex of water hyacinth biochar immobilized with algal cells was attained. The study also found that intracellularly sorbed Cd(II) accounted for 34.8% of the total metal ions adsorbed. Recovery tests showed that both the algal cells and biochar pellets could be recycled and reused. In another study, a continuous fixed-bed biosorption process using *Scenedesmus obliquus* immobilized in loofa sponge was found to be feasible in removing Cd(II) (Chen et al. 2014). Repeated adsorption/desorption cycles showed that the fixed-bed system could be reused.

Saeed and Iqbal (2006) reported that biosorption of Cd(II) by *Synechococcus* sp. immobilized in loofa sponge (*Luffa* sp.) could be enhanced by 21% compared to free biomass. In another study, immobilized *Chlorella vulgaris* in Ca-alginate beads was found to have higher biosorption capacity in removing Fe(II), Mn(II) and Zn (II) compared to free-suspended cells (Ahmad et al. 2018). Immobilized cells of *Chlamydomonas reinhardtii* in Ca-alginate was found to be efficient in biosorption of Hg(II), Cd(II) and Pb(II) (Bayramoğlu et al. 2006). Similarly, Wan Maznah et al. (2012) found that *Chlorella* sp. immobilized in sodium alginate beads showed higher biosorption capacity in removing Cu(II) and Zn(II) than free biomass. In another study, Mohapatra and Gupta (2005) assessed the ability of immobilized cells of *Oscillatoria angustissima* to remove Co(II), Cu(II) and Zn(II) in single, binary and ternary metal systems. The affinity of single metal removal could be ranked as follows, Cu(II) > Co(II) > Zn (II), while in binary system, Cu(II) inhibited the sorption of both Zn(II) and Co(II).

Cheng et al. (2016) evaluated the biosorption capacity and kinetics of Cd(II) by both living and non-living biomass of *Chlorella vulgaris*. The study showed that both living and dead cells of *Chlorella vulgaris* had high adsorption capacity for Cd (II), removing 96.8% and 95.2% of the metal, respectively. The biosorption efficiency of Cd(II) in natural water by the alga was lower, with values of 61.6% and 81.5% for living and dead biomass, respectively. In another study, Yang et al. (2015) reported that removal of Cd, Cu, Mn and Zn by living cells and lyophilized biomass of *Chlorella minutissima* was mainly through intracellular accumulation and partly by extracellular adsorption. In another study, Wehrheim and Wettern (1994) showed that whole cells of *Chlorella fusca* accumulated more metal ions than isolated algal cell walls.

Torres et al. (2014) reported that *Phaeodactylum tricornutum* has the potential for bioremediation of Cd in saline habitats. The diatom is highly tolerant to cadmium

and could remove Cd(II) through biosorption and bioaccumulation. In another study, gelatinous colonies of mixed population of cyanobacteria from paddy fields were found to be efficient for the adsorption of Cu (II), Cd (II) and Pb(II) (Tran et al. 2016). The cyanobacterial biomass could be reutilized for three biosorption-desorption cycles with only slight decrease in their biosorption capacity. Gupta and Rastogi (2008b) assessed the biosorption capacities of dried biomass of two filamentous microalgae, *Oedogonium* sp. and *Nostoc* sp., in removing Pb(II). The study showed that *Oedogonium* sp. was more efficient than *Nostoc* sp. in removing Pb(II), and regeneration of both biosorbents with recovery up to 90% could be attained using 0.1 M HCl. Regeneration of the biosorbent is important to ensure the reusability of the biomass and recovery of the sorbate. For instance, Chen et al. (2012) showed that cadmium-loaded biomass of *Scenedesmus obliquus* could be regenerated, with 0.1 M HCl giving higher desorption efficiency than 0.1 M $CaCl_2$. However, treatment with $CaCl_2$ was preferred as the regenerated biosorbent retained good adsorption capability even after five consecutive adsorption/desorption cycles.

There are only few reports on the use of microalgae for biosorption of metals from real industrial effluents. In one study, freely suspended and immobilized *Chlorella vulgaris* was shown to be efficient in removing Fe(II), Mn(II) and Zn(II) from palm oil mill effluent (POME) by biosorption (Ahmad et al. 2018). Chojnacka et al. (2004) reported that *Spirulina* sp. could remove trace elements, especially Hg and Cd, from industrial effluent from copper smelter and refinery by biosorption and bioaccumulation. In another study, El-Sheekh et al. (2005) showed that *Nostoc muscorum* and *Anabaena subcylindrica* were able to grow in effluent from salt and soda factory and sewage wastewater, removing metals such as Cu, Co, Pb and Mn.

4.2 Biosorption of Dyes

A wide range of microalgae, including both unicellular and filamentous species, have been shown to have good biosorption capacity in removing various dyes, especially malachite green and methylene blue (Table 23.2). Most of the studies were done using non-viable algal biomass on synthetic dye solution. In one study, algal biomass of *Microspora* sp. after lipid extraction was found to be an efficient biosorbent for methylene blue, removing the dye up to 100% in 24 h when agitated at 150 rpm (Maurya et al. 2014). Defatted algal biomass from an oleaginous microalga, *Scenedesmus dimorphus*, was also evaluated for its efficiency in removing methylene blue by biosorption (Sarat Chandra et al. 2015). The maximum adsorption capacity of the defatted algal biomass was comparable to raw and acid-pretreated biomass. Waste residue from algal biodiesel industry has been shown to be useful as biosorbent for dye removal. For instance, Nautiyal et al. (2017) reported that biochar derived from *Spirulina platensis* after oil extraction for biodiesel was found to be an efficient biosorbent for methylene blue. In another study, Chen et al. (2018) showed that biochar derived from residual biomass of *Ulothrix zonata* after pigment extraction could be used as a low-cost biosorbent for malachite green, crystal violet and Congo red.

Table 23.2 Summary of studies on the use of microalgae for biosorption of dyes

Microalgae	Dye	Concentrations tested	Biomass used	Test system	Findings	References
Chlorella sp.	Malachite green	2.0–20.0 mg/dm^3	Waste biomass from algae-manufacturing industry	3 L stirred batch biosorption apparatus with four baffles	Biosorption capacity –9.45–33.7 mg/g Kinetic of removal followed pseudo-second-rate model	Tsai and Chen (2010)
Chlorella pyrenoidosa	Methylene blue in simulated textile wastewater	10–60 mg/L	Harvested from cultures grown in textile wastewater Wet and dried biomass	250 mL conical flask containing 100 mL textile wastewater added with methylene blue; 0.15 g biomass	Adsorption isotherms Langmuir and Freundlich Kinetics Pseudo-second order Adsorption capacity Dry biomass 7.2–29.2 mg/g Wet biomass 5.6–18.24 mg/g	Pathak et al. (2015)
Chlorella vulgaris	Remazol Black B (RB) Remazol Red RR (RR)	20–800 mg/L	Cultured in defined medium before harvesting, drying and homogenizing the biomass for the study	10 mL dried biomass suspension added to 90 mL dye solution; biosorbent load: 1 g/L	RB – adsorbed most effectively (419.5 mg/g) Biosorption capacity increased with increasing initial dye conc.	Aksu and Tezer (2005)

(continued)

Table 23.2 (continued)

Microalgae	Dye	Concentrations tested	Biomass used	Test system	Findings	References
	Remazol Golden Yellow RNL (RGY)				Biosorption equilibrium models – Freundlich, Langmuir, Redlich-Peterson and Koble-Corrigan	
					RB – sorption increased with increasing temperature up to 35 °C	
					RR and RGY – sorption decreased with increasing temperature	
Chlorella vulgaris	Textile dyes (Supranol Red 3BW, Lanaset Red 2GA and Blue EBNA) and textile wastewater	0–60 mg/L	Cells immobilized in 1% κ-carrageenan and 2% alginate	Immobilized cells were grown in flasks containing Bold's Basal Medium added with dye or 40% textile wastewater supplemented with 1.47 mM $NaNO_3$	Highest % colour removal from Lanaset Red 2GA (44.0%)	Chu et al. (2008)
					% colour removal from Supranol Red 2GA and Lanset Red 2GA	
					Cells immobilized in 1% κ-carrageenan > cells in 2% alginate	
					% colour removal from textile wastewater	
					Immobilized > suspension cultures	

Chlorella vulgaris	Textile wastewater (TW) and Supranol Red 3BW	TW: 36 L Supranol Red: 20 mg/L	Live cultures – 10% inoculum	High rate algae ponds (HRAP)	Colour removal up to 50%, with removal of NH_4–N (44.4–45.1%), PO_4–P (33.1–33.3%) and COD (38.3–62.3%) Sorption of dye – Langmuir and Freundlich models	Lim et al. (2010)
Microspora sp.	Methylene blue	20–2500 mg/L	Collected from coastal lagoons, used after oil extraction	500 mL flasks containing 200 mL solution	Dye removal – 5–7 g/L	Maurya et al. (2014)
				Fixed-bed class column (1.2 × 15 cm) packed with 0.3 g algal biomass	Dye removal –107.57 to 139.11 mg/g	
Pithophora sp.	Malachite green	20–100 mg/L	Collected from the wild (fountain) Thermally activated at 300 °C	30 mL dye in 50 mL beaker	Dye removal Raw algae 64.4 mg/g Thermally activated 117.6 mg/g	Kumar et al. (2005)
Scenedesmus dimorphus	Methylene blue	1–5 mg/L	Harvested from high rate algae ponds Raw, defatted and acid-pretreated biomass	2 L flasks	Maximum biosorption capacity Raw 6.0 mg/g Defatted – 7.73 mg/g Acid-pretreated 7.80 mg/g	Sarat Chandra et al. (2015)

(continued)

Table 23.2 (continued)

