Biofuel and Biorefinery Technologies 2

Navid R. Moheimani
Mark P. McHenry
Karne de Boer
Parisa A. Bahri *Editors*

Biomass and Biofuels from Microalgae

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Navid R. Moheimani · Mark P. McHenry
Karne de Boer · Parisa A. Bahri
Editors

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Editors
Navid R. Moheimani
School of Veterinary and Life Sciences, Algae R&D Centre
Murdoch University
Murdoch, WA
Australia

Karne de Boer
School of Engineering and Information Technology
Murdoch University
Murdoch, WA
Australia

Mark P. McHenry
School of Engineering and Information Technology
Murdoch University
Murdoch, WA
Australia

Parisa A. Bahri
School of Engineering and Information Technology
Murdoch University
Murdoch, WA
Australia

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Preface

Microalgae cultivation has an advancing role in solving some of the future limitations of traditional biomass production and markets (i.e., food, feed, energy, emission mitigation, chemicals, materials, etc.). In conjunction with conventional growth systems, new biomass industries such as microalgae must be developed in order to produce large-scale sustainable products cost-effectively. This book presents some of the most promising existing microalgal biomass growth technologies and summarizes some of the novel methodologies for sustainable and commercial microalgae production.

There are many different and unrelated microalgae taxonomic groups that directly or indirectly utilize solar energy to produce organic compounds. At present only a little over a dozen species are medium-term candidates for large-scale cultivation. However, even an ideal microalga must sustain high productivity under varying environmental and production conditions, and produce commercially sought services and products.

To enable such an industry, both advancements in microalgae biological sciences and biomass production and processing engineering systems are a major parallel focus of this book. We are very pleased to have 17 excellent chapters detailing some of the latest research and developments in microalgae cultivation and processing techniques, heterotrophic production methods, introduction of wastewaters, the effect of CO_2 injection, flocculation and auto-flocculation, in addition to species-specific extraction methods and bioproduct optimization. The chapters also include the production of a range of fuels, including anaerobically digested microalgae for biogas, and biodiesel, bioethanol, and also hybrid chemical and electric production systems. Furthermore, innovations in production via genetic engineering for microalgae strain improvement, synthetic biology approaches, genomic, and metabolic modeling approaches are analyzed and discussed in detail. In terms of large-scale production, selected chapters discuss the economics of harvesting and downstream processing, energy and economic modeling for large-scale facilities, and research on life-cycle environmental impact of microalgae biofuel and co-products.

As the book developed we were amazed at the breadth and detail of the ongoing advances in both the biological and engineering elements that surround modern microalgae production. We trust the reader will enjoy the book as much as we enjoyed writing and editing it.

The editors would like to thank all of the authors for their excellent and timely contributions. Considering the professionalism and the experience of the contributing researchers, the editing of this book was a relative pleasure. We also thank the many people directly and indirectly involved in the production of this book, including the publisher, and also our families who supported us through our various endeavors.

Navid R. Moheimani
School of Veterinary and Life Sciences, Algae R&D Centre,
Murdoch University, Australia

Mark P. McHenry
School of Engineering and Information Technology,
Murdoch University, Australia

Karne de Boer
School of Engineering and Information Technology,
Murdoch University, Australia

Parisa A. Bahri
School of Engineering and Information Technology,
Murdoch University, Australia

Contents

Chapter 1
Past, Present and Future of Microalgae Cultivation Developments

Navid R. Moheimani, David Parlevliet, Mark P. McHenry, Parisa A. Bahri and Karne de Boer

Abstract Microalgae cultivation is a promising methodology for solving some of the future problems of biomass production (i.e. renewable food, feed and bioenergy production). There is no doubt that in conjunction with conventional growth systems, novel technologies must be developed in order to produce the large-scale sustainable microalgae products. Here, we review some of the most promising existing microalgae biomass growth technologies and summarise some of the novel methodologies for sustainable microalgae production.

1.1 Introduction

There has recently been extensive research focus on biology, physiology, engineering and their integration for microalgae cultivation to produce sustainable products such as biofuel, food, feed and high-value products. Algae belong to many different and unrelated taxonomic groups that all contain chlorophyll *a* and are able to utilise solar energy and fix CO_2 to produce organic compounds (Borowitzka 2012). More than a dozen algal species have been mentioned in the literature as potential candidates for large-scale cultivation. However, conclusive information obtained through commercial trials is not yet available to assess suitability of most of these species. The ideal microalga must be able to grow very well even under high biomass concentration and varying environmental conditions. It must be able to produce high concentration of product of interest (i.e. high-value products, lipids and hydrocarbons). However, it is unknown how many species of algae exist, with

N.R. Moheimani (✉)
Algae R&D Center, School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150, Australia
e-mail: n.moheimani@murdoch.edu.au

D. Parlevliet · M.P. McHenry · P.A. Bahri · K. de Boer
School of Engineering and Information Technology, Murdoch University, Murdoch, WA 6150, Australia

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Table 1.1 Main microalgae species tested for medium- to large-scale biomass production

Chlorophyceae	*Neochloris oleoabundans; Scenedesmus dimorphus; Botryococcus braunii; Dunaliella tertiolecta; Nannochloris* sp.; *Chlorella protothecoides; Ankistrodesmus braunii*
Euglenophyceae	*Euglena gracilis*
Prasinophyceae	*Tetraselmis* spp. (i.e. *T. chuii and T. suecica*)
Haptophyceae	*Chrysotila carterae*; *Isochrysis galbana*
Eustigmatophyceae	*Nannochloropsis* spp. (e.g. *N. salina*, *N. oculata*, *N. gaditana*)
Bacillariophyceae (diatoms)	*Cyclotella cryptica*; *Chaetacerous* sp.; *Skeletonema* sp.
Cyanobacteria (blue-green algae)	*Arthrospira* (*Spirulina*) *platensis*

estimates ranging between several hundred thousand and several million different species—with new types identified all of the time (Guiry 2012). Only a small portion of microalgal species (several thousand) can be kept alive in culture, and only a handful of them have been successfully grown commercially. Table 1.1 summarises the main microalgae species tested for medium- to large-scale production (especially for feed, high-value products and biofuel).

However, to date, only a few of these species were successfully grown in large scale. Commercial large-scale production of microalgae for bioproducts began in early 1960s and 1970s with *Chlorella* and *Spirulina* and followed in the 1980s with production of β-carotene from *Dunaliella salina* (Borowitzka 2013a). All three species were successfully grown in mixed or unmixed open ponds (Craggs et al. 2013). The ability to grow at highly selective environments is the main reason for the successful growth of these species (*Spirulina* = high pH and high HCO_3^-, *D. salina* = high salinity and *Chlorella* = high nutrients) (Craggs et al. 2013). Moheimani and Borowitzka (2006) also showed that *Chrysotila carterae* reliable long-term culture in raceway pond is successful due to the ability of this alga to grow at very high pH. Other species that do not have this selective advantage may need to be grown in closed photobioreactors. The selection of growth technologies or production systems for microalgae will need to be based to a large extent on the microalga of choice and cultivation system.

1.2 Microalgae Cultivation and Production Process

A conventional microalgae production system consists of (a) growth and cultivation of microalgae, (b) biomass harvesting and dewatering and (c) extraction/conversion of the biomass to the product of interest (Fig. 1.1). It is to be noted that for this process to be energetically, environmentally, and economically sustainable, it is critical to recycle medium (water and fertilisers) in harvest and extraction stages of production (Fig. 1.1).

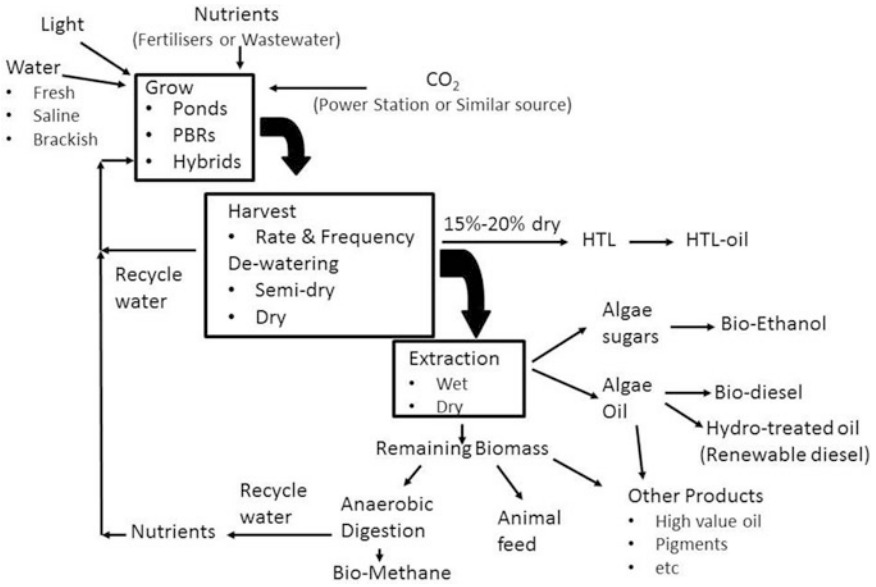

Fig. 1.1 Microalgae production flow sheet (modified and redrawn from Fon Sing et al. 2013)

1.2.1 Growth Technologies

There are two main types of microalgae cultivation systems: open ponds and closed photobioreactors (Moheimani 2012; Moheimani et al. 2011).

1.2.1.1 Closed Photobioreactors

Closed algal cultures (photobioreactors) are not exposed to the atmosphere and are covered with a transparent material or contained within transparent tubing. Photobioreactors have the distinct advantage of preventing evaporation (Dodd 1986; Moheimani et al. 2011). Culturing microalgae in these kinds of systems have the added benefit of reducing the contamination risks, limiting the CO_2 losses, creating reproducible cultivation conditions, and flexibility in technical design (Jeffery and Wright 1999). Closed and semi-closed photobioreactors are mainly used for producing high-value algal products (Becker 1994). In closed photobioreactors, the main challenge is being less economical than open ponds (Borowitzka 1996; Moheimani and McHenry 2013; Moheimani et al. 2013c; Pulz and Scheibenbogen 1998). A number of researchers have endeavoured to overcome a number of the limitations in closed including:

- reducing the light path (Borowitzka 1996; Janssen et al. 2002; Miron et al. 1999)
- solving shear (turbulence) complexity (Barbosa et al. 2003; Borowitzka 1996; Miron et al. 2003)
- reducing oxygen concentration (Acién Fernández et al. 2001; Kim and Lee 2001; Rubio et al. 1999; Weissman et al. 1988), and
- temperature control system (Becker 1994; Borowitzka 1996; Carlozzi and Sacchi 2001; Morita et al. 2001; Rubio et al. 1999; Zhang et al. 1999).

Currently, the main disadvantages of closed systems are the high cost of construction, operation both for energy (pumping and cooling) and maintenance [such as cleaning and sterilization (Borowitzka 1996)], and scaling up difficulties (Grima et al. 2000; Janssen et al. 2002; Miron et al. 1999). However, if these difficulties can be overcome, these controlled closed systems may allow commercial mass production of an increased number of microalgal species at a wider number of locations.

1.2.1.2 Open Ponds

Open ponds are the most usual setting for large-scale outdoor microalgae cultivation (Fon Sing et al. 2013; Jeffery and Wright 1999). The major commercial production of algae is today based on open channels (raceway) which are less expensive, and easier to build and operate compared with closed photobioreactors (Borowitzka 2013b; Tredici and Materassi 1992). In addition, the growth of microalgae meets is less challenging in open than closed cultivation systems; however, just a few species of microalgae (e.g. *Chlorella*, *D. salina*, *Spirulina.* sp., *Chlorella* sp. and *P. carterae*) have been successfully grown in open ponds (Moheimani and Borowitzka 2006; Tredici and Materassi 1992). Large-scale outdoor commercial microalgal culture has been methodically developed over the last sixty years (Borowitzka and Moheimani 2013a). Profitable production of microalgae, at present, are limited to a comparatively few small-scale (<10 ha) plants producing high-value health foods, most located in south-east Asia, Australia and the USA (Benemann 1992; Borowitzka and Borowitzka 1990; Richmond 1992). Two major types of large-scale open cultivation systems have been developed and have been used on a commercial basis. These are (a) unstirred ponds and (b) stirred ponds (circular and raceway) (Borowitzka 1993a, b; Borowitzka and Moheimani 2013b). The most common commercial microalgal culture system in use today is the paddlewheel-driven raceway pond (Richmond et al. 1993). The advantages and disadvantages of growing microalgae in open ponds and closed photobioreactors are summarised in Table 1.2. Relatively low cost of construction and operation are the main reasons for culturing algae in open ponds (Tredici and Materassi 1992). However, the high contamination risks and low productivity, induced mainly by poor mixing regime and light penetration, are the main disadvantages of open systems.

Table 1.2 Open versus closed photobioreactors

	Open ponds	Closed photobioreactors
Light efficiency	Fairly good	Excellent
Temperature control	None	Some
Gas transfer	Poor	Better
Oxygen produced	High	Higher
Hydrodynamic stress on algae	Low	High, very high
Surface/volume ratio	Moderate	High
Species control	Challenging	Achievable
Sterility	None	Achievable for short periods
Volumetric productivity	Low	High
Cost to scale up	Low	High

1.2.2 Harvesting and Dewatering

Energy-efficient and cost-effective microalgae dewatering, nutrient recycling and effluent water quality control are some of the major challenges facing industrial-scale microalgae production for commodity feeds and fuels (Benemann 2013; Borowitzka and Moheimani 2013b; Wyman and Goodman 1993a). Irrespective of the cultivation system, the biomass concentration of the algae culture is generally low (a few mg L^{-1} in open ponds to a few g L^{-1} in intensive closed photobioreactors). Dewatering is therefore critical for producing any materials from microalgae. The objective of harvesting and dewatering is to raise the concentration of the microalgal biomass by more than two orders of magnitude to over 10 % solids, sufficiently concentrated for subsequent processing or drying. It is widely believed that this is best achieved using a combination of technologies in a two-stage process (Benemann et al. 1982; Shelef et al. 1984; Vandamme et al. 2013), such as flocculation followed by centrifugation. This necessitates that large volumes of water need to be processed to harvest the biomass. This concentration process is typically energy intensive and results in high harvesting, thickening and dewatering costs (Mohn 1988). Available harvesting and dewatering process selection often interacts with both up- and downstream process steps in microalgae production, such as strain selection and medium composition, biomass fractionation (e.g. in a biorefinery) and water or nutrient recycling (de Boer et al. 2012; Wijffels et al. 2010).

1.2.3 Extraction/Conversion

Post-dewatering, the microalgae biomass can be used directly as a source of animal feed or human food. The cultural and economic development of society has resulted in changes in human lifestyles with developed countries' diets highly caloric, rich in

saturated fats and sugars, with lower consumption of complex carbohydrates and dietary fibre. This has brought about a greater interest in new foods that can contribute to improve nutritional health and well-being (Plaza et al. 2008). Microalgae are certainly candidates for producing high protein (*Spirulina*), high carbohydrate (*Chlorella*) and high essential oil similar to fish oil (Diatoms). Furthermore, microalgae biomass can be converted to renewable fuels. The three different pathways that can be used to extract and convert microalgae wet biomass (20 % solid) into bioenergy are summarised in Fig. 1.2. To date, hydrothermal liquefaction seems to be the most energetically positive method for biofuel production from microalgae (de Boer et al. 2012). However, extensive research and development is still required to determine the most energetically favourable and economically feasible process for extracting and converting the algal biomass for renewable bioenergy.

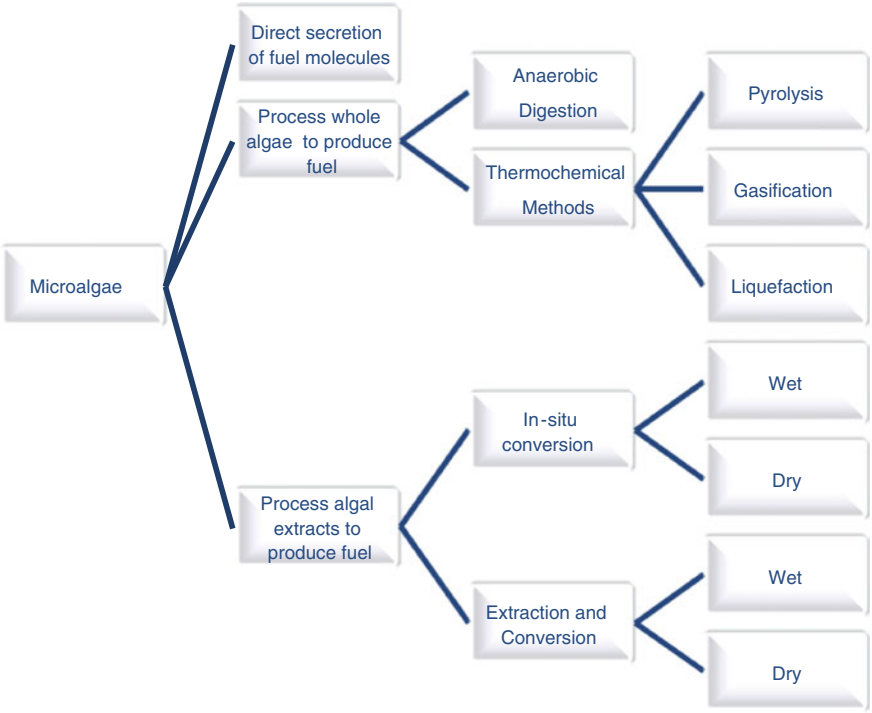

Fig. 1.2 Classification of conversion pathways for microalgae to fuel (de Boer et al. 2012)

1.3 Novel Methodologies

1.3.1 Conversion of Solar Energy to Biomass and Electricity

Photosynthesis is the driving mechanism behind microalgae biomass production but only requires a small fraction of the incident solar energy, primarily in the blue and red portions of the solar spectrum. In conventional cultivation of microalgae, the remainder of the incident solar energy simply heats the algae ponds, causing the water in them to evaporate and increase salinity which is a significant problem in biomass production. With microalgae cultivation often occurring in hot, semi-arid locations, this incidental heating is essentially a waste of the solar energy. Instead, it would be advantageous to be able to capture this unused portion of the solar spectrum and convert it to electricity for use at the cultivation site (Moheimani and Parlevliet 2013).

Figure 1.3 illustrates how the solar spectrum can be divided between the growth of microalgae and the production of electricity by a photovoltaic device (solar cell). Irradiance falling on the Earth's surface is well defined in the standard ASTM G-173-03 (ASTM 2008). This is the AM1.5 solar spectrum as shown in Fig. 1.3. Of this spectrum, only a fraction is used by photosynthesis by a microalgae culture. Some 48.7 % of the incident solar energy is considered to be photosynthetically active radiation (PAR) in the region between 400 and 700 nm (Zhu et al. 2008). However, it is clear from the absorption spectra of Nannochloropsis that some parts of the spectrum are absorbed more strongly than others. As such, the growth and performance of photosynthetic organisms are strongly linked to the quality and quantity of available light (Lindström 1984; Smith 1983) with only some parts of the spectrum being used in photosynthesis. In comparison, highly efficient crystalline silicon solar cells can absorb light strongly across the solar spectrum as shown by the spectral response of a PERL cell (Zhao et al. 1996) shown in Fig. 1.3. This suggests that although these consumers of solar energy (microalgae and solar cells) would appear to compete for the same resource, if the irradiance could be split between the two, the full utilisation of the solar spectrum would be possible. The shaded regions in Fig. 1.3 illustrate the portions of the solar spectrum that can be delivered to electrical generation and to microalgae cultivation without reducing the productivity of the microalgae. This would allow the production of biomass and electricity from the one facility.

The concept of the coproduction of electricity and agricultural production has been previously used in photovoltaic greenhouses. These are a building integrated photovoltaic system whereby solar modules are integrated into the structure of the building (Parida et al. 2011). Photovoltaic greenhouses use photovoltaic modules in the parts of the greenhouse whereby any reduction in overall PAR would not alter the growth of the plants, while the use of semi-transparent or opaque elements on the greenhouse can reduce the PAR and result in decreased productivity (Pérez-Alonso et al. 2012). This would be due to a reduction in the irradiance the plants required for photosynthesis. To overcome this issue, we propose the use of a

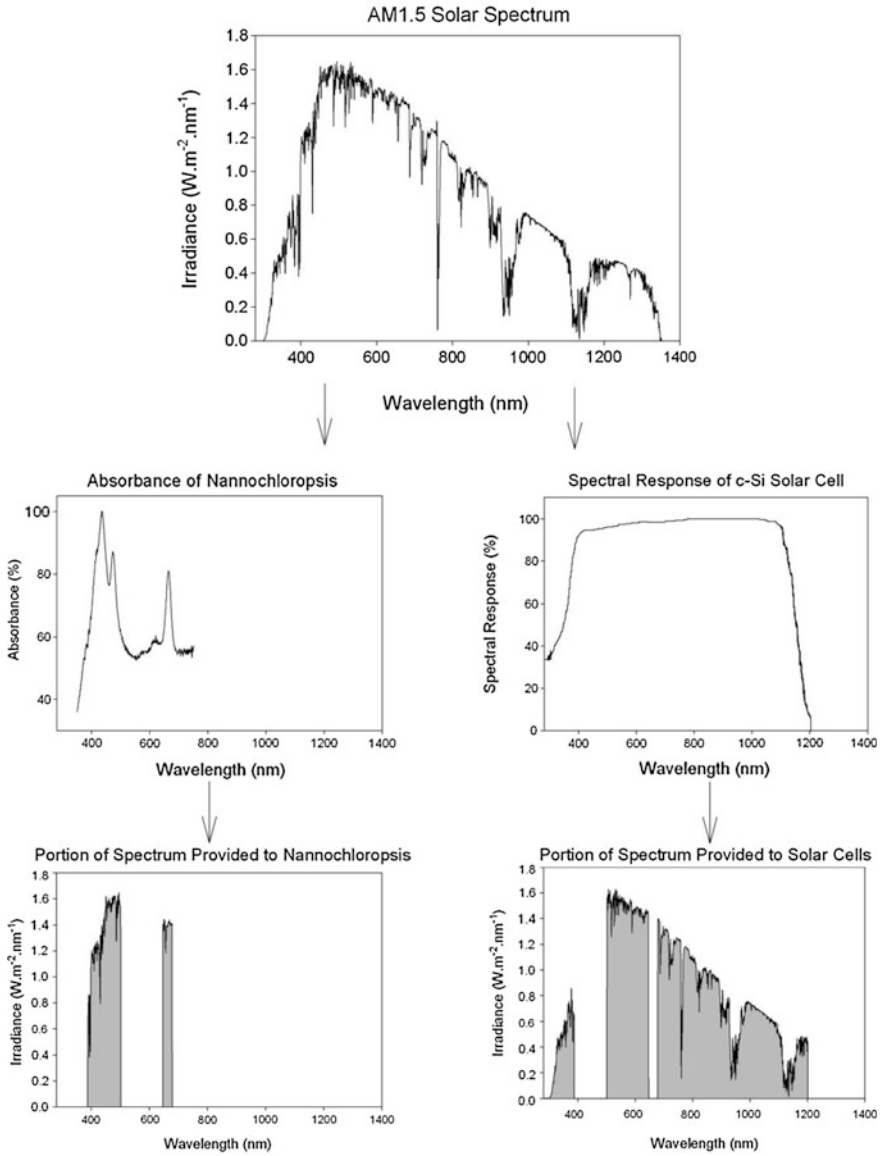

Fig. 1.3 Splitting the solar spectrum for the coproduction of biomass and electricity

semi-transparent solar module that is specifically designed to transmit the irradiance required by the microalgae and convert the remainder to electricity via a photovoltaic system. This solar module or filter can be located above the microalgae ponds (Moheimani and Parlevliet 2013).

There are a number of advantages to this system. By reducing the total irradiance incident upon the microalgae pond, the temperature of the culture would be reduced which would result in lower evaporation and a more stable salt content in the pond. As the microalgae are still receiving the portion of the spectrum required for photosynthesis, there would be no reduction in productivity. The electricity generation by the photovoltaic aspects of the system can be used on site to power motors and electronic systems to reduce the running costs of a facility. Alternatively, the electricity can be used to power additional lighting to increase the period of illumination on the microalgae or to increase the irradiance in specific parts of the solar spectrum. Using additional lighting powered by the otherwise wasted portions of the solar spectrum can increase the productivity of the microalgae. The style of system we have proposed (Moheimani and Parlevliet 2013) can improve the viability of microalgae growth for industrial purposes.

1.4 Non-destructive Extraction (Bio-oil and Bio-ethanol)

The economic viability of the current microalgae to fuel (or chemical) processes (summarised in Fig. 1.1) is limited by the high cost and energy burdens for growth inputs, capital and operating costs for dewatering, and the operating and capital costs of the growth system (Clarens et al. 2010; Lardon et al. 2009; Stephenson et al. 2010). Advances in growth, harvesting and extraction systems provide incremental improvement to these systems. The persistence of companies/research institutions and governments in continuing to pursue these systems indicates that many believe that such incremental improvement over time will ultimately result in an economically viable process. Others believe that a step change is required and are pursuing an entirely different biofuel production model in which the product of interest is continually secreted by the microalgae. This novel method is generally referred to as 'milking', as the product of interest is 'milked' from the algae without the need to destroy it and subsequently regrow it.

1.4.1 Hydrocarbons from Botryococcus Braunii

Hydrocarbons are able to be 'milked' (without cell death) from *Botryococcus braunii* using a solvent added to the growth medium (Moheimani et al. 2013a). Advances in this research further demonstrated that hydrocarbons could be repeatedly extracted (milked) from *B. braunii* using the solvent every 5 days for a total of 70 days with no addition of fertilisers (N and P) to the culture (Moheimani et al. 2013b). In this experiment, the cells were not dividing and therefore, nutrients were not required for the production of proteins and other cell elements. Instead, the majority of the light energy was used to convert CO_2 to hydrocarbons to replace those previously milked.

1.4.2 Ethanol from Blue-Green Algae (Cyanobacteria)

Algenol (Florida, USA) has been continually refining a process for continuous growth and harvest of ethanol excreted by modified cyanobacteria. In this process, ethanol is released by the organism in the vapour phase and then captured for extraction using a novel distillation process, eventually the spent microalgae biomass is converted to fuel using a variant of the HTL process. Algenol claims the following figures for their pilot plant (http://www.algenol.com/):

- **Yield**: 8000 gallons per acre of total liquid fuel production (80,000 L/ha) of which 85 % is ethanol and the remaining 15 % is hydrocarbons
- **Cost**: $1.27 per gallon

Two other companies pursuing milking-based projects are Joule unlimited and Proterro who are focused on chemicals/fuels and sugars as feedstock to traditional biofuel processes, respectively. In all of these approaches, the process is fundamentally different as the milking process extracts the oils, ethanols or other chemicals of interest from the growth medium without killing the microalgae. As a comparison, the traditional microalgae production systems 'kill' the 'cow' (microalgae) to extract the 'milk' (oil) rather than keeping the cow (microalgae) productive and continually harvesting the milk (oil) (Moheimani et al. 2013a). Milking addresses the shortfalls of the existing production systems in two major ways:

- **Nutrients**—Only the products of interest are removed (which typically contain very low N and P), and as a result, there is a limited requirement for fertilisers. Only water, CO_2 and sunlight are required to continually produce the compounds
- **Dewatering**—Microalgae are typically not removed from the culture to be milked to limit the need for dewatering (Moheimani et al. 2013b).

These systems are currently at various stages of early development. Despite this, the potential of these novel approaches to address the major issues with traditional methods warrants their continued investigation.

1.5 Wastewater as a Source of N and P

The main advantages of microalgae growth compared to land plants are the ability to grow on arid land using saline water (Fig. 1.1). This means that microalgae cultures will not compete with food crops over agricultural land and freshwater. However, microalgae, the same as any other photosynthetic organism, would still require fertilisers (especially nitrogen and phosphorous) to grow. If grown in sea water, macronutrients are necessary to be added to the culture to achieve high growth rate. Borowitzka and Moheimani (2013a) indicated that for producing 100,000 bbl of algal oil year^{-1}, there is a need for 14,447 and 219 tons of nitrogen

(as $NaNO_3$) and phosphorous (as NaH_2PO_4), respectively. Such a high volume of fertilisers will significantly affect the overall cost of production. Furthermore, phosphorous is a non-renewable resource, and at current rates of extraction, global commercial phosphate rock reserves may be depleted in less than 100 years (Cordell et al. 2009). That means that algae cultures, irrespective of their product, will be in direct competition with food crops over fertilisers. Obviously, one very important consideration in developing any potential large-scale algae production facility is the recycling of the medium (Fig. 1.1). Recycling medium especially post-extraction/conversion would allow the recycling of a large amount of fertilisers especially if the wet biomass is being converted to biodiesel and biomethane (Fig. 1.1). Furthermore, there is a possibility of combining microalgae cultivation with wastewater treatment. Combining microalgae cultures with wastewater treatment plants (domestic or animal waste) can provide microalgae with required nutrients and result in lower cost wastewater treatment than traditional approaches.

The potential of combining microalgae cultures and domestic wastewater treatment was first proposed in 1960s with the main interest to produce biofuel (Oswald and Golueke 1960). There are currently some facilities around the world (i.e. New Zealand, USA) using high rate algal ponds (HRAPS) for treating tertiary domestic wastewater. In general, microalgae growth in tertiary-level wastewater treatment can significantly reduce the electromechanical cost of treatment (Craggs et al. 2013). Another advantage of using microalgae in the domestic wastewater treatment process is more efficient nutrient removal and sunlight-driven disinfection (Davies-Colley et al. 2005). Animal waste (i.e. piggery waste) can also be treated using microalgae cultures. The environmental impacts of intensive pig production can be significant. A poorly managed piggery may risk wastewater pollution to local waterways, produce odour emissions and release greenhouse gases into the atmosphere (Maraseni and Maroulis 2008). Wastewater generated through high-intensity pig production is high in ammonia and phosphorous while also having high chemical and biological oxygen demands (Olguín et al. 2003). High phosphorous levels have been shown to correlate to high turbidity levels giving the effluent a dark colour (Ong et al. 2006). One wastewater treatment system that is gaining acceptance in Australian piggeries is anaerobic digestion ponds. These systems typically consist of a covered pond containing wastewater which is biologically treated by heterotrophic microorganisms in the absence of oxygen. The covered digesters allow the production and capture of biogas including methane and carbon dioxide. The benefits obtained from these ponds are the removal of solids through settling, capture of biogas for use as a biofuel and the reduction of odour emissions. The utilisation of methane as a fuel source can effectively reduce dependence on energy sources from outside the piggery. One challenge is that the anaerobic digestion effluent from piggeries is very high in ammonium (toxic to most organisms). If a process incorporating CO_2 uptake such as algae culture was to be adopted, ideally CO_2 (generated via burning CH_4 or separated from the raw biogas stream) will be captured and reused within the piggery. A recent review of wastewater management in Australian piggeries recommended that along with anaerobic digestion, microalgae culture systems should be investigated further as a

potential component of the Australian piggery wastewater management strategy (Buchanan et al. 2013).

To date, all trials on culturing microalgae on undiluted and untreated anaerobic digestion piggery effluent (ADPE) have failed to gain widespread acceptance in the industry. On the other hand, there are reports of the successful microalgal cultivation on piggery anaerobic digestate after dilution with freshwater (Park et al. 2010). Interestingly, in some cases, the digestate was diluted more than 15 times with freshwater. In the context of an Australian piggery system, such a method would never be practical due to the shortage of freshwater. Ayre (2013) isolated three microalgae capable of growing on undiluted, sand-filtered, piggery anaerobic digestate. This proof-of-concept study clearly illustrated the potential for culturing microalgae in such effluent with a high ammonium content. The produced algae biomass on piggery anaerobic digestate will sequester carbon and remove nutrients (i.e. nitrogen and phosphorous). The produced biomass could alternatively be used as pig feed, although the biomass pathogen load would need to be closely monitored (Buchanan et al. 2013). Another potential application for the biomass is the co-anaerobic digestion with the piggery waste.

1.6 Microalgae Growth in Saline to Hypersaline Water

The growth of algae, irrespective of cultivation system, requires large volumes of water. Almost all areas with high solar energy also have a high evaporation rate. Therefore, it is logical to use sea water for large-scale algae biomass production. As highlighted previously, it is also critical to recycle the culture medium to reduce the nutrient use. Sea water must also be used to replace evaporative loss. This means that the salt concentration in the pond will gradually increase over the time. For instance, in conditions with evaporations rate of 2 m $year^{-1}$, productivity of 20 g m^{-2} day^{-1} and 80 % medium recycling, the medium salinity will rise from 3.5 % NaCl to 25 % NaCl in 490 days. Salinity is usually growth-limiting at the extremes of salt tolerance in some microalgal species, and every microalga has an optimum salinity range (Borowitzka and Moheimani 2013a). The effect of salinity on microalgal growth relates to osmoregulation, which in microalgae is achieved through diverse strategies. Osmoregulatory metabolites are organic substances produced by microalgae that, when the latter are exposed to water stress conditions, respond appropriately to the changes in extracellular water activity. Microalgae main osmoregulators (function as intracellular osmotic regulators) are as follows: (a) polyhydric alcohols (i.e. glycerol, mannitol or sorbitol), (b) variety of glycosides (i.e. galactosyl glycerides, floridoside and isofloridoside) and (c) amino acids (i.e. glutamic acid and proline).

Freshwater algae grow between 0 and 1–2 % NaCl; hypotonic algae grow between 3.0 and 5–5.5 % NaCl; halotolerant algae grow between 6–7 and 14–15 % NaCl; and halophylic algae can grow above 15–16 % NaCl. The majority of microalgae can grow in freshwater and hypotonic conditions. Some microalgae

(i.e. diatom, chlorophyta and cyanobacteria) are capable of growth under halotolerant conditions. However, only a few species of microalgae are hypersaline (i.e. *D. salina*). There is no single strain of algae capable of optimal growth in the whole range of salinity from sea water to saturation. Interestingly, almost all companies interested in large-scale algae production focus on growing either freshwater or hypotonic algae which will not be sustainable (Moheimani et al. 2013c). Halotolerant algae can normally grow under optimal condition in a wider range of salinities (Fon Sing 2010).

An alternative method of cultivation is to mix microalgae while salinity is being increased. In such a method, a new species can be introduced to the culture of a mono-species while the salinity is rising. That is, a halotolerant microalga species will be introduced to the culture of hypotonic algae when salinity is above the optimum growth condition of the hypotonic algae. If the halotolerant alga can flourish while salinity is increased, the medium use can be maximised. The same technique can be applied for mixed cultures of halotolerant and halophylic microalgae. Such a mixed cultivation technique is yet to be tested at the laboratory or outdoor conditions. One very important advantage of mixed microalgae cultivation is avoiding unnecessary water and nutrient discharge. Considering the species change throughout the cultivation, less negative effect of autoinhibitors is also expected. When the salinity of the culture becomes very high, one option is to have large evaporation ponds for the hypersaline wastewater. Alternatively, this hypersaline water can also be used in salt gradient solar ponds for generating additional energy.