Microalgae	Dye	Concentrations tested	Biomass used	Test system	Findings	References
Spirulina platensis	Methylene blue	30–200 mg/L	Residual biomass after oil extraction, sun and oven dried, followed by physical activation in a muffle furnace at 450 °C for 2 h (biochar)	250 mL flask	>80% dye removal in <5 min	Nautiyal et al. (2017)
				Biosorbent dosage: 1 g/100 mL	Uptake	
					Biochar – 4.60 mg/g	
					Raw biomass – 4.17 mg/g	
Spirogyra sp.	Azo dye	5, 10 and 15 mg/L	Collected from a eutrophic lake, dried under sunlight, biomass treated with 0.1 N HCl	100 mL dye solution in 250 mL glass bottles	Dye removal efficiency: 35.3–64.0%	Mohan et al. (2008)
				0.1 g biosorbent	Increased with increasing temperature (10–50 °C)	
					Increased with decreasing pH (2.0–10)	
					Isotherm: Langmuir model	
Spirogyra sp.	Reactive Yellow 22	Simulated synthetic dye effluent – 25, 50 and 100 mg/L	Collected from a pond, viable algae	100 mL simulated dye solution in bottle	Initial acclimatization of 12-h contact time required	Venkata Mohan et al. (2002)
				Biosorbent dosage: 0.5, 1.0 and 2.5 g/100 mL	Maximum dye uptake – 72 h of contact time	
					Average removal capacity	
					0.4 g dye/g biomass (dry weight)	

Spirogyra sp.	Textile wastewater (TW) containing 0.22% Synazol Red dye	Raw TW	Collected from pond, dried at 80 °C for 20 h, pretreated by autoclaving (121 °C, 20 min)	100 mL TW in 250 mL flasks 0.2 g algal biomass	Maximal dye removal: 85% Three repeated runs using the same biosorbent showed the similar biosorption rate	Khalaf (2008)
Ulothrix zonata	Malachite green, crystal violet and Congo red	Malachite green 500 mg/L Crystal violet 200 mg/L Congo red 200 mg/L	Collected from the wild, biomass used was after pigment extraction, dried at 105 °C for 12 h, subjected to pyrolysis at 400 °C to produce biochar	10 mL dye solution in 30 mL glass reactor added with 0.005 g algal biochar	Maximal dye adsorption capacity Malachite green – 5306 mg/g; Equilibrium isotherm Freundlich model Kinetics: Pseudo-second-order model	Chen et al. (2018)

Devi et al. (2014) reported that the biosorption capacity of dried biomass of *Spirulina platensis* in removing reactive blue 19 dye (96.9 mg/g) was higher than that of the seaweed *Gracilaria edulis* (82.3 mg/g). In addition, *Chlorella*-based biomass derived from algae-manufacturing waste was found to be an efficient low-cost biosorbent for the removal of malachite green (Tsai and Chen 2010). Pretreatment of the algal biomass has been shown to enhance biosorption of dyes in several studies. For instance, thermally activated *Pithophora* sp. at 300 °C could enhance the sorption capacity of the biomass in removing malachite green compared to raw algae (Kumar et al. 2005). Pretreatment by autoclaving is another method to enhance the colour removal capacity of algal biomass, as indicated in the studies on the removal of Synazol reactive dye by *Spirogyra* sp. (Khalaf 2008) and malachite green by *Cosmarium* sp. (Daneshvar et al. 2007).

Living microalgae and cyanobacteria have also been shown to be able to efficiently remove colour from dyes and to treat dye-containing effluent. For instance, three cyanobacteria, *Anabaena flos-aquae* UTCC64, *Phormidium autumnale* UTEX1580 and *Synechococcus* sp. PCC7942, were evaluated for their efficiency in degrading textile dyes (indigo, RBBR and sulphur black) and dye-containing effluent (Dellamatrice et al. 2017). The study showed that the cyanobacteria could be used for bioremediation of textile effluent, particularly in removing the colour and reducing the toxicity of the dyes. It is noteworthy that *Phormidium autumnale* UTEX1580 could completely degrade indigo dye after 19 days of incubation.

In another study, *Chlorella vulgaris* grown in HRAP was found to remove up to 50% of the colour from textile wastewater and also reduced the load of other pollutants such as ammonia and phosphate (Lim et al. 2010). In addition, colour reduction up to 50% was achieved in the ponds containing textile dye alone (Supranol Red), which was attributed to sorption by the algal cells. The study concluded that the HRAP system growing *Chlorella vulgaris* could be an efficient system for the polishing of textile wastewater before discharge. Another study which highlighted the potential use of microalgae for bioremediation of textile wastewater and removal of dyes was by Pathak et al. (2015). In that study, *Chlorella pyrenoidosa* was found to be able to grow in textile wastewater, reducing phosphate, nitrate and BOD by 87%, 82% and 63%, respectively. Both wet (living) and dried (non-living) algal biomass from the cultures were further assessed for their ability to remove methylene blue in simulated textile wastewater. Dried biomass showed higher sorption efficiency due to its large surface area and high binding affinity for methylene blue compared to wet biomass. Recently, Dhaouefi et al. (2018) reported on the potential of an anoxic-aerobic photobioreactor for the treatment of synthetic textile wastewater involving microalgae-bacteria symbiosis. Efficient removal of carbon, nitrogen and phosphorus and heavy metals, as well as decolourization of the textile wastewater, was attained. However, the involvement of biosorption in the treatment system was not assessed in that study.

Apart from suspension cultures, immobilized microalgae have been shown to be efficient in removing colour from textile dyes and textile wastewater. For instance, *Chlorella vulgaris* immobilized in 2% alginate could remove 44% of the colour from the textile dye Lanaset Red 2GA at an initial concentration of 7.25 mg/L (Chu et al.

2008). The study also found that immobilized cells in alginate removed higher percentage of colour (48.9%) from the textile wastewater than the suspension cultures (34.9%).

5 Mechanisms and Equilibrium Modelling of Biosorption

Biosorption involve mechanisms such as adsorption, ion exchange and complexation/coordination (Gadd 2009). Ion exchange is the major mechanism of biosorption, which occurs through different functional groups present on the surface of the biomass (Verma et al. 2008). Through ion exchange, a readily exchangeable ion on the sorbent is replaced by another. Due to the weak attachment with the biomass, monovalent ions (H^+ and Na^+/K^+) are replaced with divalent ions of metals (Verma et al. 2008).

Variation in functional groups on the surface of the cell wall gives rise to the difference in biosorption mechanisms (Volesky 2007; Vijayaraghavan and Yun 2008; Wang and Chen 2009). Functional groups on cell wall of algae that are important for biosorption include carbonyl (ketone), carboxyl, sulfhydryl (thiol), sulfonate, thioether, amine, secondary amine, amide, imine, imidazole, phosphonate and phosphodiester (Volesky 2007). Eukaryotic algal cell wall consists of mainly cellulose, with potential metal-binding chemical groups including carboxylate, amine, imidazole, phosphate, sulfhydryl, sulphate and hydroxyl (Crist et al. 1981). The binding of metals with cell wall constituents of algae involves mainly ionic charge bonding while there is also covalent bonding with constituent proteins (Crist et al. 1981).

Recently, Ahmad et al. (2018) demonstrated through scanning electron microscopy (SEM)/energy-dispersive X-ray (EDX) analysis that sulphate, carboxyl and hydroxyl groups were involved in biosorption of metals by *Chlorella vulgaris*. In comparison, Fourier transform infrared (FTIR) spectra showed that binding of Cd (II) by *Chlamydomonas reinhardtii* involved mainly complexation to carboxylic functional groups (Adhiya et al. 2002). In another study, FTIR analysis revealed that the presence of amino, carboxyl, hydroxyl and carbonyl groups were responsible for the sorption of Pb(II) by the biomass of *Spirogyra* sp. (Gupta and Rastogi 2008a). Similarly, the main chemical interactions involved in the biosorption of Cd(II) by *Scenedesmus obliquus* were ion exchange between the metal ions and the hydrogen atoms of carboxyl, hydroxyl and amide groups of the algal biomass (Chen et al. 2012). Pores present on the surface of the algal biomass are also important in facilitating the sorption of metal ions. In another study, Li et al. (2006) investigated the process and mechanism of Cr(III) uptake using biomass of *Spirulina platensis*. The study showed that initially, Cr(III) was adsorbed to the unoccupied, negative sites on the surface of algal cell wall by electrostatic attraction. This was followed by chemical complexation and ion exchange and then binding to algal cell components such as proteins, polysaccharides and lipids.

In relation to biosorption, metal ions can be divided into A, B and borderline subcategories depending on their affinities to bind to cellular ligands, which are classified into Categories I, II and III (Volesky 2007; Wang and Chen 2009). Metal ions of Class A (e.g. Al, La and Ca) bind to Category I ligands through an oxygen atom. In comparison, metal ions Class B (e.g. Cu, Co, Zn and Cd) connect to ligands of Category II (e.g. RNH_2, -CO and $=N^-$) and Category III (e.g. S^{2-}, CO and CN^-). Borderline cations (e.g. Fe^{2+} and Mn^{2+}) can bind to various atoms of ligands from Categories I, II and III.

The cell wall biosorptive component of cyanobacteria consists of mainly peptidoglycan, with some species also produce extracellular mucilaginous polysaccharides (Fomina and Gadd 2014). Negatively charged exopolysaccharides produced by cyanobacteria have been shown to be an important chelating agent in removing positively charged metal ions from aqueous solution (De Philippis et al. 2011). For instance, Okajima et al. (2009) developed the megamolecular polysaccharide sacran, containing carboxylate and sulphate groups from the extracellular matrix of *Aphanothece sacrum*, for biosorption of metals such as indium and lead.

The mechanisms involved in dye biosorption include surface adsorption, chemisorption, diffusion and adsorption-complexation (Crini and Badot 2008). Amongst these, chemisorption involving the exchange of electrons is the main mechanism involved in the adsorption of anionic dyes in acidic conditions. Various kinds of interactions, such as chemical bonding, ion exchange, hydrogen bonds, hydrophobic interactions, van der Waals force, physical adsorption, aggregation mechanisms and dye-dye interactions, may also be involved. According to Crist et al. (1981), dye removal by adsorption on an adsorbent material may involve the following four steps: (1) bulk diffusion (migration of dye from the bulk of the solution to the adsorbent surface), (2) film diffusion (diffusion of dye through the boundary layer to the adsorbent surface), (3) pore diffusion or intraparticle diffusion (transport of the dye from the surface to within the pores of the particle) and (4) chemical reaction (adsorption of dye at an active site on the surface of the sorbent via ion exchange), complexation and/or chelation.