1.7 Hybrid Microalgae Culture and Desalination

The requirements for microalgal dewatering, nutrient recycling and control of effluent wastewater are becoming major challenges to producers (Borowitzka and Moheimani 2010; Charcosset 2009; Clarens et al. 2010; Wyman and Goodman 1993b; Xiong et al. 2008). It is crucial for industrial microalgae production to avoid becoming a net energy-intensive process generating effluent waste at risk of variable profitability (Borowitzka et al. 2010; Borowitzka and Moheimani 2010; Charcosset 2009; Clarens et al. 2010; Wyman and Goodman 1993b; Xiong et al. 2008). As freshwater and energy are essential commodities, finding low-cost and high energy-efficient means to process water and utilise both waste energy and wastewater streams is important (Gude et al. 2010; McHenry 2013). As open pond microalgae production can become expensive due to variable capital, operational, and down-stream processing costs derived from the low microalgae cell densities (Lee 2001; McHenry 2010), and if not optimised, industrial microalgae production will consume large volumes of water through evaporative loss (Chisti 2007; Clarens et al. 2010), generate effluent and become an energy-intensive process (Borowitzka and Moheimani 2010; Charcosset 2009; Clarens et al. 2010; McHenry 2013; Wyman and Goodman 1993b; Xiong et al. 2008). In parallel, conventional

industrial and mining process contaminated wastewater streams are also utilising desalination technologies to reduce environmental contamination and associated costs. With new methods of desalting and water processing, including reverse osmosis (RO), forward osmosis (FO) and membrane distillation (MD), desalination technologies are decreasing water processing costs markedly (Bennett 2011; Bourcier and Bruton 2009; Nicoll et al. 2011). This nascent area will require advances in technical performance, reliability and cost of water processing technology to become commercially viable (Banat and Jwaied 2008).

The introduction of microalgae to water processing is a major potential field of exploration in water processing and efficiency circles. As microalgae species can maintain high growth rates in poor-quality or contaminated water and salinities higher than sea water (Amin 2009; Beer et al. 2009; Hightower 2009), they are able to expand the existing tranche of development possibilities and enable new intensive production options (Cantrell et al. 2008; Gross 2007; Hankamer et al. 2007). Industrial-scale microalgae production will likely require large and intensive water processing technologies for both culturing and biomass recovery. Yet, achieving energy-efficient and cost-effective microalgae dewatering and water management are major challenges. Progressive vertical integration of energy and water-intensive technologies (including large-scale algae) may enable higher aggregated net industrial efficiencies and potentially create new major resources as by-products including minerals, animal feeds, fertilisers, freshwater, electricity, and biofuel. The integration and colocation of industrial microalgae production (e.g. photobioreactors and open ponds) with desalination and other industrial operations are a growing potential area. The theoretical basis behind higher aggregated efficiencies is essentially vertical integration of infrastructure, energy and material flows, reducing total costs, net waste and associated potential environmental contamination. In particular, the judicial use of fast advancing technical capabilities to process water at high efficiency or using waste heat and wastewater through colocating with other industrial facilities may effectively cross-subsidise microalgae energy and water use (McHenry 2013).

1.8 Conclusion

There is no doubt that finding alternative renewable sources of food and energy for future generations is needed. Algae may be a promising answer for the future of biomass production and a carbon-neutral fuel source, considering that microalgae produce significantly higher areal biomass than traditional terrestrial crops. Still, it is unreasonable to think that there is a ‘silver-bullet’ answer to microalgae large-scale biomass production. The conventional (mixed and unmixed open ponds) microalgae to biomass production systems have only been economical for high-value products. The cost of production of microalgae in closed photobioreactors is also most likely to be to very high and not sustainable. Some alternative cultivation methods such as biofilm (see Chap. 2) and mixotrophic growth (see Chaps. 3 and 4), milking

(Moheimani et al. 2013a, b) and combining solar panels with microalgae cultivation system (see Chap. 15) can bring down the cost of production. Combining microalgae cultivation with CO_2 bioremediation (see Chap. 7) and wastewater treatment (see Chaps. 5 and 6) can also add value to the whole algae biomass production. Furthermore, developing novel and economical dewatering systems will positively reduce the overall cost of production (see Chaps. 12, 13 and 14). Last but not least, modifying the algal species of interest can also reduce the cost of production (see Chaps. 8, 9, 10 and 11). Chapter 17 summarises the technoeconomics of the microalgae to biofuel production.

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Chapter 2
Application of Various Immobilization Techniques for Algal Bioprocesses

Ela Eroglu, Steven M. Smith and Colin L. Raston

Abstract Immobilized cells entrapped within a polymer matrix or attached onto the surface of a solid support have advantages over their free-cell counterpart, with easier harvesting of the biomass, enhanced wastewater treatment, and enriched bioproduct generation. Immobilized microalgae have been used for a diverse number of bioprocesses including gaining access to high-value products (biohydrogen, biodiesel, and photopigments), removal of nutrients (nitrate, phosphate, and ammonium ions), heavy metal ion removal, biosensors, and stock culture management. Wastewater treatment processes appear to be one of the most promising applications for immobilized microalgae, which mostly involve heavy metal and nutrient removal from liquid effluents. This chapter outlines the current applications of immobilized microalgae with an emphasis on alternative immobilization approaches. Advances in immobilization processes and possible research directions are also highlighted.

2.1 Introduction

Algal bioprocesses are advantageous in integrating wastewater treatment processes with valuable biomass production. Algal biomass can be further exploited for various purposes such as biofuel generation in the form of biodiesel, biohydrogen, or biogas;

E. Eroglu (✉) · S.M. Smith
School of Chemistry and Biochemistry, The University of Western Australia, Crawley, WA 6009, Australia
e-mail: elaeroglu@gmail.com

E. Eroglu · S.M. Smith
ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, Crawley, WA 6009, Australia

C.L. Raston
Centre for NanoScale Science and Technology, School of Chemical and Physical Sciences, Flinders University, Bedford Park, SA 5042, Australia

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food additives; slow-release fertilizers or soil conditioners; cosmetics; pharmaceuticals; and several other valuable chemicals (Johnson and An 1991; Mallick 2002; Mulbry et al. 2005). Microalgal cells have several other advantages in not requiring many resources to generate their biomass, providing an economical operation at lower costs, with the dissolved oxygen released by the algae being useful to elevate the oxygen levels of water effluents, and can be utilized for the reduction of carbon dioxide emissions using CO_2 for their biomass and/or energy production.

Harvesting and dewatering of algal biomass from its liquid environment is one of the major challenges of algal bioprocesses. Several studies have focused on harvesting of microalgae using a wide range of technologies from sand filtration to high-speed centrifugation (Mallick 2002; Oswald 1988). Some of the most recent technologies for algal dewatering are further discussed in Chaps. 12, 13, and 14 of this book. Immobilization of algal cells has been proposed mainly to overcome the burdens of difficult harvesting and dewatering stages, in addition to providing the retention of the high-value-added algal biomass for further processes (de la Noue and de Pauw 1988; Mallick 2002).

Immobilization of various cells in either polymeric or biopolymeric matrices has several advantages over their free-cell counterparts, since immobilized cells occupy less space, are easier to handle, and can be used repeatedly for product generation (Mallick 2002). Immobilization of cells has also been proposed to increase the biosorption capacity and bioactivity of the biomass (Akhtar et al. 2004; de-Bashan and Bashan 2010). It allows bioprocesses with higher cell densities and also easy harvesting of biomass from its liquid environment (Mallick 2002). Immobilization processes have several other advantages as being resistant to harsh environments such as salinity, metal toxicity, and pH; protecting the aging cultures against the harmful effects of photoinhibition; yielding higher biomass concentrations; recovering the cells in a less-destructive way; and enhancing the cost-effectiveness of the process by reusing the regenerated biomass (Bailliez et al. 1986; Hall-Stoodley et al. 2004; Liu et al. 2009). Given the use of large-scale bioreactors represents a significant challenge associated with algal biomass recovery, immobilization systems are becoming attractive alternatives for scale-up processing (Christenson and Sims 2011; Hoffmann 1998).

Various immobilization processes are in use, such as adsorption, confinement in liquid–liquid emulsions, capturing with semipermeable membranes, covalent coupling, and entrapment within polymers (de-Bashan and Bashan 2010; Mallick 2002). Among others, the most common immobilization processes are the entrapment of the cells within polymeric matrices and self-adhesive attachment of cells onto the surfaces of solid-supports (Godlewska-Żyłkiewicz 2003). Both synthetic and natural polymers can be applied as the immobilization matrix (de-Bashan and Bashan 2010).

Important criteria for successful entrapment are to set the algal cells free within their partition, while pores inside the gel matrix allow the diffusion of substrates and the metabolic products toward and from the cells (Mallick 2002). Nevertheless, entrapment systems still hold some drawbacks in reducing the mass transfer kinetics of the uptake of metal ions (Aksu et al. 2002). However, these can be avoided by

careful choice of the immobilization method and the nature of the matrix, which will be further discussed in detail.

Key applications suggested for immobilized algal cells include removal of nutrients from aqueous solutions (Chevalier and de la Noüe 1985), biodiesel production (Bailliez et al. 1985; Li et al. 2007), biosorption of heavy metals from wastewaters (de-Bashan and Bashan 2010), photoproduction of hydrogen and photopigments (Bailliez et al. 1986; Laurinavichene et al. 2008), providing an alternative technique to the common cryopreservation processes (Chen 2001; Faafeng et al. 1994; Hertzberg and Jensen 1989), and also toxicity testing (Bozeman et al. 1989). These processes will also be discussed in detail in the following sections of this review article.

2.2 Immobilization Techniques and Applied Matrices

Entrapment is one of the most common immobilization methods which consists of capturing the cells in a three-dimensional gel lattice, made of either natural (agar, cellulose, alginate, carrageenan) or synthetic (polyacrylamide, polyurethane, polyvinyl, polypropylene) polymers (de-Bashan and Bashan 2010; Hameed and Ebrahim 2007; Liu et al. 2009). Synthetic polymers are reported to be more stable in wastewater samples than the natural polymers, whereas natural polymers have higher nutrient/product diffusion rates and are more environmentally friendly (de-Bashan and Bashan 2010; Leenen et al. 1996).

Polysaccharide gel-immobilized algal cells have often been used for the removal of nitrate, phosphate, and heavy metal ions from their aqueous environment, in providing an alternative to the current physicochemical wastewater treatment technologies (Bayramoğlu et al. 2006). Microalgae cells entrapped within either alginate or carrageenan beads were shown to have sufficient immobilization and significant nutrient removal efficiencies from aqueous environments (Chevalier et al. 2000). Aguilar-May et al. (2007) reported that the immobilization of *Synechococcus* sp. cells in chitosan gels had a positive effect on protecting the cell walls from the toxic effect of high NaOH concentration, with immobilized cells displaying higher growth than their free-cell counterparts.

Alginate beads are one of the most common encapsulation matrices, being an anionic polysaccharide found mostly in the cell walls of brown algae (Andrade et al. 2004). Major advantages of alginate gel are it being nontoxic, easy to process, cost-effective, and transparent and permeable (de-Bashan and Bashan 2010). Despite these advantages, alginate beads have some drawbacks such as not retaining their polymeric structure in the presence of high phosphate concentrations or high content of some cations such as K^+ or Mg^{2+} (Kuu and Polack 1983). Faafeng et al. (1994) observed the degradation of sodium alginate beads, used for the immobilization of *Selenastrum capricornutum*, after keeping them in polluted wastewater with high phosphorous (P) and nitrogen (N) content for longer than two weeks. This degradation problem can be minimized if the stability of the target gel

is enhanced. In this context, Serp et al. (2000) found that the mechanical resistance of alginate beads was doubled after mixing them with chitosan. Japanese konjac flour was also used to increase the stability of chitosan gels during tertiary treatment of wastewaters with high phosphate concentrations (Kaya and Picard 1996). Kuu and Polack (1983) suggested that increasing the gel strength of carrageenan and agar gels by integrating them with polyacrylamide results in a more rigid support for microorganisms.

Most of the entrapment processes have a similar protocol, namely mixing the microalgal suspension with the monomers of the selected polymer, followed by solidification of the resulting algae/polymer mixture by some physical or chemical process such as cross-linking of the monomers of the polymer with di- or multivalent cations (Cohen 2001; de-Bashan and Bashan 2010). As an illustration, a general procedure for the entrapment of microalgae within alginate beads includes the following steps: (1) mixing of algal suspension with sodium alginate solution, (2) placing the homogenously distributed algae/alginate mixture in a vessel with a small orifice, such as a syringe, (3) gently dripping the mixture from the syringe as small droplets/beads into a cross-linking solution such as calcium chloride, (4) optimizing the time for algae/alginate beads inside the cross-linking solution to form cross-linked/hardened beads, (5) collecting the final algae/alginate beads, and rinsing them with deionized water several times (Smidsrød and Skjåk-Bræk 1990). Since a manual dripping process for bead production is not practical for larger scale processes, automated prototypes were also proposed for the mass production of gel beads (de-Bashan and Bashan 2010; Hunik and Tramper 1993).

There are some drawbacks of cellular entrapment due to limitations of the oxygen and/or carbon dioxide transfer from the liquid environment through the immobilization matrix, which would cause difficulties mainly for aerobic microorganisms (Toda and Sato 1985). Co-immobilization of the target microorganism with microalgal cells has been proposed as an interesting alternative to overcome any oxygen transfer limitations. Since microalgae are capable of generating oxygen from the photolysis of water, they function as ideal oxygen generators for their surrounding microenvironments (Adlercreutz et al. 1982; Chevalier and de la Noüe 1988). Selected microalgae–bacteria pairs have already been shown to benefit from each other, with microalgal cells generating oxygen and some organic compounds that are assimilated by bacteria. On the other hand, bacteria release some vitamins and phytohormones or provide an additional CO_2 source that can enhance the algal growth (de-Bashan et al. 2005; Gonzalez and Bashan 2000; Mouget et al. 1995). Mouget et al. (1995) also found that *Pseudomonas diminuta* and *Pseudomonas vesicularis* bacterial cells isolated from the algal cultures of *Chlorella* sp. and *Scenedesmus bicellularis* stimulate the growth of those microalgal cells.

Previous attempts to immobilize viable algal cells inside gels faced other limitations, as the volume-to-surface ratios of spherical encapsulating materials are usually orders of magnitude larger than that of thin films. As a consequence, algal viability is a concern since the nutrients or reactants have to diffuse far into these materials to reach the algal cells. In order to overcome these problems, several other immobilization matrices have been proposed in the recent literature. Three different

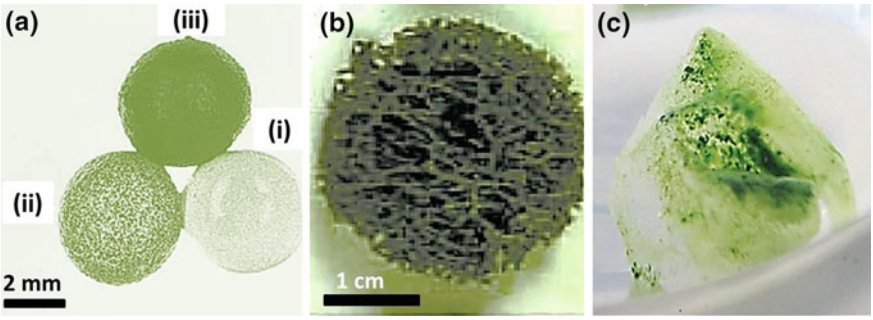

Fig. 2.1 **a** Alginate beads containing different amounts of immobilized *Scenedesmus quadricauda*: (*i*) ca. 2500; (*ii*) ca. 20,000; (*iii*) ca. 90,000 algal cells (modified from Chen 2001), **b** *Chlorella sorokiniana* cells covering the surface of a *Luffa cylindrica* sponge (modified from Akhtar et al. 2008), **c** *Chlorella vulgaris* cells attached on the surface of a chitosan nanofiber mat (3 × 2 cm) floating inside the algal growth media (modified from Eroglu et al. 2012)—reproduced by permission of The Royal Society of Chemistry

immobilization matrices with different geometries and chemical properties are given in Fig. 2.1.

Algal biofilms are one of the alternatives to overcome the harvesting problems of algae in larger scale processes, where microalgal cells stick to each other on external surfaces (Chevalier et al. 2000; Wuertz et al. 2003). Microorganisms form a biofilm as a response to several factors, such as the cellular recognition of the specific functional groups on the targeted surfaces (Karatan and Watnick 2009). Microorganisms forming a biofilm on a surface secrete extracellular polymeric substance, which is mainly composed of phospholipids, proteins, polysaccharides, and extracellular DNA (Hall-Stoodley et al. 2004; Qureshi et al. 2005). Polystyrene disks (Przytocka-Jusiak et al. 1984), textured steel surfaces (Cao et al. 2009), aluminum disks (Torpey et al. 1971), and polystyrene surfaces (Johnson and Wen 2010) are some examples of biofilm surfaces used for algal growth for the primary application of nutrient removal from wastewaters.

The shape of algal cell composite material has two components, a global geometrical form and the surface detail which determines the texture of the surface, with nanomaterial processing techniques being the useful approaches for creating different shapes, from fibers to spheres and flat membranes (Crandall 1996). Various nanofabrication processes have featured in recent research from the authors' laboratories, albeit in using more unconventional types of immobilization matrices for the immobilization of *Chlorella vulgaris* cells, such as electrospun nanofibers (Eroglu et al. 2012), laminar nanomaterials such as graphene and graphene oxide nanosheets (Wahid et al. 2013a, b), microfibers of ionic liquid-treated human hair (Boulos et al. 2013), and magnetic polymer matrix composed of magnetite nanoparticles embedded in polyvinylpyrrolidone (Eroglu et al. 2013). Electrospinning processes can create nanofiber mats with high porosities and surface-to-volume ratios and are generated by forcing a charged polymer solution through a very

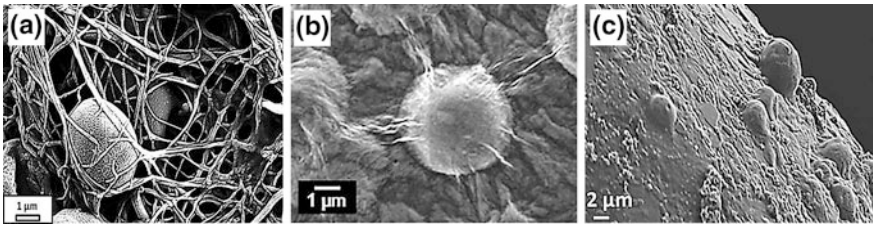

Fig. 2.2 Scanning electron microscopy images of **a** chitosan nanofibers (modified from Eroglu et al. (2012)—reproduced by permission of The Royal Society of Chemistry); **b** multilayer graphene oxide nanosheets (modified from Wahid et al. (2013a)—reproduced by permission of The Royal Society of Chemistry); **c** microfibers of ionic liquid-treated human hair (modified from Boulos et al. (2013)—reproduced by permission of The Royal Society of Chemistry), surrounding *Chlorella vulgaris* microalgal cells

small-sized nozzle while applying an electrical field (Kelleher and Vacanti 2010). On the other hand, a recently developed vortex fluidic device has been successfully used for the exfoliation of laminar materials within the dynamic thin films formed on the walls of this microfluidic platform (Wahid et al. 2013a, b). Scanning electron microscopic images of different nanomaterial matrices, used for the immobilization of *C. vulgaris* microalgal cells, are given in Fig. 2.2.

2.3 Wastewater Treatment Using Immobilized Algae

Wastewater treatment consists of the removal of unwanted chemicals, or biological contaminants from impure water sources, such as from the liquid wastes released by houses, industrial operations, or agricultural processes. Conventional wastewater treatment methods include physical processes such as filtration and sedimentation; chemical processes such as flocculation and chlorination; and biological processes such as generation of activated sludge (Metcalf and Eddy 2003). However, these methods are mainly based on the separation of pollutants from the wastewater with a requirement for a further stage to eliminate these pollutants. This brings a need for an integrated wastewater treatment process that eliminates the undesired portion of the wastewater while converting them into valuable products, which can be successfully achieved by applying a selected immobilization process. Immobilized algal systems are particularly effective for the removal of nutrients (i.e., phosphate and nitrate) and various metals from wastewaters, which will be discussed in the following sections.

2.3.1 Nutrient Removal from Wastewater

Several studies have demonstrated the potential of microalgae for the removal of nitrogen and phosphorus elements from wastewater effluents, with cells taking them

up as their nutrient sources. Some of those studies are listed in Table 2.1, while some are further described in the following text. It should be noted that the direct comparison of the nutrient removal efficiencies from various experiments is inherently difficult, because of variations in the initial nutrient concentration, duration of the experiment, pH of the working solution, selected algal species, and the type of immobilization matrix.

The most common algal species used for the removal of nitrates and phosphates are *Chlorella*, *Scenedesmus*, and *Spirulina*. Various open and closed bioreactors have been used for the removal of nutrients by algae, ranging from tubular photobioreactors to corrugated raceways and high-rate algae ponds (Borowitzka 1999; Cromar et al. 1996; Olguín et al. 2003). Increased nutrient removal efficiencies with immobilized algae are usually related, with the dual effect of the enhanced photosynthetic rate of the cells and the ionic exchange between the nutrient ions and the immobilization matrix. Gels which are anionic in nature, such as carrageenan, are usually associated with the adsorption of cations (such as ammonium (NH_4^+)), while cationic gels such as chitosan yield adsorption of anions (phosphate (PO_4^{-3}), nitrate (NO_3^-), nitrite (NO_2^-)) with higher efficiencies (Mallick and Rai 1994). Moreover, calcium ions of the alginate or chitosan gels are particularly efficient for the precipitation of PO_4^{-3} ions from wastewaters (Lau et al. 1997).

Immobilization of *C. vulgaris* cells within sodium alginate beads showed higher nutrient removal efficiencies from sewage wastewater compared to their externally immobilized counterparts on polyurethane foam (Travieso et al. 1996). de-Bashan et al. (2002b) obtained higher ammonium and phosphate removal efficiencies after co-immobilization of *C. vulgaris* microalgae with plant growth-promoting bacterium *Azospirillum brasilense* in alginate beads, relative to immobilized *C. vulgaris* cells alone. Tam and Wong (2000) obtained 78 % ammonium and 94 % phosphate removal efficiencies with immobilized *C. vulgaris*, entrapped in calcium alginate beads, compared to the 40 % ammonium and 59 % phosphate removal with free cells. Lau et al. (1997) also observed significantly higher ammonium (95 %) and phosphate (99 %) removal efficiencies for *C. vulgaris* cells immobilized in alginate beads relative to their free counterparts, resulting in only 50 % nitrogen and 50 % phosphate removal. In contrast, free cells of *Nannochloropsis* sp. cells yielded higher total phosphorus removal with respect to their immobilized cells within calcium alginate beads (Jimenez-Perez et al. 2004).

Pretreatment of the cells by starving them in a saline solution for three days was found to increase the cellular growth and phosphate removal efficiencies of the independently co-immobilized *Chlorella sorokiniana* & *A. brasilense* and *C. vulgaris* & *A. brasilense* pairs entrapped in alginate beads (Hernandez et al. 2006). Kaya et al. (1995) observed higher nutrient removal rates using *S. bicellularis* cells when they were immobilized on flat-surface alginate screens compared to their encapsulated form inside alginate beads.

Canizares et al. (1993) used immobilized *Spirulina maxima* cells in kappa-carrageenan gel beads for nutrient removal from swine waste. This immobilized system achieved around 90 % total phosphorus and ammonium-nitrogen removal, while it also allowed processing swine waste at higher concentrations. Chevalier

Table 2.1 Examples of studies on nutrient removal using immobilized algae

Immobilization matrix	Algal species	Targeted pollutant	Reference
Alginate beads	*Chlorella vulgaris*	Ammonium, phosphate	Tam and Wong (2000)
	Nannochloropsis sp.; *Scenedesmus intermedius*	Total phosphorous, total nitrogen	Jimenez-Perez et al. (2004)
	Chlorella vulgaris and *Azospirillum brasilense* (co-immobilization)	Ammonium, phosphate	de-Bashan et al. (2002b)
	Chlorella sorokiniana and *A. brasilense* (co-immobilization)	Phosphate	Hernandez et al. (2006)
Carrageenan beads	*Spirulina maxima*	Total phosphorus, ammonium	Canizares et al. (1993)
	Scenedesmus acutus; *Scenedesmus obliquus*	Ammonium, phosphate	Chevalier and de la Noüe (1985)
Agar beads	*Chlorella vulgaris*; cyanobacterium *Anabaena doliolum*	Phosphate, nitrate, nitrite	Mallick and Rai (1994)
Alginate beads			
Carrageenan beads			
Chitosan beads			
Chitosan beads	*Scenedesmus* sp.	Phosphate, nitrate	Fierro et al. (2008)
Flat-surface alginate screens	*Scenedesmus bicellularis*	Ammonium, phosphate	Kaya et al. (1995)
Alginate beads			
Filter paper	*Trentepohlia aurea*	Ammonium, nitrate, nitrite	Abe et al. (2003)
Twin-layer system composed of nitrocellulose membrane, and glass fibers	*Chlorella vulgaris*, *Scenedesmus rubescens*	Phosphate, ammonium, nitrate	Shi et al. (2007)
Polyvinyl foams	*Scenedesmus obliquus*	Nitrate	Urrutia et al. (1995)
Polyurethane foams			
Alginate beads	*Chlorella vulgaris*, *Chlorella kessleri*, *Scenedesmus quadricauda*	Ammonium, phosphate	Travieso et al. (1996)
Carrageenan beads			
Polystyrene foams			
Polyurethane foams			
Chitosan nanofibers	*Chlorella vulgaris*	Nitrate	Eroglu et al. (2012)
Graphene nanosheets			Wahid et al. (2013b)
Graphene oxide nanosheets			Wahid et al. (2013a)

and de la Noüe (1985) investigated *Scenedesmus acutus* and *Scenedesmus obliquus* cells individually immobilized in kappa-carrageenan beads for nutrient removal from a secondary effluent. Immobilized cells showed similar cellular growth and ammonium or phosphate uptake rates compared to their free-living cell counterparts. They observed around 90 % ammonium removal within the first 4 h, while all traces of phosphate were removed within 2 h (Chevalier and de la Noüe 1985).

C. vulgaris and *Anabaena doliolum* cells immobilized in chitosan have higher phosphate, nitrate, and nitrite removal efficiencies than when they were immobilized within agar, alginate, or carrageenan (Mallick and Rai 1994). In addition, the phosphate removal capacity of the immobilization process was increased when phosphate-deprived cells were initially entrapped within chitosan. Fierro et al. (2008) investigated the nitrate and phosphate removal efficiencies of individually entrapped *Scenedesmus* sp. cells within chitosan beads. Immobilized cells achieved approximately 94 % phosphate and 70 % nitrate removal within the first 12 h after incubation, whereas by themselves chitosan beads removed 60 % phosphate and 20 % nitrate by the end of the experiment. The reason for yielding a significant phosphate removal rate (60 %) by chitosan beads alone was explained by the increased pH values, which eventually triggered the release of some calcium ions from chitosan polymer, resulting in the precipitation of phosphate ions (Fierro et al. 2008; Tam and Wong 2000).

Other immobilization matrices have also been proposed as alternatives to the gel beads. Immobilized cells of *Trentepohlia aurea* microalgal cells on a filter paper formed a biofilm layer that reduced the concentration of ammonium, nitrate, and nitrite ions, for around 40 days (Abe et al. 2003). Shi et al. (2007) proposed a twin-layer system, where the microalgal cells are attached on an ultrathin and microporous "substrate layer" composed of a nitrocellulose membrane, which is surrounded by a "source layer" of macroporous glass fiber providing the growth medium (Shi et al. 2007). They observed phosphate, ammonium, and nitrate removal when *C. vulgaris* and *Scenedesmus rubescens* microalgal cells were entrapped in this twin-layer system.

In a recent study, *C. vulgaris* cells immobilized on electrospun chitosan nanofiber mats yielded an efficient nitrate removal rate (87 %) as a result of the dual action of nitrate removal by the microalgal cells and electrostatic binding of the nitrate ions on chitosan nanofibers (Eroglu et al. 2012). In other studies from the authors' laboratories, the resulting microalgal composites with multilayer graphene (Wahid et al. 2013b) or graphene oxide sheets (Wahid et al. 2013a) also achieved significant nitrate uptake rates, without being toxic for the microalgal cells.

2.3.2 Metal Removal from Wastewater

Conventional methods used for the removal of heavy metal ions include chemical precipitation, adsorption, chemical oxidation/reduction, membrane filtration, ion exchange, and electrochemical processes. However, these techniques have some

drawback, such as partial removal of metal ions, costly installation requirements, high energy demands, and the generation of toxic waste products which require additional elimination stages (Aksu et al. 2002).

Both live and dead cells can be successfully used for the biosorption of metal ions, while uptake of metal ions by living microorganisms, referred to as bioaccumulation, occurs when an active metabolic process is involved (Aksu et al. 2002; Brady and Duncan 1994; Moreno-Garrido et al. 1998). Biosorption is a reversible process, since it is possible to desorb the metal ions bound to the surfaces of cells by a simple acid treatment, whereas bioaccumulation processes are only partially reversible (de-Bashan and Bashan 2010; Dönmez and Aksu 2002; Velásquez and Dussan 2009).

Compared to the other organisms used for biosorption processes, namely fungi, cyanobacteria, and bacteria, algal cells have higher heavy metal biosorption capacities which relates to the different structure and composition of their cell wall (Bayramoğlu et al. 2006; Gekeler et al. 1988). Cell walls of different microorganisms have different functional groups which are involved in metal ion binding, such as amino, amide, carbonyl, carboxyl, hydroxyl, imidazole, phosphate, sulfate, sulfhydryl, and phenol moieties (Barkley 1991; Schiewer and Volesky 2000). Depending on the variations in the cell wall composition, there will also be differences in the metal ion binding mechanisms and affinities (Godlewska-Żyłkiewicz 2003; Leusch et al. 1995).

The chemical characteristics of the functional adsorbent (i.e., functional groups, polarity, and solubility) are responsible for determining the binding mechanism and the nature of the adsorption process. Different physicochemical forces, such as covalent bonding, van der Waals bonding, ion exchange, and dipole/dipole interactions can be responsible for the uptake of ions on the adsorbents (Aksu et al. 2002).

Free cells have some disadvantages when used for large-scale applications of metal ion biosorption studies, due to the otherwise risk of clogging problems on the filters and flow lines. Nevertheless, this problem was overcome by using immobilized cells in natural matrices such as carrageenan, alginate, chitosan, agarose; polymeric supports such as polyacrylamide, polypropylene, and polysulfone; crosslinked copolymers; or biomatrices such as sponges (Akhtar et al. 2003a, b; Robinson 1998). Some of those studies are highlighted in Table 2.2.

The presence of more than one type of metal ion within the wastewater might have a negative effect on the adsorption of one type of metal ion over another. Mehta and Gaur (2001) observed nearly complete removal of copper and nickel metals by alginate-entrapped *C. vulgaris* cells when they were in separate solutions. On the other hand, the presence of copper in the nickel solution inhibited the biosorption of both metals either by immobilized or free cells, due to the competition of different metal ions on the same active sites of microalgae. da Costa and Leite (1991) used alginate-immobilized *Chlorella homosphaera* for the removal of cadmium and zinc metals. They also observed that the biosorption of cadmium and zinc alone was much higher than the case when these two metal ions were combined.

Table 2.2 Examples of studies on metal removal using immobilized algae

Immobilization matrix	Algal species	Targeted metals	Reference
Alginate beads	*Chlorella vulgaris*	Copper, nickel	Mehta and Gaur (2001)
	Chlorella homosphaera	Cadmium, gold, zinc	da Costa and Leite (1991)
	Chlamydomonas reinhardtii	Cadmium, lead, mercury	Bayramoğlu et al. (2006)
	Chlorella vulgaris; cyanobacterium *Anabaena doliolum*	Chromium	Mallick and Rai (1993)
	Dunaliella salina; *Nannochloropsis gaditana*; *Rhodomonas salina*; *Thalassiosira pseudonana*; *Tetraselmis chui*; *Porphyridium cruentum*	Cadmium, copper	Moreno-Garrido et al. (2005)
Carrageenan beads	*Chlorella vulgaris*; *Scenedesmus acutus*	Cadmium, chromium, zinc	Travieso et al. (1999)
Polyurethane foam			
Agarose beads	*Chlorella emersonii*	Mercury	Wilkinson et al. (1990)
Agar beads			
Alginate beads			
Polyacrylamide gels	*Chlorella* sp.	Uranium	Nakajima et al. (1982)
Silica gel	*Stichococcus bacillaris*	Lead	Mahan and Holcombe (1992)
	Pilayella littoralis	Aluminum, cobalt, copper, iron	Carrilho et al. (2003)
Capron fibers	*Chlorella* sp. and *Scenedesmus obliquus* and *Stichococcus* sp. in a mixed group of microalgae-bacteria system	Copper, iron, manganese, nickel, zinc	Safonova et al. (2004)
Ceramics			
Cellex-T, anion-exchange resin	*Chlorella vulgaris*	Palladium, platinum	Dziwulska et al. (2004)
Amberlite, ion-exchange resin	*Spirogyra condensate*	Chromium	Onyancha et al. (2008)
	Rhizoclonium hieroglyphicum		
Controlled-pore glass	*Chlamydomonus reinhartii*; *Selenestrum capricornutum*	Chromium, copper, silver	Elmahadi and Greenway (1991)
Luffa cylindrica sponge	*Chlorella sorokiniana*	Cadmium	Akhtar et al. (2003b)
		Chromium	Akhtar et al. (2008)
		Lead	Akhtar et al. (2004)
		Nickel	Akhtar et al. (2003a)

Biological materials were also used as the immobilization matrices for microalgal cells. da Costa and de França (1996) attached the microalgae *Tetraselmis chuii* and cyanobacteria *Spirulina maxima* on the surface of two different seaweeds (*Sargassum* sp. and the *Ulva* sp.), which eventually increased the overall cadmium biosorption efficiencies. In series of studies by Akhtar et al., *C. sorokiniana* algal cells were immobilized on a biological matrix of *Luffa cylindrica* sponge for the removal of nickel (Akhtar et al. 2003a), cadmium (Akhtar et al. 2003b), chromium (Akhtar et al. 2008), and lead (Akhtar et al. 2004) ions from liquid effluents. *L. cylindrica* sponge was chosen as the immobilization matrix due its rigid structure, low cost, and high porosity, while its fibrous network provides an efficient contact between the immobilized cells with their surrounding aqueous environment (see Fig. 2.1b). They reported high maximum adsorption capacities in a continuous liquid flow column, as 192 mg cadmium and 71 mg nickel per gram of immobilized biomass. They also achieved successful desorption of cadmium and nickel metal ions with HCl solution, and the regenerated immobilized samples were reusable with a similar biosorption efficiency.