Maurya et al. (2014) demonstrated that the biosorption of dye by de-oiled algal biomass involved chemisorptions via surface active charges in the initial phase followed by physical sorption by occupying pores of the biomass. Similarly, Tsai and Chen (2010) concluded that the biosorption of malachite green by *Chlorella* biomass was due to the electrostatic interactions between the negatively charged surface area and positively charge dye molecule. In addition, the removal of azo dye by biomass of *Spirogyra* sp. was suggested to be due to the combined effect of chemical and ion-exchange sorption phenomena (Mohan et al. 2008).

While most studies tested on pure dye solution, Venkata Mohan et al. (2002) assessed the ability of *Spirogyra* to remove Reactive Yellow 22 azo dye from simulated dye effluent. The authors suggested that the mechanisms involved not only biosorption but also bioconversion and bioagulation. After being adsorbed onto the cell surface, the dye molecules diffuse into the algal cells and undergo subsequent bioconversion. The dye molecules in the aqueous phase coagulate with the biopolymers released as metabolic intermediates during metabolic conversion of the

dye. In another study, Chen et al. (2018) showed that dye adsorption by algae can be enhanced by subjecting the biomass to pyrolysis (800 °C), which could be due to the increased porosity and surface area values. At high temperature, the algal biomass is well carbonized because volatile matter such as cellulose and hemicellulose is removed.

A biosorption isotherm, the plot of uptake (Q) versus the equilibrium solute concentration in the solution (C_f), is often used to evaluate the sorption performance (Vijayaraghavan and Yun 2008). Two most commonly used modelling to explain, represent and predict the experimental behaviour of biosorption are the Langmuir and the Freundlich isotherms. The Langmuir isotherm is represented as follows: $Q = (Q_{\max} b_L C_f)/(1+b_L C_f)$, where Q_{max} = maximum achievable uptake by a system, b_L = affinity between the sorbate and sorbent and C_f = equilibrium solute concentration. The Langmuir constant ($Q_{\max}$) is often used to compare the performance of biosorbents. According to this model, there are a finite number of uniform adsorption sites and absence of lateral interactions between adsorbed species. In comparison, the Freundlich isotherm is represented as $Q = K_F C_f^{1/\mathrm{nF}}$, where K_F corresponds to the binding capacity, while nF characterizes the affinity between the sorbent and sorbate. The isotherm is used to characterize the sorption to heterogenous surfaces or surfaces supporting sites with various affinities (Gadd 2009). The biosorption isotherms may vary with the type of algal biomass used. For instance, the biosorption equilibrium of metal biosorption by *Synechococcus* sp. fitted the Langmuir adsorption isotherm (Saeed and Iqbal 2006). In comparison, Aksu (2001) showed that biosorption of Cd (II) by *Chlorella vulgaris* fitted well both the Langmuir and Freundlich models.

6 Factors Affecting Biosorption of Metals and Dyes by Microalgae

Factors that influence biosorption process include physical and chemical properties of metal ions (e.g. molecular weight, ionic radius and oxidation state), properties of biosorbent (e.g. structure of the biomass surface) and the process parameters (e.g. pH, temperature and concentrations of biosorbent and sorbate) (Davis et al. 2003). Amongst these factors, pH is a key factor that influences the dissociation of sites, solution chemistry of metal ions, hydrolysis, complexation by organic and/or inorganic ligands, redox reactions and precipitation as well as the speciation and the biosorption affinity of metal ions.

Maximum adsorption capacity of metals by algal biomass occurs mainly at acidic pH. For instance, non-viable biomass of *Nostoc muscorum* removed highest amount of [Cr(VI)] at pH 3.0 (Gupta and Rastogi 2008c). Similarly, the maximum biosorption of Cd(II) by both free and immobilized cells *Synechococcus* sp. was at pH 4.0 (Saeed and Iqbal 2006). In comparison, maximum removal of Hg(II), Cd (II) and Pb(II) by immobilized cells of *Chlamydomonas reinhardtii* occurred at pH 5.0–6.0 (Bayramoğlu et al. 2006). The biosorption of Cd(II) by *Chlorella vulgaris*

increased with pH up to 4.0 and then decreased with further increase in pH (Aksu 2001). The low biosorption at extremely low pH (2–3) was postulated to be due to the association of hydronium ions (H_3O^+) with cell wall ligands, which restrict the binding of metal ions because of the repulsive force. In general, increasing pH enhances sorption of cationic dyes or basic dyes but reduces that of anionic metals or acidic dyes (Vijayaraghavan and Yun 2008). For instance, sorption of azo dye (an acidic dye) by non-viable biomass of *Spirogyra* sp. was found to be higher at lower pH (Mohan et al. 2008).

Increasing temperature generally enhances biosorption by increasing surface activity and kinetic energy of sorbate, but this may also damage the physical structure of the biosorbent (Park et al. 2010). For instance, Aksu (2001) showed that biosorption capacity of *Chlorella vulgaris* in removing Cd(II) decreased from 85.3 to 51.2 mg/g with an increase in temperature from 20 to 50 °C. It was postulated that as Cd(II) biosorption is normally an exothermic process, the capacity is higher at low temperature. In contrast, temperature variations from 5 to 40 °C did not affect the biosorption capacities of immobilized cells of *Chlamydomonas reinhardtii* in removing Hg(II), Cd(II) and Pb(II) ions from aqueous solution (Bayramoğlu et al. 2006). In addition, increased agitation speed may enhance biosorptive removal rate of the pollutant by minimizing mass transfer resistance (Park et al. 2010). However, when the mixing speed is too high, it may reduce the biosorption capacity. For instance, uptake of Cd(II) by *Parachlorella* sp. decreased when mixing speed was increased to 250 rpm due to damage of the algal cells. Apart from pH and temperature, culture age may affect biosorption capacity of microalgae in removing metal ions. For instance, Mehta et al. (2002a) found that older cultures of *Chlorella vulgaris* showed higher Cu(II) adsorption capacity than exponentially growing cultures, suggesting that there may be new/additional sites in older cells.

The increase of initial pollutant concentration may increase the quantity of biosorbed pollutant per unit weight of biosorbent (Fomina and Gadd 2014). For instance, the total amount of Cd(II) removed by *Desmodesmus pleiomorphus* increased with increasing initial metal concentration (Monteiro et al. 2009). Similarly, the amounts of Cu(II) adsorbed by *Ulothrix zonata* increased with increasing concentration of the sorbate (Nuhoglu et al. 2002). In addition, the presence of other pollutants may affect biosorption efficiency, as they may compete for the binding sites. The biosorptive removal of the target pollutant may be reduced due to increasing concentration of competing pollutants. The competitive effect of multi-metal ions on biosorption capacity may be influenced by the concentration of algal biomass tested. For instance, competitive effects on biosorption of Cu(II) and Cd (II) were only observed in *Scenedesmus abundance* at the lowest algal concentration tested (15.1 mg/L).

The concentration of algal biomass used may affect the removal of heavy metals by biosorption. For instance, the capacity of *Scenedesmus abundance* in removing Cd (II) and Cu(II) increased with decreasing concentration of algal biomass (Terry and Stone 2002). Similarly, the amount of Cu(II) adsorbed to *Ulothrix zonata* increased from 38 to 160 mg/g with the decrease of algal biomass from 1.0 to 0.1 g/L (Nuhoglu et al. 2002). In another study, the adsorption capacity of *Scenedesmus obliquus* for Cd

(II) decreased with increasing adsorbent dosage, but the removal efficiency was nearly 100% at dosage higher than 0.6 g (Chen et al. 2012). Similarly, the removal of Pb (II) by *Spirogyra* sp. increased from 31.2% to 80% with the increase of adsorbent dose from 0.05 to 10 g/L (Gupta and Rastogi 2008a).

Maurya et al. (2014) assessed the influence of multiple factors on biosorption efficiency of de-oiled algal biomass in removing methylene blue based on an artificial neural network model. The results showed that the relative importance and ranking of the input variables for dye removal efficiency are as follows: temperature > agitation speed > contact time > pH > initial dye concentration > adsorbent dose. In another study, efficiency in the removal of Acid Red 274 dye by *Spirogyra rhizopus* was influenced by initial pH, temperature and initial dye and algal biomass concentrations (Özer et al. 2006). The optimum conditions for dye removal by the alga were at initial pH 3.0, temperature 30 °C and algal concentration 0.5 g/L.

The removal of azo dye (Reactive Yellow 22) by *Spirogyra* was also found to be dependent on the concentrations of dye and algal biomass (Venkata Mohan et al. 2002). An incremental increase of 10% dye removal was observed for every 0.5 g increase of biomass, which could be attributed to the more surface area for sorption with the increased biomass. In addition, the authors observed that there was increase and then decrease in pH during the dye removal process, which could be attributed to the interaction between the cell surface and dye molecule. In comparison, highest sorption of Synazol (anionic dye) from textile wastewater by *Spirogyra* sp. occurred at pH 3.0, with very little removal between pH 6.0 and 8.0 (Khalaf 2008). Similarly, highest sorption capacity of dried biomass of *Chlorella vulgaris* in removing three vinyl sulfone-type reactive dyes (Remazol dyes) was at pH 2.0 (Aksu and Tezer 2005). The sorption capacity was also affected by temperature, with maximum capacity at 25 or 35 °C, depending on the type of dyes.