The biosorption of lead (Pb) ions by *C. sorokiniana* cells immobilized on *L. cylindrica* sponge was another efficient method, with 96 % adsorption efficiency of the metal ions within the first 5 min of the experiments (Akhtar et al. 2004). They also observed a maximum adsorption of lead ions at around pH 5.0. Higher removal rates were associated with the fibrous structure of the immobilization matrix, increased surface area, and easier access of the targeted metal ion to the sorption sites (Akhtar et al. 2003a, b).

Leusch et al. (1995) used two marine brown algae, *Sargassum fluitans* and *Ascophyllum nodosum*, for the biosorption of cadmium, copper, nickel, lead, and zinc heavy metal ions. They observed the highest metal uptakes when the cells were cross-linked with glutaraldehyde, followed by cross-linking with formaldehyde. Both species had the highest biosorption efficiencies for lead and the lowest for zinc. Introducing formaldehyde possibly involves cross-linking of the hydroxylic groups with the sugars of the cell wall, while glutaraldehyde cross-links mostly with the amino groups (Leusch et al. 1995).

Significant amounts of pollutants were removed using a mixed-immobilization of selected consortium of several microalgal species (*Chlorella* sp., *S. obliquus*, *Stichococcus* sp.) and several bacteria (*Rhodococcus* sp., *Kibdelosporangium aridum*) inside a highly contaminated pond, after the separate immobilization of microalgae and bacteria in solid carriers such as capron fibers and ceramics. They established 62 % copper, 62 % nickel, 90 % zinc, 70 % manganese, and 64 % iron removal efficiencies (Safonova et al. 2004).

Bayramoğlu et al. (2006) used immobilized *Chlamydomonas reinhardtii* cells in calcium alginate beads for the removal of mercury, cadmium, and lead ions from aqueous solutions. They observed the highest adsorption capacities for immobilized cells for a pH in the range 5.0–6.0, achieving mercury, cadmium, and lead ion adsorption capacities of 89.5, 66.5, and 253.6 mg g^{-1} dry adsorbent, respectively. On the other hand, control samples composed of only calcium alginate beads provided less metal-binding sites and yielded lower adsorption capacities of

mercury, cadmium, and lead ions at 32.4, 27.9, and 173.9 mg g^{-1} dry adsorbent, respectively. Acidic pH conditions were not optimal due to the protonation of the cell wall components. In contrast, mildly acid conditions (pH range 5.0–6.0) allowed sufficient interaction of the heavy metal ions with the carboxylate and phosphate groups of the algal cell wall (Bayramoğlu et al. 2006). Neutral pH was found to be the optimal condition for an efficient chromium biosorption by immobilized *C. vulgaris* and freshwater cyanobacterium *A. doliolum* cells in alginate (Mallick and Rai 1993).

Barkley (1991) investigated the utilization of immobilized algae in a permeable polymeric matrix for the adsorption of mercury ions from groundwater in both laboratory and pilot-scale field tests. Their resulting immobilization product (AlgaSORB) was quite robust and can be packed within adsorption columns, having sufficient porosity to allow easy diffusion of the ions toward the cells. Field test results showed that AlgaSORB was a highly reasonable alternative to the conventional ion-exchange resins (Barkley 1991).

Nakajima et al. (1982) achieved the removal of uranium ions from both freshwater and seawater samples using the immobilized cells of *Chlorella* sp. in polyacrylamide gels. They also reported that this system can be used several times by applying consecutive adsorption and desorption stages.

Recovery of precious metals with immobilization methods can be a highly cost-effective process. da Costa and Leite (1991) used immobilized *C. homosphaera* cells within alginate beads for the adsorption of gold metal, which achieved a very high absorption yield of around 90 % of the initial quantity of gold present in solution.

Due to their exclusive catalytic properties, corrosion, and oxidation resistivity, palladium and platinum noble metals have been widely used in various areas from metallurgical processes, chemical synthesis, petroleum processing, electronics to automotive industry (Dziwulska et al. 2004). As a result of the high emission risks of these metals into the environment, it has become important to monitor their concentration in environmental samples. Thus, several microorganisms have been investigated for the separation and preconcentration of some trace metals such as palladium, platinum, copper, cadmium, lead, and gold via biosorption processes, which then allows the use of analytical methods such as atomic absorption spectrometry and inductively coupled plasma optical emission spectrometry (Carrilho et al. 2003; Dziwulska et al. 2004; Elmahadi and Greenway 1991; Godlewska-Żyłkiewicz 2003).

Dziwulska et al. (2004) demonstrated the selective biosorption of palladium and platinum ions from strong acidic solutions (pH below 2), using immobilized *C. vulgaris* cells on anion-exchange resin Cellex-T. This technique was also used for the preconcentration and analysis of these noble metals for graphite furnace atomic absorption spectrometry in different environmental samples including wastewater, tap water, and grass. Elmahadi and Greenway (1991) used *Chlamydomonus reinhartii* and *S. capricornutum* algal cells immobilized on controlled-pore glass for the preconcentration of copper, silver, and chromium metals for atomic adsorption spectrophotometric detection. In their work, they also found that

the presence of some compounds, such as sodium chloride, humic acid, and sodium bicarbonate, can interfere with metal biosorption process by competing for the metal ions. Silica gel was used as the immobilization matrix for *Stichococcus bacillaris* microalgae for lead preconcentration (Mahan and Holcombe 1992), while silica gel-entrapped *Pilayella littoralis* brown microalgae was used for the preconcentration of copper, iron, aluminum, and cobalt ions for their detection by inductively coupled plasma optical emission spectrometry (Carrilho et al. 2003).

2.4 Secondary Products Using Immobilized Algae

Photosynthesis is responsible for the conversion of light into chemical energy which can be used for biofuel production, including biohydrogen, biodiesel, bioethanol, and biomethane generation (Hankamer et al. 2007). Immobilized microalgal cultures have also been in use for the enhancement of these secondary product formations, as explained further below.

2.4.1 Biohydrogen Production

Several unicellular green algae are capable of generating hydrogen through their [FeFe]-hydrogenase enzyme by reducing water protons to molecular hydrogen. However, given the sensitivity of [FeFe]-hydrogenase to oxygen, which is generated by photosystem II (PSII), new approaches have been developed for increasing the practical application of microalgae for biohydrogen production (Laurinavichene et al. 2008). For instance, higher hydrogen production efficiencies were achieved by growing the microalgal cells under sulfur-deprived conditions (Melis et al. 2000). Sulfur deprivation causes partial inactivation of PSII, which is responsible for O_2 generation, resulting an enhanced synthesis of [FeFe]-hydrogenase enzyme (Laurinavichene et al. 2008).

Immobilization processes have been proposed by several researchers for enhancing hydrogen production by sulfur-deprived microalgae and also allowing an easy exchange step between "sulfur-replete" and "sulfur-depleted" stages of the experiment (Laurinavichene et al. 2006, 2008). Several challenges require addressing for scaling up the current hydrogen production systems, while immobilization processes offer an alternative approach to the current technology. Immobilized cells were also reported to have higher light utilization efficiencies per area and higher cell densities (Kosourov and Seibert 2009).

In a study by Kosourov and Seibert (2009), *C. reinhardtii* cells were immobilized inside alginate films for the photoproduction of hydrogen. The cells were previously deprived of sulfur and phosphorus nutrients before being entrapped inside the alginate films. They observed higher cell densities and specific hydrogen production rates after the immobilization process. An immobilization strategy also

provided easy protection of the hydrogenase enzyme from oxygen inhibition, yielding higher hydrogen production rates compared to the free cells.

Laurinavichene et al. (2006, 2008) used immobilized *C. reinhardtii* cells on a fiber glass matrix under sulfur-deprived conditions and observed a prolonged hydrogen production phase for the immobilized cells, while the specific hydrogen production rate was similar to the free-cell counterparts. In another study, algal cells were immobilized on fumed silica particles, which had similar hydrogen production rates with the suspended cultures (Hahn et al. 2007). Song et al. (2011) recently used agar-immobilized *Chlorella* sp. cells for a two-stage cyclic hydrogen production involving the oxygenic photosynthesis followed by anaerobic incubation under sulfur-deprived conditions.

2.4.2 Biodiesel Production

Biodiesel is a diesel fuel consisting of mono-alkyl esters of long-chain fatty acids that are generally made by the transesterification of lipids in animal fat or vegetable oils such as soybean, sunflower, rapeseed, and oil palm (Hankamer et al. 2007; Li et al. 2007; Ma and Hanna 1999). As an alternative, microalgae have become popular for the renewable generation of hydrocarbon-based biofuels with high biofuel yields relative to those from plants (Eroglu and Melis 2009; Li et al. 2007).

Immobilization of the hydrocarbon-rich microalgae, *Botryococcus braunii* and *Botryococcus protuberans*, in alginate beads yielded a significant increase in the chlorophyll, carotenoids, cellular growth, and lipid contents of the cells during their stationary growth phase (Singh 2003). Bailliez et al. (1985) also observed enhanced hydrocarbon production for the *B. braunii* cells immobilized in calcium alginate gel as a result of enhanced photosynthetic activity.

In a study by de-Bashan et al. (2002a), *C. vulgaris* and *C. sorokiniana* microalgal cells were individually co-immobilized with *A. brasilense* growth-promoting bacterium in alginate beads. They found that the presence of the growth-promoting bacterium within the immobilization matrix significantly enhanced the metabolism of *Chlorella* strains and yielded higher lipid and fatty acid production.

Li et al. (2007) used immobilization technology for the transesterification of algal oils, using immobilized lipase enzyme from *Candidia* sp. Initially, they grew *Chlorella protothecoides* cells in large-scale photobioreactors at three different sizes (5, 750, 11,000 L) yielding high lipid contents in the range 44–49 % per dry cell weight. Then, immobilized lipase enzyme from *Candidia* sp. was used to catalyze the transesterification of the lipids from *C. protothecoides*, yielding biodiesel production rates of 7.02, 6.12, and 6.24 g L^{-1} from 5, 750, and 11,000 L bioreactors, respectively. They also highlighted that the quality of this *Chlorella* biodiesel was comparable to that for conventional diesel fuels (Li et al. 2007).

2.4.3 Pigment Production

Bailliez et al. (1986) found that the immobilized *B. braunii* cultures in calcium alginate beads had higher chlorophyll and photosynthetic activities compared to their free cells. *S. obliquus* cells immobilized in alginate (Brouers et al. 1983), and *C. vulgaris* and *Anacystis nidulans* in agar (Kayano et al. 1981; Weetall and Krampitz 1980), also showed a significant increase in their chlorophyll content. Enhanced chlorophyll and photosynthetic activity was explained by the protection of immobilized cells from photoinhibition due to the self-shadowing effect, and a possible increase in the concentrations of particular ions in the microenvironment of cells which can improve photosynthesis (Bailliez et al. 1986; Tamponnet et al. 1985).

Individually co-immobilized cells of *C. vulgaris* and *C. sorokiniana* with *A. brasilense* growth-promoting bacterium also yielded higher chlorophyll *a* and *b*, violaxanthin, and lutein accumulation compared to the immobilized algal cells without any bacterium (de-Bashan et al. 2002a).

Lebeau et al. (2000) reported that the immobilization of the marine diatom *Haslea ostrearia* in agar had a positive effect on the continuous production of the marennin pigment, which is primarily used for the oyster-breeding industry.

Some potential limitations of the secondary product formation by immobilized cells are the commonly reported slower growth rates of the microorganisms compared to their free-cell suspension systems and slower diffusion rates of the target-products (i.e., hydrogen) from the cells into their environment. Resolving these issues with the combination of optimized immobilization matrices and innovative bioreactor designs (e.g., some attempts include membrane-based cell recycle bioreactor (Chang et al. 1994); dual-layer coaxial hollow fiber-type bioreactor (Yang et al. 2006); and a multimembrane bioreactor in a pressure cycling mode (Efthymiou and Shuler 1987), aiming to increase the nutrient transfer to the cells) can potentially bring other dimensions to the research areas of those aforementioned bioprocesses.

2.5 Biosensors

Biosensor research often focuses on the application of enzyme sensors for the detection of toxic chemicals (Dennison and Turner 1995; Shul'ga et al. 1994). Due to the drawbacks of this technology, such as enzyme stability, cost of the process, and difficulty to prepare multienzymatic biosensors, immobilized cells have been proposed as an alternative biosensor technology. Using the entire cells has the advantage of involving various enzymes at the same time, which allows establishing information about the toxicological effects of different pollutants directly on the selected organisms. Immobilized cells had more stable metabolic activities than free cells during the long testing periods (Lukavský et al. 1986) and also higher resistivity to turbid/colored effluents (Bozeman et al. 1989).

The generation or consumption of charged chemicals during bioreactions results in a significant change in the ionic composition of the test sample that can be detected by conductometric biosensors. For this reason, Chouteau et al. (2004) investigated the development of conductometric biosensors using immobilized *C. vulgaris* cells for alkaline phosphatase analysis and cadmium ion detection. *C. vulgaris* cells were immobilized inside bovine serum albumin membranes that were cross-linked with glutaraldehyde vapors.

Frense et al. (1998) used immobilized *Scenesdesmus subspicatus* algal cells as optical biosensors for the determination of the herbicide content in wastewater samples. The algal cells were initially immobilized on a filter paper, which was then covered by alginate and then cross-linked with $CaCl_2$ solution. They used a fiber optics-based electronic device for measuring the chlorophyll fluorescence of algal cells as a response to the presence or absence of the toxic substances in the liquid sample.

C. vulgaris cells immobilized in a membrane of oxygen electrode has been used as a biosensor for the detection of perchloroethylene aerosols by monitoring the photosynthetic activity of the microalgae through oxygen production (Naessens and Tran-Minh 1999). Shitanda et al. (2005) also immobilized alginate-entrapped *C. vulgaris* cells on the surface of an indium tin oxide electrode, for the monitoring toxic compounds such as atrazine, toluene, benzene, and 3-(3,4-dichlorophenyl)-1, 1-diethylurea (DCMU).

Immobilized algal cells of *S. capricornutum* in alginate beads were used for the toxicity testing of various chemicals, such as cadmium ions, copper ions, pentachlorophenol, sodium dodecyl sulfate, and herbicides (glyphosate, hydrothol, paraquat) (Bozeman et al. 1989). In subsequent studies, alginate-immobilized *S. capricornutum* cells were also successively used for the toxicity testing of various pesticides, herbicides, and fungicide (Abdel-Hamid 1996; Van Donk et al. 1992). The immobilization process reduced the toxic effect of these tested chemicals on the algal cells compared to their free-cell equivalents.

2.6 Stock Culture Management

Some researchers have applied immobilization technologies to the stock culture management as an alternative to the common cryopreservation processes, since entrapment processes are cheaper and easier (Chen 2001; Faafeng et al. 1994; Hertzberg and Jensen 1989). Immobilization can also provide protection of the cells toward being consumed by any zooplankton present in the same aquatic ecosystems.

Chen (2001) observed that the immobilized *Scenedesmus quadricauda* cells in alginate beads can preserve their physiological activities for a long time, even after three years of storage in darkness at 4 °C. This observation was explained by the entrapped cells self-consuming their own pyrenoid reserves. Transmission electron microscopic images of immobilized *S. quadricauda* cells showed that they lose

their pyrenoids after extended storage, which is then rebuilt when the cultures are placed back into their nutrient media under light conditions.

Lebeau et al. (1998) also established the ability to store marine diatom *H. ostrearia* cells for nearly 2 months after their entrapment in calcium alginate beads and later used them as a substrate source for the greening of oysters. As a follow-up study, the same group achieved a longer term storage when *H. ostrearia* diatom cells were entrapped in alginate beads and kept at 4 °C (Gaudin et al. 2006). Chen (2003) stored *Isochrysis galbana* marine microalgal cells for more than a year after immobilizing them in alginate at 4 °C in dark conditions, and the cells were then used for feeding clam cultures.

2.7 Other Applications

In some studies, more than one culture was immobilized to achieve a multifunctional immobilization matrix. For example, Adlercreutz et al. (1982) co-immobilized mixed cultures of algae (*Chlorella pyrenoidosa*) and bacteria (*Gluconobacter oxydans*) inside calcium alginate beads for the continuous production of dihydroxyacetone. They did not observe any significant loss of activity within the first six days of this bioprocess. They used the algal cells as an in situ oxygen supplier, which was directly used by the bacteria during the conversion of glycerol to dihydroxyacetone (Adlercreutz et al. 1982). Co-immobilization of microalga *S. obliquus* with *Bacillus subtilis* bacteria in carrageenan beads was studied inside air-lift reactors, for enhancing the production of alpha-amylase enzyme (Chevalier and de la Noüe 1988). Microalgal cells were again used as an in situ oxygen generator for the bacterial cells, which were mainly responsible for the synthesis of alpha-amylase enzyme. Co-immobilization overcame the existing oxygen diffusion problems and yielded higher alpha-amylase activity by a factor of around 20 %. They also observed higher growth rates for the algal cells when co-immobilized with bacteria, compared to the immobilization with algal cells alone (Chevalier and de la Noüe 1988).

Immobilization of *Dunaliella tertiolecta* in alginate (Grizeau and Navarro 1986) and *Dunaliella salina* in agar-agar (Thakur and Kumar 1999) increased the amount of glycerol production. Immobilized algae were also used for the generation of keto acids from amino acids (Wikström et al. 1982).

Luan et al. (2006) achieved successful removal (90 %) of a highly toxic tributyltin using alginate-immobilized *C. vulgaris* cells. They observed that less than 10 % of the tributyltin was accumulated inside the cells, while the remainder was adsorbed by both the immobilization matrix and the cell walls.

He et al. (2014) recently constructed an algal fuel cell with immobilized *C. vulgaris* cells in sodium alginate placed inside a cathode chamber of the fuel cell. The aim was to achieve a complete process that combines biomass production, electricity generation, and wastewater treatment all at the same time. They observed a significant chemical oxygen demand (COD) removal efficiency of 92.1 %.

2.8 Conclusions and Future Directions

immobilization of cells brings several advantages over current suspension bioprocessing, such as (1) providing flexibility to the photobioreactor designs; (2) increasing reaction rates arising from higher cell density; (3) enhancing operational stability; (4) avoiding cell washouts; (5) facilitating cultivation and easy harvesting of microorganisms; (6) minimizing the volume of growth medium as the immobilized cellular matter occupies less space; (7) easier handling of the products; (8) permitting the easy replacement of the algae at any stage of the experiment; (9) protecting the cell cultures from the harsh environmental conditions such as salinity, metal toxicity, variations in pH, and any product inhibition; and (10) allowing continuous utilization of algae in a non-destructive way. Enhanced survival rates of immobilized cells in toxic environments provide a significant alternative to achieve sufficient bioremediation of chemically contaminated environments. It is also important to stress that continuous biomass production, opportunity for product recycling, and nearly spontaneous biomass harvesting will have the potential to outweigh the difficulties and added costs associated with applying the technology on a larger scale.

Conventional wastewater treatment methods are mostly focused on the separation of pollutants from the liquid effluents with a requirement for a further stage to eliminate them. Developing integrated wastewater treatment processes that eliminate the undesired portion of the wastewater while converting it into valuable products is important in developing sustainable processes for the future. immobilization of algal cells is important in the development of an integrated process while simplifying the harvesting of biomass and providing the retention of the high-value algal biomass for further processing.

There are, however, technical issues to address, such as the hybridization of different polymers for creating more efficient and stronger immobilization matrix for algal cells. Immobilization of viable algae inside three-dimensional gel lattices also faces several limitations given that the encapsulating materials can have high volume-to-surface ratios. As a consequence, algal viability decreases since the light, nutrients, or reactants have to diffuse far into these materials to reach the algal cells. One of the other restrictions for the gel-entrapped cultures is their lower growth rates compared to their free-living counterparts. Such drawbacks can be addressed by optimizing the immobilization processes, that is, by choosing different encapsulating materials with lower volume-to-surface ratios such as thin films. Overcoming the difficulties of the current technology will increase the applicability of immobilized algae systems for various industrial applications.

Current immobilization projects have been often confined to the laboratory in providing an effective proof-of-concept rather than quick-install industrial prototypes. For larger scale wastewater treatment and biofuel production bioprocesses, the cost of immobilization matrix becomes a significant parameter that needs to be improved by further innovative designs and additional profits through generating valuable by-products.

Discovering the optimal microalgae–bacteria combinations for co-immobilization processes can also be a good alternative for large-scale wastewater treatment practices, since algal cultures in nature are usually associated with bacteria.

Application of innovative composite materials for use as the algal immobilization matrices can have a significant contribution to the economic and environmental development by sustainable utilization and recovery of the local resources, while bringing valuable strategies for solving important environmental issues.

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Chapter 3
Potentials of Exploiting Heterotrophic Metabolism for Biodiesel Oil Production by Microalgae

James Chukwuma Ogbonna and Navid R. Moheimani

Abstract The current prices of microalgae oils are much higher than oils from higher plants (vegetable oils) mainly due to the high cost of photoautotrophic cultivation of microalgae. However, many strains of microalgae can also grow and produce oil using organic carbons, as the carbon source under dark (heterotrophy) or light conditions (mixotrophy). Lipid productivities of most strains of microalgae are higher in culture systems that incorporate heterotrophic metabolisms (presence of organic carbon source) than under photoautotrophic conditions. This is because for many strains, cell growth rates and final cell concentrations are higher in heterotrophic cultures than in photoautotrophic cultures. Furthermore, in some cases, the oil contents of the cells are also higher in cultures incorporating heterotrophic metabolisms. It has also been reported for some strains that the quality of oil produced in the presence of organic carbon sources are more suitable for biodiesel oil production than those produced under photoautotrophic conditions. Thus, heterotrophy can be used to reduce the cost of biodiesel oil production, but the effectiveness of the various organic carbons in supporting cell growth and oil accumulation depends on the strain and other culture conditions. Use of wastewaters for cultivation of microalgae can further substantially reduce the cost of production (since they contain carbon, nitrogen, and other nutrients) and also reduce the requirement for freshwater. Generally, many factors such as nitrogen limitation, phosphate limitation, silicon limitation, control of pH, and low temperature can be used to increase oil accumulation, although their effectiveness depends on the strain and other culture conditions.

J.C. Ogbonna (✉)
Department of Microbiology, University of Nigeria, Nsukka, Nigeria
e-mail: james.ogbonna@unn.edu.ng

N.R. Moheimani
Algae R&D Center, School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150 Australia

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3.1 Introduction

Interest in production of biodiesel continues to be sustained because, unlike fossil diesel which is non-renewable and associated with various environmental problems, biodiesel is biodegradable, renewable, non-toxic, and emits less gaseous pollutants. The cost of biodiesel will determine to what extent it will be able to replace or complement fossil diesel production. Vegetable oil remains a major source of oil for large-scale industrial biodiesel production. However, the cost of vegetable oil is high, and waste oils often contain large amounts of free fatty acids which are difficult to convert to biodiesel through transesterification. Microalgae oil has a high potential for biodiesel production as it contains large proportions of fatty acid triglycerides, and the composition of the oil can be controlled by varying the culture conditions (Jiang and Chen 2000; Widjaja et al. 2009; Wen and Chen 2001a, b; Zhila et al. 2005). Microalgae oil is characterized by lower oxygen content, higher calorific value, and higher H/C ratio which make it more suitable for biodiesel, as compared to terrestrial plant oils (Miao and Wu 2004, 2006).

However, the cost of microalgae biodiesel is still too high to compete with the fossil diesel. The cost of microalgae cultivation accounts for 60–75 % of the total cost of the microalgae biodiesel fuel (Krawczyk 1996). It has been estimated that the cost of production of a liter of oil ranges from $1.40 to $1.81, depending on the type of photobioreactor used, and assuming that the biomass contains 30 % oil by weight (Azimatun-Nur and Hadiyanto 2013). Reduction in the cost of microalgae oil requires improvement in growth rate, oil content of the cells, and reduced cost of construction and operation of bioreactors. Reports from various studies have shown that it is already very difficult to increase cell growth rates and productivities in photoautotrophic cultures. However, many strains of microalgae can grow heterotrophically, using various organic carbons in dark. Heterotrophic cultures can be used to overcome most of the problems associated with photoautotrophic cultures. Generally, in comparison with photoautotrophic cultures, higher cell densities are achieved in heterotrophic cultures, with consequent reduction in the cost of downstream processing. Thus, heterotrophic cultures can be used to significantly reduce the cost of microalgae biodiesel production. The feasibility of exploiting heterotrophy for efficient biodiesel oil production is discussed in this chapter.

3.2 Microalgae with Heterotrophic Metabolism

Many strains of microalgae are known to grow either heterotrophically or mixotrophically. They include *Haematococcus pluvialis*, *Chlamydomonas reinhardtii*, *Chlamydomonas globosa*, *Scenedesmus acutus*, *Selenastrum capricornutum*, *Scenedesmus bijuja*, *Ankistrodesmus* sp., and many strains of *Chlorella* (Salim 2013; Ogbonna et al. 1998; Chojnacka and Marquez-Rocha 2004; Chojinacka and Noworyta 2004; Chen 1996). Growth of cyanobacteria can also be enhanced by mixotrophic culture, depending on the carbon source used (Lodi et al. 2005).

However, only a few of these strains that can utilize organic carbon sources accumulate more than 20 % oil under normal growth conditions. The oleaginous strains (those that accumulate more than 20 % oil) include *Chlamydomonas*, *Dunaliella*, *Botryococcus*, *Chlorella*, *Phaeodactylum*, *Thalassiosira*, *Nannochloropsis*, and *Isochrysis*. Out of these, many are known to be capable of growing heterotrophically. The choice of the strain to be used for heterotrophic or mixotrophic oil production depends on the oil productivity (a product of the cell growth rate and oil contents of the cells), and the quality of the oils.

Many reports have shown that photoautotrophic and heterotrophic culture conditions result in different biomass and lipid yields for the same microalga strain (Xu et al. 2006; Cheng et al. 2009). *Chlorella emersonii* and *C. protothecoides* gave the highest average lipid and biomass yield among many strains of microalgae tested (Suali and Sarbatly 2012). Liang et al. (2009) also reported very high oil productivities with *Chlorella vulgaris*, while Mandal and Mallick (2009) reported that *Scenedesmus obliquus* has very high potential for oil production. Heterotrophically cultivated *C. protothecoides* was reported to be composed of 40–60 % lipid, 10–28 % protein, 11–15 % carbohydrate, and 6 % ash (Xu et al. 2006; Miao and Wu 2004; Zhang et al. 2008). In view of their lipid content, *C. vulgaris*, *C. protothecoides*, and *C. zofingiensis* were reported as candidates for biodiesel production under photoautotrophic or heterotrophic culture conditions (Liu et al. 2008; Miao and Wu 2006; Hsieh and Wu 2009). Park et al. (2012) found that under mixotrophic conditions, oleic acid is comprised of 41–62 % fatty acid in many strains of microalgae, but in some *Chlamydomonas* isolates, oleic acid comprised only 9–16 % fatty acid, while palmitic and linoleic acid constituted 47–49 % of the total fatty acid content. Although *C. vulgaris* and *C. minutissima* are capable of producing high lipid contents, the triglyceride content is low, making them unsuitable for biodiesel production (Stephenson et al. 2010). The biodiesels produced from some *Chlorella* species were acid methyl esters, linoleic acid methyl esters, and oleic acid methyl esters (Gao et al. 2009). Unsaturated fatty acid methyl esters comprised over 82 % of the total biodiesel content of *Chlorella* species (Xu et al. 2006; Cheng et al. 2009). Therefore, the properties of the biodiesel produced from *Chlorella* comply with ASTM 6751, the US Standard for biodiesel (Li et al. 2007). From various reports on the potentials of several strains of microalgae for oil production, no strain can be selected as the best for biodiesel oil production, since both their oil contents and the composition of the oils vary with culture conditions. The choice, therefore, depends on the culture condition and the composition of the culture medium.

3.3 Use of Mixed Cultures

Depending on the type of reactor and the substrate used, it can be difficult to maintain monocultures of high-lipid-producing strains. For example, mixotrophic cultivation in open ponds, using wastewater as the nutrient source, will result in a

significant reduction in the cost of production. However, under such conditions, monocultures of oleaginous strains are likely to be outcompeted by faster growing species of microalgae or cyanobacteria. Therefore, it is important to explore the use of naturally occurring mixed cultures in wastewaters. Lipid content of mixed cultures in municipal wastewater was reported to be 11.3 %, and as high as 29 % when grown with anaerobic digester effluent (Woertz et al. 2009). Griffiths (2009) reported a fatty acid methyl ester content of as high as 23.4 % after in situ transesterification of a mixed culture grown in municipal wastewater.

Mixed cultures of selected strains of microalgae, as well as mixed cultures of microalgae and yeasts have also been investigated. Mixed cultures can be designed for efficient absorption of light (combining strains with different light absorption spectrum) or better nutrient utilization (combining strains with different nutrient preference). Competition for nutrients can lead to a limitation of nutrients, such as P and N, which can induce oil accumulation. Mixtures of *Chlamydomonas and Chlorella*, *Scenedesmus and Chlorella,* or a combination of three strains *Chlamydomonas, Chlorella*, and *Scenedesmus* were investigated for efficient oil production (Bhatnagar et al. 2011). In a mixed culture of *Chlorella* and yeast under mixotrophic cultivation with molasses as the carbon source, the biomass, lipid content per cell, and lipid productivities were higher than the values obtained in a monoculture of either of the strains (Leesing et al. 2012).

3.4 Organic Carbon Sources for Heterotrophic and Mixotrophic Cultures

Many organic carbon sources have been investigated for biodiesel production. These include various sugars such as glucose, galactose, fructose, and even some disaccharides. Polyhydric alcohols, such as glycerol, and some organic acids, such as acetate and propionate, have also been investigated. For example, *Chlorella protothecoides* can grow on a variety of carbon sources such as glucose (Shen et al. 2010; Xiong et al. 2008; Xu et al. 2006), fructose (Gao et al. 2009), sucrose (Gao et al. 2009), glycerol (Heredia-Arroyo et al. 2010), acetate (Heredia-Arroyo et al. 2010), and reducing sugars from Jerusalem artichoke and sugarcane (Cheng et al. 2009). Many species have also been reported to grow heterotrophically on ethanol (Ogbonna et al. 1998; Yokochi et al. 1998), lactose, galactose and mannose (Liang et al. 2009), and molasses (Andrade and Costa 2007). *Schizochytrium limacinum* produced palmitic acid (16:0) as 45–60 % of their dry weight when supplied with glucose, fructose, or glycerol (Yokochi et al. 1998; Chi et al. 2009). The effectiveness of these carbon sources varies with the species as well as on the culture conditions such as the light intensity, the pH, dissolved oxygen concentration, and on the presence of other carbon sources. Some carbon sources are good for mixotrophic culture, but not in heterotrophic cultures. For example, according to

Ceron Garcia et al. (2006), *Phaeodactylum tricornutum* UTEX-640 did not grow heterotrophically in media containing 0.005–0.2 M of fructose, glucose, mannose, lactose, or glycerol. However, addition of any of these organic carbons in mixotrophic culture increased the biomass concentration and productivity relative to photoautotrophic controls. The biomass, lipids, eicosapentanoic acid (EPA), and pigment contents were considerably enhanced with glycerol and fructose in relation to photoautotrophic controls. The EPA content was barely affected by the sugars, but was more than twofold higher in glycerol-fed cultures than in photoautotrophic controls (Ceron Garcia et al. 2006).

Liu et al. (1999) compared several carbon sources and concluded that glucose was the best in terms of cell growth rate. This contradicts the work of Chen and Walker (2011) who reported that crude glycerol gave the highest growth of *Chlorella protothecoides*, followed by pure glycerol, while the least biomass concentration was obtained with glucose. In the case of *Chlorella vulgaris*, Kong et al. (2011) reported that glucose was the best carbon source for mixotrophic cultivation, followed by sucrose and then glycerin, while sodium acetate did not support good growth. Effectiveness of carbon source in supporting cell growth may depend on their energy content (Chojnacka and Marquez-Rocha 2004; Wang et al. 2012). For instance, glucose produces about 2.8 kJ/mol of energy compared to 0.8 kJ/mol for acetate (Boyle and Morgan 2009), and glucose was more effective as a substrate for mixotrophic cultivation of *Phaeodactylum tricornutum* than acetate (Wang et al. 2012). The carbon source that gives high biomass productivity may not be the one that gives high oil production. Although for many strains, glucose has been reported to be the best in terms of cell growth, Das et al. (2011) ranked the effectiveness of different organic substrates in terms of intracellular lipid contents in mixotrophic culture in the following order: glycerol > sucrose > glucose.

The cost is another major factor determining the choice of carbon source for mixotrophic/heterotrophic cultures. The present cost of microalgae oil at US$2.4/L (Li et al. 2007; Xu et al. 2006) is 3–4 times higher than that of plant oils. However, Liu et al. (2010) estimated oil production cost of US$0.9/L for heterotrophic cultures of *Chlorella zofingiensis* using sugar as substrate. In heterotrophic/mixotrophic cultures, the cost of organic carbon represents a very high percentage of the total production cost. Economic analysis shows that organic carbon source contributes 45.4 %; inorganic chemicals, 3.2 %; electricity, 30.6 %; steam, 14.2 %; and aseptic air, 6.6 % of the total production cost (Li et al. 2007; Xu et al. 2006). The cost of glucose has also been estimated to be about 80 % of the total medium cost (Li et al. 2007). Thus, there is a need to drastically reduce the cost of the organic carbon source. Many cheap carbon sources such as non-sugar carbon sources (Heredia-Arroyo et al. 2011), corn powder hydrolysate, impure glycerol, and molasses have been investigated.

Currently glycerol is an inexpensive and abundant carbon source generated as a by-product of biodiesel fuel production. About 0.45 kg of glycerol is produced per 4.5 kg of biodiesel, and the price of crude glycerol is now about 0.025USD/0.45 kg

(Chen and Walker 2011). It has been reported that crude glycerol is better than pure glycerol and glucose (Chen and Walker 2011; Liang et al. 2010) because of the residual nitrogen in crude glycerol. The use of corn powder hydrolysate has also been widely investigated, and it has been reported that it is superior to glucose solution since it contains some other components that are beneficial for cell growth. For example, *C. protothecoides* produced 55.2 % crude lipids in a medium containing corn hydrolysate, with a cell dry weight concentration of 15.5 g/L (Xu et al. 2006), which is higher than the values reported for glucose. Li et al. (2007) noted that if hydrolyzed starch is used as a carbon source for *Chlorella*, the cost of medium can be reduced to about 60–70 %. Cheng et al. (2009) used hydrolysate of Jerusalem artichoke tuber as a carbon source for heterotrophic cultivation of *C. protothecoides,* and the resulting biomass contained 44 % lipid. The lipid content of microalgae cultivated in the presence of the enzymatic hydrolyzates of sweet sorghum (which contains 10 g/L of reducing sugars) was 52.5 %. This is 35.7 % higher than the value obtained by cultivation using glucose (Gao et al. 2009). Anaerobically digested dairy manure (Wang et al. 2010) and wastewater containing 85–90 % carpet mill effluents (Chinnasamy et al. 2010) were used as carbon sources for production of lipids for biofuel. Many agro-industrial wastes such as dry-grind ethanol thin stillage (TS) and soy whey (SW) have been used as nutrient feedstock for mixotrophic/heterotrophic cultivation of *Chlorella vulgaris* (Mitra et al. 2012). Both the cell concentration (9.8 g/L) and oil content (43 %) obtained with TS were higher than those obtained with modified basal medium containing glucose as the carbon source (8 g/L and 27 %, respectively) under mixotrophic conditions.