7 Living Versus Non-living Algal Biomass for Biosorption

The use of non-living algal biomass is preferred to living cells for removal of metals and dyes by biosorption. It is advantageous to use non-living biomass as there is the absence of toxicity limitations, absence of requirements for growth media and nutrients, easy recovery of the sorbates, easy regeneration and reuse of biomass, possibility of easy immobilization of dead cells and easier mathematical modelling of uptake of sorbates (Dhankhar and Hooda 2011). However, there may be a need to use living cells for overall removal of heavy metals and other pollutants. For instance, the use of metal-resistant microalgae is desirable to ensure better removal of metals and other pollutants, involving a variety of processes including bioprecipitation, biosorption and continuous uptake of metals after physical adsorption (Malik 2004). Such processes may lead to simultaneous removal of toxic metals, organic pollutants and other inorganic impurities. Metabolic processes are important in bioremediation systems such as sewage treatment, biofilm reactors for pollutants and anaerobic digestion, where biosorption is a component of the overall process (Gadd 2009).

There have been reports on the use of both living and non-living algal biomass for biosorption of heavy metals. For instance, Kızılkaya et al. (2012) and Alam et al. (2015) showed that living biomass of *Scenedesmus quadricauda* and *Neochloris pseudoalveolaris* was effective in removing Co(II), Cr(III), Pb(II), Cd(II), Ni(II) and Mn(II) from aqueous system by biosorption. Adhiya et al. (2002) reported that lyophilized and living cells of *Chlamydomonas reinhardtii* showed similar ATR-FIR spectra, suggesting that lyophilization did not change the chemical composition of the cell surface, including cell wall. However, thermally inactivated cells of *Desmodesmis pleiomorphus* showed lower Cd(II) adsorption than living cells at the highest metal concentration tested (5 mg/L) (Monteiro et al. 2009). In another study, living cells of *Scenedesmus abundans* were found to be more efficient in removing Cu(II) and Cd(II) than non-living algae (Terry and Stone 2002). The use of microalgae with self-flocculating ability as biosorbent is advantageous as it reduces the harvesting costs. For instance, Alam et al. (2015) reported the potential use of a self-flocculating strain of *C. vulgaris* as an efficient biosorbent for the removal of Cd (II), with a maximum sorption capacity (144.93 mg/g), which was much higher than the non-flocculating strain (84.03 mg/g).

Both living and non-living algal biomass have also been assessed for their ability to remove dyes (Daneshvar et al. 2007). For instance, decolourization of malachite green dye by living cells of *Cosmarium* sp. was mainly by biodegradation (Daneshvar et al. 2007). However, autoclaved dead biomass could also remove the dye by 63%, comparable to living cells (74%). It was suggested that autoclaving ruptured the cells, increasing the surface area for sorption and exposing more binding sites on the cell wall.

8 Scaling Up and Commercialization of Biosorption Technologies Based on Microalgae

Biosorption technologies based on microalgae for removal of metals and dyes have not been fully developed for large-scale applications in industries (Ghosh et al. 2016). Most studies on biosorption were conducted using synthetic solution under controlled laboratory based on single species of algae. Actual industrial effluent is much more complex, consisting of metals and dyes together with other organic compounds and salts. The test algae may not be efficient in removing the dyes or metals on a larger scale using actual effluent. Lim et al. (2010) evaluated the potential application of *Chlorella vulgaris* for bioremediation of textile wastewater using HRAP. Apart from 41.8% to 50.0% of colour removal, there was also significant reduction in other pollutants such as ammoniacal nitrogen, phosphate and carbon oxygen demand (COD) from the wastewater. The use of HRAP to produce high-density cultures is well established as an efficient system for treating agroindustrial effluents (Phang et al. 2001, 2015; Mustafa et al. 2011).

Batch culture studies are important to gather sufficient data before scaling up of the biosorption system (Kumar et al. 2016). Continuous flow studies, such as those conducted using packed bed column, appears to be more efficient and economically feasible than batch operation for metal sorption. For instance, Saeed and Iqbal (2006) reported that 63.7% removal of Cd(II) could be attained using immobilized *Synechococcus* sp. packed in a fixed-bed column bioreactor with continuous liquid flow system. Fixed-bed column containing algal biomass can be used for biosorption of heavy metals and/or dyes for final polishing of industrial effluents before discharge. The effluent is passed through a column with biosorbent, which can be regenerated when the maximal sorption capacity is reached (Zabochnicka-Świątek Magdalena 2014). The columns can also be arranged in parallel for sorption and desorption processes to occur without interruption in a continuous flow system (Gadd 2009). Fixed-bed column systems have been used mainly with seaweed rather than microalgal biomass. For instance, Ibrahim and Mutawie (2013) developed a fixed-bed column using biomass from red seaweeds for the removal of Cu(II), Zn(II), Mn(II) and Ni (II) from industrial effluent from chemical fertilizer factory. Batch and continuous stirred up tank reactors are also used in large-scale biosorption systems (Ghosh et al. 2016). In continuous stirred tank reactors, the inflow and outflow of medium and substrate are kept equal, while agitation and aeration are applied to mix the medium content.

Despite the extensive research, commercialization of biosorption technologies based on microalgae is still limited (Fomina and Gadd 2014). In the 1990s, a biosorbent, AlgaSORB™, based on *Chlorella* immobilized in silica or polyacrylamide gels was developed and commercialized for wastewater treatment (Garnham et al. 1997). The biosorbent can efficiently remove metal ions from dilute solution of 1 to 100 mg/L, reducing the concentration to below 1 mg/L. The biosorbent resembles an ion-exchange resin, which can undergo more than 100 biosorption/desorption cycles. Another biosorbent that has been commercialized is Bio-fix, which consists of a variety of biomasses, including *Sphagnum* peat moss, algae, yeast, bacteria and/or aquatic flora immobilized in high-density polysulphone. The granular Bio-fix has been tested for the treatment of acid mine waste, particularly for removal of Zn(II) (Garnham et al. 1997).

9 Future Directions of Research

Integrating biosorption technologies with other applications of microalgae, especially for biofuel production and CO_2 fixation, would be a way forward in attempts to develop cost-effective and environment-friendly bioremediation system for removing heavy metals and dyes from industrial effluents. Along this line, Yang et al. (2015) showed that *Chlorella minutissima* was effective in removing Cd, Cu, Mn and Zn ions from artificial wastewater. The elevated levels of Cd and Cu also induced lipid accumulation, generating useful algal biomass for biofuel production. The potential use of residual algal biomass after lipid extraction as biosorbents

should be further worth explored. For instance, waste biomass of green algae after oil extraction has been shown to be useful as a low-cost biosorbent for Pb(II), Cu(II) and Co (II) (Bulgariu and Bulgariu 2012). In addition, de-oiled biomass of *Microspora* sp. has been shown to be useful as biosorbent for dye, utilizing the waste stream from algal biofuel production (Maurya et al. 2014). Microalgae which are able to fix CO_2 efficiently are advantageous for use as biosorbents. For instance, Chen et al. (2012) showed that an efficient CO_2-fixing microalga, *Scenedesmus obliquus* CNW-N, could be used as a useful biosorbent to remove Cd(II) from aqueous solution, with a maximum capacity of 68.6 mg/g. The microalga could be first grown with continuous feeding of 2.5% CO_2 to generate high-density biomass as biosorbent for Cd(II) removal.

Most of the reported biosorption studies on microalgae were based on unialgal cultures or biomass derived from single species. Although such studies are useful in providing insights into the mechanistic aspects of biosorption, there is a need to assess the efficiency of the microalgae in removing heavy metals or dyes from real industrial effluents. Efficient treatment of industrial effluents involves not just a single algal species but requires the symbiotic involvement of microalgae with other microorganisms such as bacteria (Dhaouefi et al. 2018). Thus, there is a need for more biosorption studies based on consortium of microalgae and other microorganisms grown in industrial effluents. The potential of co-culturing microalgae with bacteria or yeasts to generate high amounts of biomass as biosorbents should be further explored. Such concept has been proposed as a strategic approach to enhance biomanufacturing processes based on algae (Padmaperuma et al. 2017). Further, the potential application of biofilms as biosorbents is another area that needs further investigations. The biofilm community, consisting of microalgae, cyanobacteria and other bacteria, is known to produce extracellular polymeric substances (EPS), which could enhance the biosorption capacity for heavy metals (Comte et al. 2008).

Detailed economic and market analyses are required to assess the feasibility of using algae-based biosorption technology for the removal of heavy metals and dyes (Srinivasan and Viraraghavan 2010). In addition, detail life cycle analysis (LCA) is needed to assess the environmental impacts if the biosorption component is incorporated into an algal biofuel production system integrated with wastewater treatment (Mu et al. 2014). In developing further the industrial use of algae-based biosorption technology, there is a need to adopt a multidisciplinary approach in which chemists, biologists and engineers work together. Apart from engineering principles involved in designing the biosorption system, there is a need of better understanding of biological processes involved and the chemistry involved in the binding of sorbates with cell materials. Furthermore, with the advent of "omic" tools, genomic and metabolomic approaches can be applied to enhance the biosorption capacity of microalgae in removing metals and dyes. For instance, genetically engineered bacteria with modified metal-binding peptides on their cell surface have shown improved affinity and selectivity for biosorption of target metals (Mosa et al. 2016). However, such approach has not been explored yet in attempts to enhance the biosorption capacity of microalgae.

While the use of dead algal biomass as biosorbents is advantageous, the potential of hybrid technology combining both living and non-living cells should be further explored (Wang and Chen 2009). Combining biosorption with other biotechnological processes such as bioaccumulation, bioreduction and bioprecipitation is important for effective treatment of real effluents. The use of living algal cells is desirable in bioremediation of dye-containing wastewater, as efficient removal of dyes involves not only biosorption but also biodegradation (Vikrant et al. 2018). For instance, the decolourization of dyes by *Chlorella vulgaris* and *Nostoc linckia* involves azo dye reductase enzyme, which was induced when the microalgae were treated with G-Red and methyl red (El-Sheekh et al. 2009).