The optimal concentrations of these carbon sources vary with both strain and other culture conditions. Concentrations of glycerol used ranged from 3 to 12 % (Yokochi et al. 1998), while Liang et al. (2009) reported that 1 or 2 % glycerol resulted in a higher lipid content of microalgae compared to the value obtained with 5 % glycerol. The tolerable concentration of glycerol is within the range of 0.7–10 % (Chi et al. 2009). The optimum cassava starch hydrolysate concentration for cell growth and lipid accumulation was 5 g/L, but the values were not significantly different from those obtained with 10 g/L. A higher concentration of 15 g/L of hydrolysate resulted in lower biomass and lipid contents (Salim 2013). The optimum concentration of glucose for growth was 1 g/L, but there were no significant effects of varying acetate or starch concentrations between 0.5 and 5 g/L on cell growth. The highest values of the lipid content and lipid productivity with glucose in media were approximately 2.8 times (at 2.0 g/L glucose) and 4.6 times (at 1.0 g/L glucose) compared to control (photoautotrophic culture). As the content of glucose increased to 5.0 g/L, the total lipid content and lipid productivity decreased, but were still higher than the values obtained in photoautotrophic culture (Wang et al. 2012). On the other hand, starch in the medium did not influence the specific growth rate with concentrations below 1.0 g/L, but was inhibited significantly above 2.0 g/L ($p < 0.05$) (Wang et al. 2012).

3.5 Nitrogen Sources for Heterotrophic and Mixotrophic Cultivation of Microalgae

Nitrogen source is very important in mixotrophic and heterotrophic cultures of microalgae. Adequate concentration of nitrogen is required for cell growth, while nitrogen limitation is often used to enhance lipid accumulation. Inorganic nitrogen, organic nitrogen, and various waste products have been investigated for biodiesel oil production (Becker 1994). The use of ammonium as a nitrogen source for *Ellipsoidion* sp. resulted in higher growth rate and lipid content than when urea and nitrate were used (Xu et al. 2001). On the other hand, *Neochloris oleoabundans* grew faster and accumulated higher lipid with nitrate than with urea (Li et al. 2008a), but the cell grew poorly in medium with ammonium as the nitrogen source. Complex nitrogen sources are expected to be more effective than simple nitrogen sources in the heterotrophic culture of microalgae, since most of them contain amino acids, vitamins, and growth factors. However, the effectiveness of the nitrogen source depends on the species. For example, nitrate was the best, followed by urea, for the growth of *Chlorella vulgaris*, while peptone and beef extract did not improve cell growth. Furthermore, ammonium sulfate and ammonium nitrate were less effective than nitrate and urea (Kong et al. 2011). The type of nitrogen source affects not only the cell growth, but also lipid accumulation. The lipid content of *Chlorella vulgaris* in mixotrophic culture was highest for peptone, followed by beef extract, but the lipid productivities were low because of low biomass concentration. Ammonium sulfate and ammonium nitrate gave the least lipid content. Potassium nitrate and urea gave intermediate lipid content, yet had the highest productivity as a result of the high biomass content (Kong et al. 2011).

Among the organic nitrogen sources, urea is a promising nitrogen source for large-scale production because it is relatively cheap (Becker 1994; Danesi et al. 2002; Matsudo et al. 2009). With urea as the nitrogen source, the lipid contents of *Chlorella* sp. decreased with the increase in urea concentration (Hsieh and Wu 2009). The optimal concentrations differ with the nitrogen source. The optimal sodium nitrate and yeast extract concentrations for growth and lipid production by *Tetraselmis* sp. in mixotrophic culture were 4.70 and 0.93 g/L, respectively (Iyovo et al. 2010). Industrial wastewater rich in nitrogen, such as monosodium glutamate waste, has been reported to be a good and cheap source of nitrogen for cultivation of *Rhodotorula glutinis* for the production of biodiesel (Xue et al. 2006; Becker 1994). The type of nitrogen source affects the pH of the culture broth. The pH was stable when urea or potassium nitrate were used, but dropped sharply when other nitrogen sources were used.

3.6 Wastewater as a Substrate for Lipid Production by Microalgae

Aside from mineral elements and nitrogen sources, some wastewaters contain high concentrations of organic carbons for mixotrophic/heterotrophic cultivation of

microalgae. Thus, some wastewaters can be used as both carbon and nitrogen sources for cultivation of microalgae. Cultivation of microalgae in wastewater for biodiesel production is highly desirable since it leads to a significant reduction in the production costs and reduction in the demand for freshwater with the concomitant removal of various contaminants, such as phosphorus, nitrogen, heavy metals, and pathogens from the wastewater.

In addition to carbon, nitrogen, and phosphorus, microalgae also require micronutrients for growth and oil production. Micronutrients required in trace amounts include silica, calcium, magnesium, potassium, iron, manganese, sulfur, zinc, copper, zinc, nickel, lead, chromium, and cobalt (Bao et al. 2008; Ortiz Escobar and Hue 2008; Faridullah et al. 2009; Vu et al. 2009). These nutrients are usually added through the addition of commercial fertilizers, which substantially increase production costs. The concentrations of these essential micronutrients rarely limit algal growth when wastewater is used (Knud-Hansen et al. 1998). Furthermore, many wastewaters such as poultry litter, slaughter house wastes, dairy effluents, swine wastes, municipal wastewater, and effluents from anaerobic digesters are rich in organic nutrients. In addition to supplying these nutrients, the cultivation of microalgae in wastewaters is an efficient method of wastewater treatment (Ogbonna et al. 2000). Hodaifa et al. (2008) recorded 67.4 % reduction in BOD with *S. obliquus* cultured in diluted (25 %) industrial wastewater from olive oil extraction. Wang et al. (2009) also reported that wastewaters from different stages of treatment are good for cultivation of *Chlorella* sp. with efficient removal of N, P, and COD. A consortium of 15 native algal isolates removed more than 96 % nutrients from wastewater (Chinnasamy et al. 2010). However, there are variations in the composition of wastewaters and each may be suitable for cultivation of only a few strains of microalgae for some specific purposes. Furthermore, most wastewaters are opaque, limiting light penetration, and thus are not suitable for photoautotrophic culture.

Types of wastewaters investigated for microalgae cultures include municipal, industrial, and agricultural wastewaters (Jiang et al. 2011). For example, poultry litter contains approximately 3.3 % nitrogen and 2.6 % phosphorus and cell growth promoters, such as glycine, are released from poultry manure on decomposition (Schefferle 1965). The composition of poultry manure depends on the type of feed used. For example, according to Magid et al. (1995), some common nutrients in poultry manure include (g/Kg) potassium 37.5, phosphate 25.5, and nitrogen 55.7. Nitrogen is normally in the form of uric acid, and about 66 % can be available on decomposition (Ruiz et al. 2009).

It has further been reported that various species of microalgae were cultivated in POME and biomass productivity varied from 2.9 to 8.0 mg/L/day and the oil content ranged from 21.34 to 30.83 % (Putri et al. 2011; Nwuche et al. 2014). One problem of POME as a medium for microalgae is the high COD content, dark color of tannic acid, and high impurity. This could be solved by anaerobic digestion to significantly reduce COD, TS, TSS, T-nitrogen, and orthophosphate (Habib et al. 2003). Rubber Mill Effluent consists of latex washings and a solution containing proteins, sugars, lipids, and inorganic and organic salts. The high level of

ammonium and other plant nutrients make it a good medium for algal growth (Azimatun-Nur and Hadiyanto 2013). Fermented cocoa bean mill effluent, also called cocoa-sweating effluent, contains several sugar residues and micronutrients (Syafila et al. 2010). The final lipid content of the culture with feeding of effluent from stably operated anaerobic continuous-flow stirred-tank reactor was 27 ± 1.11 % after 168-h cultivation in flasks, which was higher than the value obtained with glucose of the same COD concentration (Wen et al. 2013).

3.7 Improving the Productivities in Heterotrophic and Mixotrophic Culture Systems

Various species of microalgae can be used for biodiesel oil production using either photoautotrophic, heterotrophic, or mixotrophic culture system. However, the suitability of each depends on the strain, availability of facilities, nature of organic carbon source, and other culture conditions. In a comparison of the lipid productivity in 20 species of photosynthetic microorganisms, three strains produced more lipid in heterotrophic cultures when compared to photoautotrophic cultures and 11 strains produced more in mixotrophic cultures than in photoautotrophic cultures (Ratha et al. 2013).

Lipid productivity is a product of lipid content and biomass concentration and gives the total amount of lipid formed per unit culture volume and time (g-lipid/L.d). Thus, strategies for improving productivity aims to increase lipid content without significant reduction in cell growth, increase cell growth without significant reduction in oil content, or increase both cell growth and lipid accumulation per cell. Several species of microalgae can be induced to overproduce lipids by the choice of culture system as well as by manipulations of the culture medium such as the source and concentrations of carbon, nitrogen, phosphorous, and silicate as well as by manipulation of culture conditions such as temperature, pH, and oxygen tension.

Nitrogen starvation is one of the most studied methods of inducing oil accumulation in microalgae. As a result of nitrogen starvation, the lipid content as high as 70–85 % of dry weight has been reported (Becker 1994). The effectiveness of nitrogen limitation in increasing lipid contents of microalgae has been demonstrated with many strains such as *Prophyridium cruentum* (Becker 1994), *Chlorella vulgaris* (Widjaja et al. 2009; Converti et al. 2009), *Chlorella protothecoides* (Miao and Wu 2004), as well as many strains of cyanobacteria and other green algae (Shifrin and Chisholm 1981; Illman et al. 2000; Takagi et al. 2000; Li et al. 2008a, b). However, using nitrogen limitation to increase intracellular lipid content may have negative effects on cell growth and thus lipid productivity, which has been reported for several strains (Hsieh and Wu 2009; Xiong et al. 2008). On the whole, the effectiveness of nitrogen limitation in increasing lipid productivity depends on the strain. In the case of diatoms, such as *Achnanthes brevipes* and *Tetraselmis* sp. for example, nitrogen limitation leads to accumulation of carbohydrates, rather than lipids

(Guerrini et al. 2000; Gladue and Maxey 1994). Under nitrogen starvation, accumulation of lipids has been attributed to mobilization of lipids from chloroplast membranes as chloroplastic nitrogen is relocated by 1.5-biphosphate carboxylase/oxygenase (E.C. 4.1.1.39, Rubisco) (Garcia-Ferris et al. 1996). Some other reports have shown that increased lipid content of cells at low nitrogen concentration may be due to the high C/N ratio, rather than the absolute nitrogen concentration. For example, in culture of *Chlorella sorokiniana*, a C/N ratio of 20 gave the lowest cell lipid content, but increased at both higher and lower C/N values (Chen and Johns 1991). Furthermore, in marine (*Cryptheconidium conhii*) and freshwater (*Chlorella sorokiniana*) algae, accumulation of lipids may not be dependent on nitrogen exhaustion but on an excess of carbon in the culture media. Hence, in heterotrophic cultures, lipid accumulation was attributed to consumption of sugars at a rate higher than the rate of cell generation, leading to conversion of excess sugar into lipids (Chen and Johns 1991; Ratledge and Wynn 2002; de Swaaf et al. 2003).

In the case of diatoms, lipid accumulation is related to depletion of silicates because of their dependence on silica for growth (Roessler 1988; Wen and Chen 2000a, b, 2003; Wilhelm et al. 2006). Roessler (1988) reported that silicon deficiency could induce lipid accumulation in *Cyclotella cryptica* by two distinct processes: (1) an increase in the proportion of newly assimilated carbons which are converted to lipids and (2) a slow conversion of previously assimilated carbon from non-lipid compounds to lipids.

Phosphorus starvation has also been used to induce lipid synthesis (Zhila et al. 2005; Weldy and Huesemann 2007). Khozin-Goldberg and Cohen (2006) found that phosphate limitation could cause significant changes in the fatty acid and lipid composition of *Monodus subterraneus*. However, in other species such as *Nannochloris atomus* and *Tetraselmis* sp., phosphorus deficiency led to reduced lipid content of the cells (Reitan et al. 1994).

Lipid synthesis may also be induced under other stress culture conditions such as high light intensity (Guedes et al. 2010; Khotimchenko and Yakovleva 2005; Qin 2005; Weldy and Huesemann 2007), low temperature (Renaud et al. 2002; Qin 2005), high salinity (Kotlova and Shadrin 2003; Takagi et al. 2006; Qin 2005; Wu and Hsieh 2008), pH control (Guckert and Cooksey 1990), CO_2 concentration (Chiu et al. 2009; de Morais and Costa 2007), and high iron concentration (Liu et al. 2008). The polyunsaturated fatty acid contents of microalgae tend to increase at low temperatures. The high PUFA content at low temperature might be explained by the need for the algae to produce more PUFAs to maintain cell membrane fluidity. Another reason might be that low temperature could lead to a high level of intracellular molecular oxygen and hence improve the activity of the desaturase and elongase involved in the biosynthesis of PUFAs (Jiang and Chen 2000).

Aside from lipid productivity, it is important to consider the quality of lipids produced by microalgae, since the quality of the oil influences the quality of the biodiesel. European Biodiesel standards (EN 14214 and 14213) limit the contents of fatty acid methyl esters with four and more double bonds to a maximum of 1 % (mol/mol). According to the EN 14214, for example, the linolenic acid (C18:3) should not exceed 12 % (mol/mol) (Knothe 2006). These oils require additional

treatment, such as partial catalytic hydrogenation (Dijkstra 2006). An advantage of microalgae oil is that the composition of the oils can be controlled by controlling the culture condition, as previously discussed. In some strains, it has been reported that nutrient limitation results in a change in lipid composition from free fatty acids to TAGs which are more suitable for biodiesel production (Widjaja et al. 2009). In some strains, nitrogen starvation might not result in an increase in total lipid content, but a change in lipid composition. Zhila et al. (2005) reported that nitrogen limitation increased oleic acid contents of *Botryococcus braunii*, yet the content of total lipids and triacylglycerols did not change. The saturation of fatty acids is directly dependent on the amount of excess sugar and the culture conditions (Tan and Johns 1991; Wood et al. 1999; Wen and Chen 2000a, b). Wood et al. (1999) noted that as the concentration of sugar increased, the fatty acid became more saturated. The type of nitrogen source also affects the quality of oil. In cultures of *N. laevis*, ammonia favored saturated and monounsaturated fatty acids (C14:0, C16:0, C16:1), and nitrate and urea promoted polyunsaturated fatty acids (C20:4 and C20:5) (Wen and Chen 2001a, b).

Genetic and metabolic engineering has high potentials for improving both the lipid quality and lipid productivity and therefore the economy of microalgae biodiesel. Much work has been done on genetic engineering of cyanobacteria, and many functional genes have been successfully cloned in cyanobacteria (Qin et al. 1999). For example, Acc1 (a kind of restriction enzyme) has been cloned from *Cylclotella cryptica* (an oceanic diatom) for the production of biofuel (Roessler 1988). Genetic engineering of diatoms has increased the lipid contents from 5–20 % to over 60 % under laboratory conditions. The improvement of lipid content in genetically engineered microalgae is mainly due to the high expression of acetyl-coA carboxylase gene, which plays an important role in the control of the level of lipid accumulation (Huang et al. 2010). Due to the various advantages of heterotrophic cultures, attempts have also been made to genetically modify obligate photoautotrophs for heterotrophic growth. For example, Glut 1 gene that encodes the glucose transporter protein was introduced into *Phaeodactylum tricornutum* (which is an obligate phototroph), thereby making it possible to grow it heterotrophically (Zaslavskaia et al. 2001).

3.8 Co-products

Many strains of microalgae are able to simultaneously produce oil and many other useful and expensive metabolites, such as antioxidants (Fujita et al. 2008; Ogbonna 2009) and bioactive compounds. Such metabolites can be separated and purified, thereby making the process profitable. After oil extraction, residual biomass can be converted to biofuels, such as bioethanol, biohydrogen, and biomethane via thermochemical and biochemical methods (Golueke et al. 1957; Gunaseelan 1997). As an example, the anaerobic digestion of microalgae produces methane, which can then be used to generate electricity. The generated electricity can be used for

microalgae cultivation, dewatering, extraction, and transesterification process. Harun et al. (2011) estimated that the electricity generated from methane reduces the cost of biodiesel production by as much as 33 %. It can also be used to produce bioplastic materials (Chiellini et al. 2008), or used as a fertilizer, soil amendment, or feed for fish or livestock (Mulbry and Wilkie 2001; Mulbry et al. 2005; Roeselers et al. 2008).

3.9 Conclusions

Although microalgae biodiesel has many advantages over plant biodiesel in terms of effects on food security and sustainability, the present cost of microalgae oil is still much higher than that of vegetable oils. This is mainly due to low cell growth rate and low final cell concentrations in photoautotrophic cultures. Cell growth rates, final cell concentrations, and in some cases intracellular oil contents are higher in heterotrophic and mixotrophic cultures than photoautotrophic cultures. These lead to increase in oil productivity and reduction in the cost of production. The qualities of oil produced under these culture conditions have also been reported to be more suitable for biodiesel oil production than photoautotrophic microalgae oils. Heterotrophic and mixotrophic culture systems should therefore be exploited for large-scale biodiesel oil production.