10 Concluding Remarks

There have been extensive studies on the use of microalgae as biosorbents for heavy metals and dyes reported in the literature, as highlighted in this chapter. However, most of the studies were based on metal or dye solution rather than real industrial effluents. Such experimental studies have provided much insights into the mechanistic aspects of biosorption of metals and dyes by microalgae, particularly on adsorption equilibrium and kinetic modelling. Despite that, commercialization of biosorption technologies based on microalgae is still limited. For efficient bioremediation of industrial effluents, especially in removing heavy metals and dyes, combination of biosorption technologies with other processes such as bioconcentration and bioconversion, using both living and non-living algal biomass would be a strategic approach. More studies focusing on the integration of biosorption technologies with other applications, particularly using the microalgae for biofuel production and CO_2 biofixation, would be the way forward.

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Chapter 24
Bioremediation and Biofuel Production from Chlorella sp.: A Comprehensive Review

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Abstract Microalgal biofuels are environmentally friendly fuels regarded as a potential alternative to fossil fuels. Algae are fast-growing photosynthetic microscopic plants compared with terrestrial ones. Microalgae exhibit an inherent potential to accumulate various metabolites inside a cell, which can be utilised for various industrial applications. Cultivation of microalgae for biofuel and high-value chemical applications is costly due to the consumption of substantial freshwater nutrients, such as nitrogen and phosphorous. Mass production of algal biomass in freshwaters is an impractical approach due to increasing demands in the future. Multi-application of microalgae for wastewater treatment for low-cost biomass for biofuel and high-value chemicals and bioremediation can be a viable alternative to various stipulations, such as lowering cost of nutrients, freshwater resources and energy. This chapter discusses the various types of wastewater remediation and industrial-scale bioreactors for biofuel production and wastewater remediation by microalgae *Chlorella* sp., respectively. In addition, the life cycle assessment of bioremediation and its future perspectives are analysed.

Keywords Microalgae · Wastewater · Chlorella sp. · Bioremediation · Biofuel

1 Introduction

The increasing human population associated with urbanisation has resulted in several hindrances to the world economy predominantly due to the preservation of the environment and energy security. The world economy primarily depends on conventional and non-renewable fossil fuels (Kassim and Meng 2017; Chen et al. 2011). The increasing use of fossil fuels has greatly affected the environmental

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deterioration and energy insecurity. The CO_2 level has increased up to 400 ppm, thereby causing greenhouse effect in the environment due to uncontrolled use of fossil fuels (Cheah et al. 2014; Farrelly et al. 2013). Thus, renewable biofuels from algal oils have been used as substitute for conventional fossil fuels. Oil-producing algae have received increasing attention as an alternative bioresource to bioethanol and biodiesel production. Algal-based cultivations are environmentally friendly solutions for addressing all environmental issues associated with fossil fuels.

1.1 Importance of Algal Cultivation in Wastewater

Growing algae for biomass production of biofuels in freshwaters is an unfeasible approach. Freshwater resources are an important commodity. Considerable wastewater resources, such as industrial effluents and domestic wastewater, are available. These resources are rich in phosphorous, nitrogen and other microelements and organic contents that are essential nutrients for algal biomass production. Utilisation of algae for bioremediation aids in wastewater treatment and results in low-cost algal biomass for biofuel and nutraceutical production. Commercially produced *Chlorella* sp., *Chlorococcum* sp. and *Scenedesmus* sp. are utilised for bioremediation applications. *Chlorella* sp. is the widely used organism in several industrial applications.

1.2 Morphology and Structure of Chlorella sp.

Chlorella cell is spherical and has a diameter of 2–10 μm (Yamamoto et al. 2004, 2005). The rigid nature of cell walls assists in preserving cell integrity and further protects against algal feeders and harmful environmental conditions. During autosporangia formation, newly synthesised cell walls are fragile with electron-dense unilaminar layer (Yvonne and Tomas 2000). The thicknesses of these layers gradually increase up to 17–21 nm after maturation. Then, microfibrillar layers are formed with chitosan-like layers composed of glucosamine (Yamamoto et al. 2005; Kapaun and Reisser 1995), which provides rigidity to the cells. The composition and thickness of cell walls are unstable because they vary on the basis of growth stage and environmental conditions. Cytoplasm is a gel-like substance that contains soluble proteins, minerals and water. This substance has several organelles, such as vacuoles, mitochondria, a small nucleus, Golgi apparatus and a single chloroplast (Kuchitsu et al. 1987). The pyrenoid contains photosynthetic proteins commonly known as ribulose bisphosphate carboxylase/oxygenase (RuBisCO), which acts as a carbon fixation centre. The chloroplast exhibits considerable merged thylakoids, wherein chlorophyll is synthesised; the dominant photosynthetic pigments amongst the other ones, such as lutein, usually synthesised in thylakoids. Under stress and harsh conditions, lipid droplets accumulate in the cytoplasm and chloroplast. *Chlorella* sp. has an autospore (non-motile reproductive cell), which is asexually

reproduced. When algae adapt to a favourable environment, they start multiplying by autosporulation after 24 h. The common reproduction in green microalgae is autosporulation, wherein the cell walls of the mother and daughter cells (4) are released into the medium after maturation of new daughter cells.

1.3 Algal Biofuels

1.3.1 Biodiesel

Algal biodiesel has received increasing attention due to its carbon neutrality and capability to substitute fossil fuels.

Transesterification reaction is the conversion of oil in the presence of alcohol (commonly methanol) and used with the aid of a catalyst (either acid or alkali). The ratio between biodiesel output and feedstock is approximately 1:1; thus, 1 kg of oil can produce nearly 1 kg of biodiesel (Mata et al. 2010). Algal biodiesel is highly suitable to be used as jet fuel in the aviation industry because it exhibits high energy densities and low freezing points compared with the first-generation biodiesel (NREL 2006). Moreover, its CO_2 emissions are lower (78%) than that of fossil fuel (Sheehan et al. 1998). The properties of algal biodiesel must be in accordance with the International Biodiesel Standard for Vehicles (EN14214) to be accepted as a substitute for fossil fuels. Some biodiesel oils from microalgae have high unsaturated fatty acid contents compared with vegetable oils, which are prone to oxidation during storage, thereby making it unsuitable for various applications.

1.3.2 Bioethanol

Bioethanol is currently considered an alternative source to fossil fuel. Ethanol is regarded as a substitute to petrol in other developed countries (Willke and Vorlop 2004) due to its properties similar to gasoline. At present, feedstocks used for the production of ethanol are corn and sugarcane. These feedstocks have a problem related to the first-generation biofuel, that is, food versus fuel disputes. Hence, these feedstocks compete with the food chain and land use, thereby limiting the production of this biofuel (Sun and Cheng 2002). Fundamentally, ethanol is produced from fermentation of sugar released from different types of biomass, such as agricultural, organic waste and energy crops (Xuan et al. 2009). Microalgae can be used as an alternative feedstock to bioethanol due to their high carbohydrate contents. The steps involved in ethanol production by microalgae consist of cultivation, biomass harvest, hydrolysis, fermentation and ethanol recovery. When cells are broken down as sugar units after hydrolysis, the yeast cells are added to the sugar syrup for fermentation, and ethanol is then purified by distillation (Amin 2009). Harun et al. (2010a, b) recently reported on fermentation for bioethanol production. Their results showed that the theoretical maximum yields of ethanol are 0.51 kg and 0.49 kg CO_2 from per

kg of the glucose unit. The biomass of *Chlorella* sp. is rich in carbohydrate contents. Microalgae *Chlorella vulgaris* has been tested for bioethanol production, and results have shown that the theoretical yield is 87.6% with a concentration of 11.7 g/L (Ho et al. 2013). Microalgal ethanol can be utilised as fuel; the liberated CO_2 can be reutilised for algal cultivation for biomass production, which can be used in anaerobic digestion (Harun et al. 2010b), thereby minimising greenhouse gas effect. However, the commercial-scale ethanol production technology from algal biomass is still under progress worldwide and needs further study in this perspective.

1.3.3 Biomethane

Biomethane can be produced by anaerobic digestion of organic matter. Anaerobic digestion primarily produces 55.0%–65.0% of methane (CH_4), 30.05–45.0% of carbon dioxide (CO_2), hydrogen sulphide, water vapour (Cooney et al. 2007) and traces of H_2 and CO (Bailey and Ollis 1976). The energy content of biogas is 16,200–30,600 kJ/m^{-3}, which also depends on the nature of the source of biomass being processed in anaerobic digestion (Chisti 2008). Biomethane can be used as fuel and in electricity generation (Holm-Nielsen et al. 2009). Moreover, residues of anaerobic digestion can be used as biofertilizers. Anaerobic digestion is useful when utilising biomass that contains 80%–90% of moisture content, and algal biomass is effectively suited for the aforementioned procedure (Brennan and Owende 2010). Microalgae with high protein content are unsuitable for this process due to increased production of ammonia, which is toxic to microorganisms and lowers the C/N ratio, which may affect the overall efficiency of the process. To overcome this issue, preadaptation microorganisms are required for an excellent conversion process (Brennan and Owende 2010). Methane production can be improved by supplementing residues from paper recycling with microalgal biomass (Yen and Brune 2007). Hence, the integrated technology for algal cultivation coupled with a wastewater treatment system for methane can be a suitable approach to lower the cost associated with the production and viability of the technology.

1.3.4 Bio-oils

At present, bio-oil is a promising alternative to fossil fuels due to low life cycle emissions of CO_2 and high energy content (Azadi et al. 2014). Two methods are used for bio-oil production, namely, thermochemical liquefaction and pyrolysis processes. The former is used to convert wet biomass to liquid fuel (Patil et al. 2008). This method is operated at a high pressure (5–20 MPa) and temperature (300–350 °C) with the aid of catalyst and hydrogen to yield bio-oil (Goyal et al. 2008). This process is expensive due to reactors used for thermochemical liquefaction and complex fuel–feed systems (McKendry 2002). However, an advantage of this technology is its capability to operate in a wet condition (Clark and Deswarte 2008). The reactions occur under high water activity in subcritical conditions to decompose biomass to short and small molecules with high energy densities (Patil

et al. 2008). This process is eco-friendly, fast (Peterson et al. 2008) and energetically efficient because no drying is required (Jena and Das 2011).

Pyrolysis is conducted at low to high temperatures (350–700 °C) in the absence of air to convert any type of biomass to oils, charcoal and syngas (Goyal et al. 2008). This method can be operated at a large scale to convert biomass to biofuels that can replace fossil fuels (Demirbas 2006). Several studies have been conducted for the pyrolysis of microalgal biomass bio-oils (Biswas et al. 2017; Grierson et al. 2009; Miao et al. 2004). An evaluation shows that microalgal species of *Chlorella protothecoides* and *Microcystis aeruginosa* yield 17.5–23.7% oils, whereas heterotrophic *C. protothecoides* produces a bio-oil yield of 57.9% (Miao et al. 2004). Pyrolysis of *Sargassum tenerrimum* with different solvents produces 22–24% of oils (Biswas et al. 2017). The property of bio-oils from microalgae is suitable for fuel use in comparison with those from the first- and second-generation feedstock.

1.4 Microalgal Biorefinery

Algal biomass can produce various products, such as energy, fuels, chemical polymers, food additive and pigments. Meanwhile, algal biorefinery is the integration of algal biomass production for biofuels with various industrially important chemicals and products for the sustainability of the algal biomass-based industry. Algal biomass can be fully utilised in integrated algal biorefineries. Fig. 24.1 shows the biorefinery approaches of algal biomass.

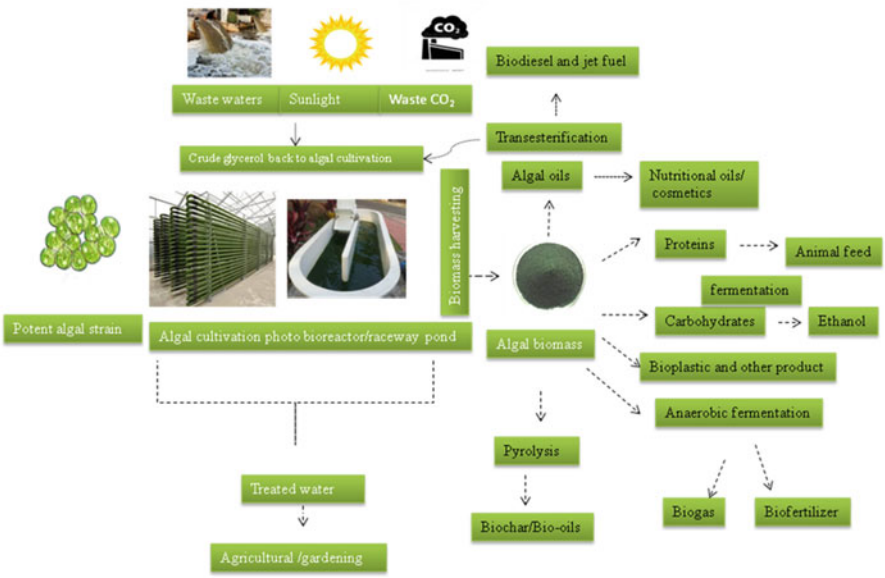

Fig. 24.1 Microalgal biorefineries coupled with wastewater treatment

Algal biomass from triglycerides can be utilised as biodiesel (fatty acid methyl ester) via transesterification. Oleaginous microalgae can be used as biodiesel feedstock due to their high oil contents (5000–100,000 L/ha^{-1}/year^{-1}; McGinn et al. 2011). Deoiled algal biomass contains carbohydrates and proteins that can be used for bioethanol and methane production. Bioethanol can be produced from the carbohydrate contents of algae, which mainly come from cytosolic starch and cellulosic polysaccharides found in the cell walls. The hydrolysis of polysaccharides from the microalgae of sugar can be further converted into bioethanol via fermentation. From the biofuel point of view, cultivation of algae associated with biorefinery can enhance energy productivity and CO_2 recycling, thereby achieving overall sustainability. Furthermore, biomass can be used for high-value products, such as cosmetics, polyunsaturated fatty acids (e.g. DHA and EPA), food supplements, animal feeds and pigments (Ho et al. 2013; Yen et al. 2011). However, many algal-based bioprocesses focus on one product without complete utilisation of biomass. Hence, integrated process for complete utilization of microalgal biomass to be processed for sustainability and ecofriently use of biomass.

2 Different Types of Chlorella Cultivation System

2.1 Phototrophic

The evolutionary advantage of algae is its ability to adapt with the nutritional mode on the basis of the accessibility of sunlight and substrate. In view of the substrate availability context, microalgae can be grown under phototrophic, mixotrophic and heterotrophic conditions. The phototrophic algae utilise inorganic CO_2, salts and light energy for photosynthesis and fix carbon for growth and multiplication. Photosynthetically produced carbon in the form of glucose serves as an energy source for algal cells. The key benefit of phototrophic algae is lipid production with the utilisation of atmospheric CO_2 and light. Photosynthesis is a major part of phototrophic algae survival, which occurs in the chloroplast conquered by light and dark reactions, whereby the conversion of sunlight and CO_2 absorbed by the cells into adenosine triphosphate (ATP) and O_2 is consumed during the respiration process to produce energy for multiplication of cells and growth (Zilinskas 1976; Falkowski and Raven 1997). During light reaction of photosynthesis, the integration of a glucose molecule requires three ATPs and two NADPH molecules, which are produced as a result of light absorption, water splitting, proton gradient and charge separation (Venkata Mohan 2010; Venkata Mohan et al. 2015). The Calvin cycle/dark reaction occurs in three phases, namely, carbon fixation, reduction and regeneration. In the first step, phototrophic carbon fixation occurs with the aid of RuBisCO, thereby forming two molecules of 3-phosphoglyceraldehyde, which is decreased to glyceraldehyde 3-phosphate (G3P). The RuBP is regenerated from G3P molecules, whereas carbohydrates, such as glucose and fructose, are produced in a form of energy. For the synthesis of one molecule of fructose or glucose from

CO_2, this cycle must operate sixfold to yield the desired hexoses and transformation of the six RuBP molecules. Then, lipid biosynthesis in algae occurs from glucose during nutrient limiting and stress conditions, which help to cope up stress. Major restraints of phototrophic cultivation require a large surface area in cultivation systems, shallow depth for better access of light and low biomass yield. Large-scale cultivation of *Chlorella* sp. is usually operated under photoautotrophic conditions (Dahmani et al. 2016; Omari et al. 2018).

2.2 Heterotrophic

Heterotrophic algae are non-photosynthetic and need supplementation of substrates (organic) and nutrients as an energy source. The heterotrophic mode of cultivation of a photosynthetic activity is shut down, and energy is obtained through organic carbon sources that convert sugar and other molecule storage compounds (e.g. lipids). This mode of algal cultivation exhibits high cell densities with elevated production yield and non-dependence on light. The heterotrophic nutritional mode of algae facilitates high biomass production, thereby providing economic possibilities for mass cultivation (Behrens. 2005; Perez-Garcia et al. 2011). The main advantages of heterotrophic cultivation are simplicity in operations, cost-effectiveness and easy maintenance (Perez-Garcia et al. 2011). If this cultivation system is improperly handled, then other microorganisms will be contaminated. *Chlorella regularis* (Fu et al. 2017), *C. vulgaris* (Xie et al. 2017) and *Chlorella sorokiniana* (Chen et al. 2018) are some examples of *Chlorella* sp. that are heterotrophically cultivated.

2.3 Mixotrophic

A mixotrophy cultivation system is used for microalgae, which can impel photoautotrophic and heterotrophic metabolism using organic and inorganic carbon sources (Kang et al. 2004). The photosynthetic process for fixing an inorganic carbon source is influenced by sunlight. The organic carbon source is absorbed through aerobic respiratory pathways, which also depend on the availability of carbon sources (Hu et al. 2012). The mixotrophic organism light is a nonlimiting factor for biomass production due to the availability of organic carbon. Mixotrophic compared with autotrophic and heterotrophic cultures show improved growth rates and diminished photo inhibition. An advantage of mixotrophic nutrition is its dependency on photosynthesis and growth substrates (Kong et al. 2013). Mixotrophic nutrition occurs in ecological water bodies, wherein the homeostatic structure and function of a living system are supported by physical, chemical and organic activities of biota, thereby balancing the ecological status (Venkata Mohan et al. 2015). Venkata

Mohan et al. (2015) effectively explained the various metabolic modes of microalgal cultivation systems. The mixotrophic cultivation system can improve growth rate and short growth periods, and biomass loss in the dark can be diminished due to respiration (Park et al. 2012), thereby enhancing protein and lipid productivity (Li et al. 2012; Abreu et al. 2012). *Chlorella* is widely used as edible microalgae due to a balanced amino acid composition in its protein contents (Liu and Chen 2016), and many authors have shown that the mixotrophic mode of cultivation enhances the protein content in *Chlorella* sp. (Wan et al. 2011, Abreu et al. 2012).

3 Bioremediation Potential of Chlorella

A cost-effective process for algal-based biofuels and nutraceuticals depends on the supply of water, inorganic nutrients and carbon resources for algal growth (Yang et al. 2006; Alam et al. 2015). The use of wastewater for *Chlorella* cultivation can be a viable alternative strategy for mass cultivation for fuel applications. Many effluents are rich in inorganic nutrients for supporting algal growth. Increasing population associated with industrialisation worldwide generates a substantial quantity of effluents rich in considerable pollutants that must be handled before being released into natural water bodies. Numerous studies have conducted wastewater treatment of effluents from industrial, municipal and agricultural fields by *Chlorella* sp. (Daneshvar et al. 2018; Lam et al. 2017; Chiu et al. 2015). Microalgae can grow in wastewater and use organic content and minerals present in the effluents for biomass production. Growing *Chlorella* sp. in wastewater results in numerous advantages, such as a decrease in organic content, oxygenated treated effluent due to photosynthetic oxygen and feasibility to obtain valuable metabolites, such as proteins, lipids and carbohydrates for chemical, pharmaceutical and fuel industries from biomass. Thus, the integrated approach for increasing the environmental sustainability of *Chlorella* cultivation and the cost-effectiveness involves combining the advantages of biofuel production, CO_2 mitigation and wastewater treatment processes.

3.1 Municipal Wastewater

Increasing urbanisation associated with population growth in various cities has generated substantial domestic/municipal wastewater, and its composition varies depending on the area. Municipal wastewater typically contains organic waste, nutrients, microorganisms, human and household and other chemical wastes. It has less nitrogen and phosphorous compared with wastewater from other sectors. Meanwhile, municipal wastewater contains considerable heavy metals, such as lead, zinc and copper from localised small factories. Reports show that *Chlorella* usually exhibits a high pollution (nutrient) removal potential. Cho et al. also revealed that

Chlorella sp. produces a biomass yield of 3.0 g/L^{-1} using anaerobic digestion water tanks (10%) and conflux line. Approximately 90% of nutrients from wastewater are used for algal growth for biomass production. Growing *Chlorella* sp. in concentrated municipal wastewater stream by a sludge thickening process produces a biomass productivity of 0.9 $g/L^{-1}/d^{-1}$ (Li et al. 2011). Cultivation of *Chlorella* sp. in the municipal effluent as a nutrient source has been extensively studied by many researchers. Growing *C. vulgaris* in nutrient-rich municipal wastewater and CO_2 supplementation shows high biomass and lipid accumulation under elevated light intensity and carbon (Ebrahimian et al. 2014). In this study, *C. vulgaris* cultivation under a mixotrophic condition with 25% of primary wastewater results in 100% COD, 100% ammonium and 82% nitrate removal efficiencies (Ebrahimian et al. 2014). Growing *Chlorella* sp. in sewage effluent is effective for nutrient removal and lipid production for biofuel applications (Kiran et al. 2014). *C. vulgaris* is used for biomass production and nutrient removal under different wastewater, such as primary and secondary ones. In addition, the supplementation of petroleum effluent shows high biomass production and can remove all nutrients present in these effluents. Results show that the total nitrogen removal efficiency rates are 85% and 100% (Znad et al. 2018). Growing *C. sorokiniana* in pretreated domestic wastewater of influent and centrate from an anaerobic tank under heterotrophic and mixotrophic conditions is effective for nutrient removal efficiency and biomass production. In this study, the removal efficiency rates of ammonium and phosphate from anaerobic centrates under the mixotrophic mode of operation were 94.29% and 83.30%, respectively (Ramsundar et al. 2017). The tolerance of municipal wastewater influent, which is used for growing ten *Chlorella*, in this effluent and its CO_2 supplementation were studied. The result showed that the biomass from four *Chlorella* sp. was considerably higher than other strains. The addition of 10% of CO_2 in the culture of the same organism revealed high CO_2 fixation efficiency (35.51%) and maximum lipid accumulation (58.48%; Hu et al. 2016). The algal morphology under electron microscopy revealed that the cells of *C. vulgaris* were normal after a 15-day batch culture. The morphology of *Chlorella* sp. was unaltered, whilst it showed tolerance to CO_2 level and effluents when cultivated under elevated CO_2 condition. Thus, this wastewater utilisation for microalgal cultivation could be a viable strategy for green process technology. From the aforementioned data, algal cultivation for wastewater treatment neutralises toxic pollution components.

3.2 Cultivation in Industrial Wastewater

Several types of industrial effluents are generated by different businesses and mode of operations with various chemicals and contaminants. Every industrial sector generates its particular combination of pollutants and chemicals. Heavy metal pollutant nitrogen and phosphorous are some contaminants present in wastewater (Chinnasamy et al. 2010). Screening and isolation of organic/metal-tolerant microalgal species are crucial for achieving high growth efficiency in wastewater

due to its complex nature (Ahluwalia and Goyal 2017). The microalgal *cultivation* of *Chlorella pyrenoidosa* collected from a starch and alcohol processing unit *in wastewater* under outdoor condition shows considerably improved lipid and biomass production. Moreover, the growth of *Chlorella* in this water effectively removes nutrients (i.e. 405 mg COD Cr/L/day, 49 nitrogen/L/day and 6.7 mg phosphorous/L/day; Tana et al. 2018). The utilisation of biogas effluent of seafood-processing wastewater has been tested for biomass production of *Chlorella* sp. for biogas manufacturing**.** A maximum biomass of 0.184 g/L has been obtained with 50% effluent. Methane yield has been improved tenfold with *Chlorella* sp. biomass compared with seafood-processing wastewater alone (Jehlee et al. 2017). A two-stage cultivation system from photoautotrophic to mixotrophic condition has been operated for maximum lipid production of algae using brewery wastewater. Brewery wastewater-born *Chlorella* sp. has high growth than wild-type *C. vulgaris. However, increased biomass and lipid contents are observed in wild-type Chlorella sp.* The endogenous *Chlorella* sp. of brewery water can remove total nitrogen and phosphorus of 90% during the first stage of photoautotrophic–mixotrophic conditions in each *microalga*. The two-stage cultivation system improves the biomass production potential of *Chlorella* sp. (Farooq et al. 2013). Hongyang et al. (2011) showed that batch and fed-batch growth of *C. pyrenoidosa* in wastewater from the soybean-processing industry indicates that algae can remove 78% of COD, 89% of total nitrogen and 70% of total phosphate after the 5th day of cultivation. After the cultivation stage, *C. pyrenoidosa* produces 0.64 $g/L^{-1}/d^{-1}$ of biomass, 37% of lipid content and 0.40 $g/L^{-1}/d^{-1}$ of lipid productivity (Hongyang et al. 2011). Growing *Chlorella* sp. under mixotrophic condition in industrial dairy waste, which is used as a carbon source, produces a maximum biomass of 3.6 g/L in comparison with a normal medium. *Chlorella* sp. is used for cultivation in wastewater from the meat industry for nutrient removal and biomass production. Results show that the biomass yield obtained in mixed wastewater is 0.7–1.5 g/L, which is higher than the wastewater alone and artificial medium (Lu et al. 2015). Wastewater mixing for algal cultivation relieves the hindrances associated with biomass yield and nutrients. Moreover, algal cultivation in mixed wastewater improves nutrient removal efficiency. Wastewater mixing for algal cultivation is a resourceful and economic approach for improving biomass yield for biofuels (Lu et al. 2015). Cultivation of *C. vulgaris* and *C. sorokiniana in* coke-making wastewater exhibits tolerance of *C. vulgaris* with the obtained maximal biomass and lipid productivity of 2.8 g/L and 32 mg/L/d, respectively (Chen et al. 2018). Algal cultivation in semi-batch operation produces constant biomass and lipid productivity of 5.18 g/L and 77.3 mg/L/d, respectively. From the preceding results, *C. vulgaris* is a potential candidate for growth in wastewater for biofuel application (Chen et al. 2018). The studies indicate that *Chlorella* sp. can adapt to any wastewater for biomass production, and its potential to remove nutrients is beneficial for the bioremediation of wastewater for improving the environment.

3.3 Cultivation in Agricultural Wastewater

The agricultural sector also contributes to the large amount of wastewater (Abdel-Raouf et al. 2012). Such water contains wastes (e.g. manure) from several activities, including animal farming, such as poultry and livestock operations. An important form of nitrogenous waste is from animal manure with ammonium, which also changes depending on activities, such as animal diet, usage, productivity and location, and is affected by the final nutrient contents of wastewater (de Godos et al. 2009; Wang et al. 2010; Zhu et al. 2013). Phosphorus and nitrogen are the major components in wastewater (Zhu et al. 2013). *Chlorella* is regarded as one of the dominant and high-tolerant organisms in freshwater environments (Álvarez-Díaz et al. 2017). Strains from this species show great acclimatisation to the changing environmental conditions. The growth potential of *C. vulgaris is evaluated in aquaculture, pulp and lake wastewater. This alga can produce 1.3 g/L biomass in mixed wastewater of pulp and aquaculture at a ratio of 60:40.* The total nitrogen and phosphorous removal efficiency rates are 55.5% and 94%, respectively (Daneshvara et al. 2018). Growing *Chlorella* sp. in piggery wastewater demonstrates growth rate and biomass productivity of 0.839 d^{-1} and 0.681 $g/L^{-1}/d^{-1}$, respectively. The maximum lipid productivity and content are 0.155 $g/L^{-1}/d^{-1}$ and 29.3%, respectively (Kuo et al. 2015). Piggery wastewater is also utilised for the culture of *Chlorella zofingiensis for biomass and lipid production in photobioreactor* (Zhu et al. 2013). *Symbiotic bioremediation methods are used for aquaculture wastewater treatment (*Lananan et al. 2014), in which algae and bacteria are used for effective treatment process. The microorganisms can produce CO_2 during respiration and consume photosynthetically produced O_2 by algae and vice versa. In addition, both organisms degrade organic matter. The removal capacity of phosphorus is 99.15% when *Chlorella* sp. and bacteria are utilised for the effective treatment of aquaculture wastewater compared with 49.73% for the conventional bioremediation with algae alone (Lananan et al. 2014). When the indigenous microalgal strain *C. vulgaris* is cultivated in swine wastewater effluent for 12 days, the removal efficiency rates of the total nitrogen and phosphorous are 90.51% and 91.54%, respectively (Wen et al. 2017).

4 Lipid Production of Chlorella Cultures in Wastewater

Studies on large-scale operations indicate that high lipid productivity and content inside the cell are important factors for biofuel production from algae. However, high lipid content is usually attained in a stressed environment, such as high light intensities, low temperature, nutritional deficiency, pH and salinity conditions (Chokshi et al. 2017; Ummalyma and Sukumaran 2015). In the context of biofuel application from algae, lipid production is an important factor to be considered for biodiesel. Lipid production from *Chlorella* sp. varies on the basis of the type of

Table 24.1 Lipid production of *Chlorella* sp. in various wastewater

Chlorella sp.	Type of wastewater	Lipid mg/L/d or mg/L	References
C. sp. MM3	Winery and piggery effluents	29–51	Ganeshkumara et al. (2018)
C. pyrenoidosa	Alcohol wastewater	20	Tana et al. (2018)
C. vulgaris,	Secondarily treated urban wastewater	17.9	Álvarez-Díaz et al. (2017)
Chlorella kessleri		18.5	
C. sorokiniana		22.4	
C. vulgaris	Dairy wastewater	48	Qin et al. (2014, 2018)
C. vulgaris	Anaerobically digested starch wastewater+alcohol wastewater	127	Yang et al. (2015)
C. vulgaris ESP31	Coke-making wastewater	47.1	Chen and Chang (2018)
C. vulgaris	Domestic wastewater	32.7	Lam et al. (2017)
C. vulgaris	Industrial (monosodium glutamate) wastewater	30	Jiang et al. (2016)
C. pyrenoidosa	Industrial (riboflavin manufacturing) wastewater	99	Sun et al. (2013)

wastewater, composition and other environmental factors. Table 24.1 shows the lipid production of *Chlorella* sp. in different types of wastewater. Cultivation of *Chlorella* sp. *MM3* in mixed wastewater of piggery and winery effluents produce lipid accumulation rates of 29% and 51% in cells, respectively (Ganeshkumara et al. 2018). A report indicates that the growth of *C. pyrenoidosa* in alcohol wastewater and anaerobically digested starch wastewater used as carbon source in outdoor condition produces a lipid content of 20% (Tana et al. 2018). Álvarez-Díaz et al. (2017) evaluated wastewater treatment and lipid production of seven microalgae. The tested algae *Chlorella* sp. showed great dominance in wastewater for nutrient removal and lipid production. Mixed wastewater from anaerobically digested starch and alcohol wastewater used for the growth of *C. pyrenoidosa* produced a maximum of 3 g/L biomass and 127 mg/L/d lipid production (Yang et al. 2015). Chen and Chang (2018) revealed that *Chlorella* sp. could tolerate coke-making wastewater and produce 4 g/L biomass and 47.1 mg/L/d lipid productivity. The growth of *C. vulgaris* in domestic wastewater with low pH (3) was evaluated, and results showed that the pH was neutralised with a lipid production of 32.7% (Lam et al. 2017). Non-sterilised piggery wastewater used for the cultivation of *C. vulgaris* CY5 produced satisfactory biomass and lipid content of 3 g/L and 30%, respectively, when compared with the same organism cultivated in a sterilised medium (Marjakangas et al. 2015). Studies on the influence of illumination on the growth of *C. vulgaris* in diluted monosodium glutamate wastewater show that 30% lipid content is achieved at an illumination intensity

of 150 μmol·m^{-2} s^{-1}, which is doubled under heterotrophic mode of cultivation (Jiang et al. 2016). Lipid production of *Chlorella* sp. varies on the basis of the wastewater type, mode of cultivation used for algae and nutrient composition in water. The preceding research works indicate that algae are suitable for lipid production in wastewater without depending on valuable freshwater resources and fertilisers for algal growth.

5 Large-Scale Cultivation System for Chlorella sp. Under Outdoor Conditions

Most reported research on wastewater treatment with *Chlorella* sp. is conducted in laboratory scale only; however, it can be valued when realised with large-scale cultivation in bioreactors, such as open raceway reactors and photobioreactors. The currently used bioreactors for the cultivation of *Chlorella* sp. can be categorised as open ponds with and without paddle wheel and closed systems in photobioreactors with different shapes and sizes. The open pond culture system is preferred due to its low-cost, economical algal cultivation systems and easy operation. However, an open pond system depends on several environmental conditions, such as the pond used for cultivation, evaporation rate and contamination with other unwanted microorganisms. Various models of open cultivation systems used for algae are slope system and raceway, circular and high rate algal ponds. Photobioreactor-based algal cultivation is extremely costly compared with an open system. However, this method is efficient for algal biomass production due to control in various cultural parameters and less contamination risk. Effective algal cultivation practices can improve biomass production and nutrient removal efficiency. Photobioreactors have been recently selected for wastewater remediation by *Chlorella* sp. The use of a small-scale photobioreactor that contains centrate wastewater and waste glycerol for growing *C. vulgaris* produces 17 g/m^{-2}/d^{-1} biomass, 23.6% lipid content, 2.4 g/m^{-2}/d^{-1} NH_4^+–N nutrient removal efficiency, 2.7 g/m^{-2}/d^{-1} total nitrogen, 3.0 g/m^{-2}/d^{-1} phosphorous and 103.0 g/m^{-2}/d^{-1} COD after a 34-day operation under a semi-continuous mode (Ren et al. 2017). Tertiary wastewater that is treated in laboratory-scale photobioreactor with *C. protothecoides* shows that low irradiance and photoperiods affect the efficiency of wastewater treatment. Alkaline pH improves the phosphorous removal rate and CO_2 concentration but affects the COD removal rate (27.97%). The optimum conditions inside a reactor show high COD (78%) and phosphorous and nitrogen removal rates (100%), which are achieved at 10th, 7th, and 6th days of cultivation, and approximately 2 g/L of maximum biomass production (Binnal and Babu 2017). Algal cultivation with anaerobic processing in circulation airlift photobioreactor and dynamic membrane reactor for *C. pyrenoidosa* has been combined with an up-flow anaerobic sludge bed reactor for treatment of starch-processing wastewater (Tan et al. 2014). The airlift photoautotrophic–

heterotrophic photobioreactor with 890 L capacity, 1.80 m length, 0.62/0.30 m breath and 1.10 m height is used. This system contains five transparent methacrylate sheets and two baffle plates. Moreover, the system has a 5.62 m^2 illumination area, 820 L working volume, heterotrophic and autotrophic zones and a chamber for gas–liquid separation (Tan et al. 2014). Circulation of liquids amongst two zones is aided by two micro-bubble air diffusers of the reactors. The aeration volume is controlled and supplied by a gas flow metre and air compressor. A heating device is used to control and maintain the temperature in the reactor (Tan et al. 2014). The optimal pollutant removal and biomass rates are obtained at temperatures between 35 °C and 38 °C. The removal rates of COD, total nitrogen, phosphorous and biomass are 65.9%, 83%, 96.97% and 0.37 $g/L^{-1}/d^{-1}$, respectively (Tan et al. 2014). Reports show that improved biomass production and different nutrient removal efficiency rates of ammonia, nitrate and dissolved phosphate and COD are obtained by using algal consortium that contains dominant *Chlorella* sp. for biomass production in grey water and scaled up in a raceway reactor with 800 L capacity (Kumar et al. 2017). Resultant biomass is used for biogas production (Kumar et al. 2017). Thus, a mixed culture of algae has the potential for biomass production for value addition and wastewater treatment.

6 Limitations of Algal Biomass Productivities in Wastewater

Algae have high biomass generation and elevated lipid contents. In addition, many reviewed studies on algal growth in wastewater have suggested that algae can be explored for low-cost biofuel production (Pittman et al. 2011; Razzak et al. 2013; Wu et al. 2014; Venkata Mohan et al. 2015). However, few limitations must be addressed for wastewater-based algal cultivation. Wastewater-grown algal biomass is only used for biofuel application and is unsuitable with other high-value chemicals. Microalgae can multiply and consume nutrients from wastewater and provide alternative low-cost algal biomass for biofuels. However, some algae are resistant to pollutions, which is an important factor to be considered for algal growth in wastewater. The existence of microbes is an important characteristic of wastewater. In some studies, algal growth in wastewater is inhibited by the presence of protozoan and other microorganisms. Hence, many reports of wastewater remediation from algae are produced from sterilised waters. For laboratory-scale studies, sterilisation of wastewater, such as nutrient medium, is easy. By contrast, large-scale operations in wastewater are costly and pose considerable challenges. For technical-level operations, sterilisation is unfeasible with a large volume of water. Therefore, adapting the process for controlling other microbial pollutants of wastewater is necessary. Sodium hypochlorite and UV sterilisation is currently used for the control of microbial pollutants in large-scale operations with *Chlorella* sp. and other microalgae.

7 Life Cycle Assessment of Bioremediation of Algae for Biofuels

Algal production systems must be evaluated for determining the overall sustainability of the technology. Algal cultivation in freshwater can also decrease the local water supply. In addition, an environmental impact assessment of building materials for photobioreactor and raceway reactors is an important factor to be considered for LCA. This condition is highly relevant in the case of photobioreactor cultivation systems, which require special materials for fabrication depending on the end user. After harvesting, algal cake is used as a protein meal or for biogas production. Biogas-produced energy can be used in various processing stages, and nutrients and CO_2 can be recycled via algal cultivation. Photosynthetically produced oxygen needs to be released through degassing column before biomass recovery. Recovered nutrients from wastewater can be used as fertiliser. Mass and energy balance calculations are needed to determine the overall energy process that can be provided from the biogas production method of algal cakes, heat power production, nutrient recovery and water recycling.

8 Future Perspective and Conclusion

Algal biomass production systems must be coupled with wastewater treatment facilities to address freshwater shortage and nutrient cost for algal mass cultivation. Many studies have focussed on wastewater-born algae and other microbe interactions for nutrient removal efficiency. Another factor to be considered is the algal feeders when cultivating *Chlorella* sp. in raceway ponds with wastewater. The application of an alternative harvesting method to enhance the auto-flocculation of *Chlorella* in large-scale cultivation systems shall be investigated to improve profitable possibilities and complete the operation of microalgal-based wastewater treatment systems. Early prevention and detection of rotifer infection and protozoan attack are needed to control algal cultivation in an open pond system. Application of strain improvement techniques in *Chlorella* sp. and genetic engineering approaches can also solve many of the current challenges. Increasing algal biorefineries for complete utilisation of biomass must also be explored in a renewable and sustainable approach. Integration of biorefineries with wastewater and CO_2 from industries will offer an economically feasible technology for the sustainable utilisation of algae for value-added products.

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