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Chapter 4
Culture Systems Incorporating Heterotrophic Metabolism for Biodiesel Oil Production by Microalgae

James Chukwuma Ogbonna and Mark P. McHenry

Abstract The feasibility of using various culture systems incorporating heterotrophic metabolism for biodiesel oil production was compared. Heterotrophic culture can be used to achieve high cell concentration, and depending on the strain and organic carbon source employed, the introduction of light (mixotrophic culture) can enhance cell growth and oil accumulation. However, mixotrophic cultures also face the problem of light limitation, and depending on the relative concentrations of the organic carbon source and light intensity, the interaction between the heterotrophic and photoautotrophic metabolic activities can have negative effects on cell growth and oil accumulation. Systems that separate the two metabolic activities in time or space, such as cyclic photoautotrophic–heterotrophic cultures, sequential heterotrophic–photoautotrophic cultures, and sequential photoautotrophic–mixotrophic cultures, can all be used to improve oil productivity. However, the effectiveness of each system depends on the strain of microalgae and other culture conditions.

4.1 Introduction

Requirement for light, and the technical problems and costs of light supply and distribution inside photobioreactors are the major challenges facing large-scale microaglae production in photoautotrophic cultures (Ogbonna et al. 1995, 1996). Much work has been carried out to optimize photoautotrophic cultures in large-scale outdoor cultures, yet it remains difficult to increase cell growth and final biomass concentrations in open pond cultures (Borowitzka and Moheimani 2013). Although higher productivities can be achieved in closed photobioreactors, such

J.C. Ogbonna (✉)
Department of Microbiology, University of Nigeria, Nsukka, Nigeria
e-mail: james.ogbonna@unn.edu.ng

M.P. McHenry
School of Engineering and Information Technology, Murdoch University, Murdoch, WA 6150, Australia

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photobioreactors are highly complex, and technically difficult and very expensive to construct and operate (Ogbonna et al. 1996; Ogbonna 2003). Many strains of photosynthetic cells can metabolize various organic carbon sources in the presence of light (mixotrophy) or in its absence (heterotrophy). Various types of efficient large-scale heterotrophic bioreactors are available and are currently used for large-scale cultivation of fungi, bacteria, and yeasts for production of various metabolites. Such bioreactors are also used for large-scale heterotrophic cultivation of microalgae. Even in mixotrophic cultures, the limitation of light is not as critical since the algae can still grow efficiently by metabolizing organic carbon sources. However, for many strains of microalgae in mixotrophic cultures, there is interaction between the photoautotrophic and heterotrophic metabolic activities (Ogbonna et al. 2002a). This interaction can positively or negatively affect both the cell growth and metabolite accumulation. Regulation of these metabolic activities is therefore required for the efficient accumulation of the desired metabolites (Ogbonna et al. 2002b). These metabolic activities can be separated in space (by employing a different bioreactor for each activity), or in time (by switching from one metabolic activity to another in the same bioreactor). Thus, many culture technical designs have been investigated to exploit heterotrophic metabolic activities in microalgae cultures. Such culture systems include heterotrophic, mixotrophic, cyclic photoautotrophic–heterotrophic, sequential heterotrophic–photoautotrophic, and sequential photoautotrophic–mixotrophic cultures. Each of these culture systems can be used for efficient biodiesel oil production, but their effectiveness is dependent on the nature of the microalgae used, the media components, and other culture conditions. The potentials and limitations of these culture systems are reviewed in this chapter.

4.2 Heterotrophic Cultures

Heterotrophic cultures use organic carbons as both sources of energy and carbon. There are many advantages of heterotrophic cultures over photoautotrophic cultures. These include the following: (i) the use conventional heterotrophic bioreactors that are simpler and easier to scale up, since the elimination of light requirements means that smaller reactor surface-to-volume ratios can be used; (ii) greater control of the cultivation process, since the cultures can be done indoors; and (iii) higher cell densities, which reduces the cost of harvesting the cells. The basic components of media for heterotrophic cultures are similar to those of the photoautotrophic media, with the addition of organic carbon sources. In addition, the growth rate and oil accumulation of heterotrophic cultures are affected by the C:N ratio in the medium.

Generally, the biomass concentrations obtained in heterotrophic cultures are much higher than those in photoautotrophic cultures (Ogbonna et al. 1998). Although the biomass concentration in most photoautotrophic cultures is less than 5 g/L, much higher concentrations of 15.5 g/L for *Chlorella protothecoides* (Xu et al. 2006), 28.8 g/L for *Traselmis suecica* (Azma et al. 2011), and even 53 g/L for

Chlorella zofingiensis (Sun et al. 2008) have been reported. Fed-batch cultures can be used to obtain even higher biomass concentrations. Furthermore, heterotrophically grown microalgae usually accumulate more lipids than those cultivated photoautotrophically, as demonstrated for *Chlorella* species (Miao and Wu 2006; Xu et al. 2006; Agwa et al. 2013; Liu et al. 2008; Hsieh and Wu 2009). In the case of *Chlorella vulgaris*, for example, Wu et al. (2012) reported an increase from 15 % under photoautotrophic condition to more than 50 % under heterotrophic condition. Compared with photoautotrophic cultures, Jiménez et al. (2009) reported an 8 times increase in oil content of *C. protothecoides* under heterotrophic condition, and a 9 times increase in lipid yield was achieved in heterotrophic cultures fed with 30 g/L of glucose (Liu et al. 2011).

The high biomass concentration and high lipid contents obtained in heterotrophic cultures result in very high lipid productivities. A lipid productivity of 179 mg/L/d in photoautotrophic culture is regarded as high (Chiu et al. 2008), but much higher productivities of 528.5 mg/L/d (Morales-Sanchez et al. 2013), 932 mg/L/d (Xu et al. 2006), 1.38 g/L/d (Liu et al. 2011), 2.43 g/L/d (Chen and Walker 2011), 3.0 g/L/d (Chen and Walker 2011), and 3.7 g/L/d (Xiong et al. 2008) have been reported for heterotrophic cultures. It is usually technically difficult to construct large-scale photoautotrophic photobioreactors; however, for heterotrophic cultures, conventional bioreactors can be used for large-scale processes. For example, a heterotrophic culture was scaled up from 5 to 750 L, and then 11,000 L, and the oil contents remained fairly stable at 46.1, 48.7, and 44.3 % of cell dry weight, respectively (Li et al. 2007).

It has also been reported that the quality of oil produced under heterotrophic cultures is more suitable for biodiesel production than those produced under photoautotrophic cultures with the same strains of microorganisms. Liu et al. (2011) reported that heterotrophic cells accumulated predominantly neutral lipids that accounted for 79.5 % of the total lipids, with 88.7 % triacylglycerol, while oleic acid accounted for 35.2 % of the total fatty acid. In contrast, photoautotrophic cells contained mainly the membrane lipids, glycolipids, and phospholipids. Furthermore, *C. saccharophila*, *C. vulgaris*, *N. laevis*, *Cylindrotheca fusiformis*, *Navicula incerta*, and *Tetraselmis suecica* accumulate more lipids under heterotrophic than under photoautotrophic conditions, mainly in the form of triglycerides (Day et al. 1991; Tan and Johns 1991, 1996; Gladue and Maxey 1994). Conversely, photoautotrophic cultures form more highly unsaturated fatty acids (polar lipids) (Tan and Johns 1991, 1996; Wen and Chen 2000a, b). Miao and Wu (2004) further noted that the oil obtained from heterotrophically grown cells possesses properties similar to those of fossil diesel in terms of oxygen content, heating value, density, and viscosity.

However, heterotrophic cultures have some limitations. Only a limited number of microalgae are capable of growing under heterotrophic conditions, and addition of an organic carbon source can significantly increase the cost of production. Furthermore, the presence of an organic carbon source further increases the risk of contamination, and depending on the species, the cell growth rate and lipid productivity in heterotrophic culture may be lower than the values obtained in

mixotrophic culture. For example, Day and Tsavalos (1996) found that heterotrophic culture of *Tetraselmis* with glucose yielded only about one-sixth of cellular lipid compared with the value obtained in mixotrophic culture.

4.3 Mixotrophic Cultures

Mixotrophic cultures are culture systems where light and organic carbons are used as the energy source, while inorganic and organic carbons are used as the carbon source. Although it is sometimes used interchangeably with photoheterotrophic culture, in a strict sense, photoheterotrophic culture involves the use of light as the energy source, while organic carbon is used as the carbon source. In other words, light is required to metabolize the organic carbon source in photoheterotrophic culture. Photoheterotrophic cultivation requires both organic carbons and light at the same time, whereas in mixotrophic culture, both are present, but either can be used without the other. From practical point of view, both mixotrophic and photoheterotrophic cultures can be regarded as culture systems where light, organic carbon, and inorganic carbon are present at the same time.

As already discussed, heterotrophic culture has many advantages over photoautotrophic cultures. However, there are many metabolites whose syntheses are promoted by light, and thus are not efficiently produced in heterotrophic cultures (Chen and Zhang 1997; Lee and Zhang 1999; Cohen 1999; Sukenik et al. 1991). This disadvantage can be overcome by mixotrophic culture which involves simultaneous use of light and organic carbon sources. Mixotrophic cultures have many advantages over other culture systems. For example, inhibition of photosynthesis by high dissolved oxygen concentration is a major problem in photoautotrophic cultures, while oxygen limitation is a major problem in heterotrophic cultures. In mixotrophic culture, dissolved oxygen concentration does not increase to inhibitory levels since it is simultaneously used for heterotrophic metabolism of the organic carbon. On the other hand, organic carbon assimilation is hardly limited by dissolved oxygen concentration since oxygen is constantly produced by photosynthetic activities. Furthermore, heterotrophic growth generates carbon dioxide which is used for photoautotrophic growth (photosynthesis).

In mixotrophic cultures, the presence of an organic substrate means that cell growth is not strictly dependent on photosynthesis, and hence, light is not an indispensable growth factor. Read et al. (1989) and Fernandez Sevilla et al. (2004) have reported that mixotrophic growth requires relatively low light intensities and, consequently, can reduce energy costs. In some strains, it has been found that mixotrophic cultures reduced photoinhibition and that the growth rates are higher than those observed in both photoautotrophic and heterotrophic cultures. Furthermore, mixotrophic cultivation reduces biomass loss at night and decreases the amount of organic substances utilized during growth (Chojnacka and Noworyta 2004).

In mixotrophic cultures of many strains of microalgae, there are additive or synergistic effects of photoautotrophic and heterotrophic metabolic activities, leading to increases in productivity. Park et al. (2012) reported higher biomass and fatty acid productivities of 14 species of microalgae in mixotrophic culture over photoautotrophic culture. Bhatnagar et al. (2011) found that the mixotrophic growth of *Chlamydomonas globosa*, *Chlorella minutissima*, and *Scenedesmus bijuga* resulted in 3–10 times more biomass production compared to those obtained under photoautotrophic growth conditions. It has also been shown that the addition of glycerol as the carbon source resulted in increased biomass productivity of *Phaeodactylum tricornutum* (Cerón García et al. 2005, 2006; Moraisa et al. 2009). One of the possible reasons for better growth in mixotrophic cultures is the stability of pH, since carbon dioxide is simultaneously assimilated and released during photosynthesis and respiration. In photoautotrophic cultures, the pH increased to more than 10, but remained stable around 7 in mixotrophic culture (Kong et al. 2011). It is important to note, however, that biomass productivity in mixotrophic cultures depends on many factors such as the strain, type, and concentration of the carbon source, and other medium components, as well as the light intensity. In some strains, for example, the addition of some carbon sources to photoautotrophic cultures inhibits growth, while others stimulate growth (Heredia-Arroyo et al. 2011). This is because photosynthesis and oxidative phosphorylation of organic carbon substrates seem to function independently in some algae, and growth rates in mixotrophic cultures are the sum of those in photoautotrophic and heterotrophic cultures. This has been reported for *Chlorella* sp., *Spirulina* sp., and *Haematococcus* (Ogawa and Aiba 1981; Marquez et al. 1993; Martinez and Orus 1991; Hata et al. 2001). Under certain culture conditions, the presence of organic carbon in some microalgae depresses photosynthetic O_2 evolution and inhibits respiration and enzymes of Calvin cycle (Liu et al. 2009). In mixotrophic cultures, photosynthetic fixation of inorganic carbon is influenced by light intensity, while the heterotrophic assimilation of carbon is influenced by the availability of organic carbon. Thus, the ratio of photoautotrophic growth to heterotrophic growth depends on the light intensity, type, and concentration of organic carbon and carbon dioxide concentration (Ogbonna et al. 2002a, b). These factors must be controlled to ensure high rates of growth and lipid accumulation.

Aside from increased biomass concentration and productivities (Lodi et al. 2005), mixotrophic cultures can lead to increases in lipid accumulation over the values obtained in photoautotrophic cultures. This has been reported for several species of microalgae such as *Chlorella* sp. (glucose), *P. tricornutum* (glycerol) (Fernandez Sevilla et al. 2004), *Nannochloropsis* sp. (glycerol) (Wood et al. 1999; Liang et al. 2009), and *C. vulgaris* (Kong et al. 2011). However, the oil contents of the cells in mixotrophic cultures are dependent on the nature of the carbon source. In some cases, the lipid contents of the cells are even lower or the same as those in the photoautotrophic cultures (Park et al. 2012). Nevertheless, because of the higher growth rate, the lipid productivities are, generally, much higher than those in photoautotrophic cultures. Ratha et al. (2013) reported that lipid production by twenty different strains of cyanobacteria and green algae was highest under

mixotrophic condition, compared to heterotrophic and photoautotrophic cultures. With either glucose, starch, or acetate, the maximum lipid productivities of *Phaeodactylumtricornutum* in mixotrophic cultures were several times higher than those obtained in the corresponding photoautotrophic control cultures (Wang et al. 2012).

Lipid productivity in mixotrophic culture is also dependent on the strain used. For example, the lipid content and lipid productivity were higher under mixotrophic conditions as compared to both photoautotrophic and heterotrophic cultures in all the members of Chlorococcales tested. Yet, the filamentous alga *Ulothrix* and all the cyanobacterial strains had slightly higher lipid content and lipid productivity in photoautotrophic cultures (Ratha et al. 2013). The increases in fatty acid productivity under mixotrophic conditions can result from the combined increases in biomass productivity and fatty acid content, or from increased biomass productivity at relatively constant fatty acid content. In some strains and under certain culture conditions, there is no positive effect of mixotrophic culture on cell lipid content; thus, the increase in lipid productivity is mainly due to increases in biomass productivity, shown for *C. vulgaris* with various carbon sources (Kong et al. 2011). In contrast to photoautotrophic cultures, where conditions that favor lipid accumulation often suppress cell growth (Chisti 2007; Hu et al. 2008), in mixotrophic cultures, there can be a linear relationship between biomass and fatty acid productivities (Griffiths and Harrison 2009; Park et al. 2012).

Mixotrophic cultivation affects the fatty acid profile of microalgae. In 10 out of 14 isolates grown under mixotrophic condition with acetate as the organic carbon source, the percentage of oleic acid content increased significantly (Park et al. 2012). However, the fatty acid profile was not affected when glycerol was used (Fernandez Sevilla et al. 2004), indicating that high oleic acid content is not a general feature of fatty acids in mixotrophically grown cells and that the carbon source is likely to be an important determinant of the fatty acid profile.

Other advantages of mixotrophic cultures include the feasibility of using open ponds for large-scale cultivation (Perez-Garcia et al. 2011), and the use of wastewaters as sources of organic carbon and other nutrients for reduced production costs (Zhao et al. 2012). When open ponds or non-sterilized bioreactors are used, the addition of the organic carbon sources must be controlled to avoid contamination by fast-growing heterotrophs. In some cases, the organic carbon substrate is only introduced during daylight hours, or alternatively is added only once toward the end of the culture to avoid bacterial contaminants from accumulating to unacceptable levels (Abeliovich and Weisman 1978; Lee 2001).

The main disadvantages of mixotrophic culture, as with heterotrophic culture, are that the cost of carbon source can be high and an excess/uncontrolled addition of organic substrates in an open system is likely to stimulate growth of invasive heterotrophic bacteria, resulting in a low microalgae biomass yield. There is also the problem of photoinhibition of organic carbon metabolism, in some cases, while maintaining an optimum balance of photoautotrophic to heterotrophic metabolic activities can be challenging.

4.4 Cyclic Photoautotrophic–Heterotrophic Cultures

Depending on the strain and organic carbon source, a major disadvantage of heterotrophic culture is that light is required for increased productivity. In some strains, for example, lipid productivity is higher under photoautotrophic cultures when compared with heterotrophic cultures. Furthermore, the cost of organic carbon source can be very high, making heterotrophic production of biodiesel oil uneconomical. Solar light energy is abundant and freely available in outdoor photoautotrophic cultures. Thus, it is desirable to use light from solar energy to reduce production costs. Depending on the location and season, however, only a few hours of the day have the high light intensity needed to support photoautotrophic growth. During the night, cells not only cease to grow, but they metabolize the already intracellularly stored energy for cell maintenance, thereby leading to a decrease in biomass concentration (Ogbonna and Tanaka 1996). Cyclic photoautotrophic–heterotrophic culture seeks to overcome this problem by cultivating cells photoautotrophically during the day, while adding the required amount of organic carbon source to grow the cells heterotrophically at night. By taking advantage of both alternating photoautotrophic and heterotrophic cultures, the cells grow continuously during both day and night, leading to increased productivity (Ogbonna and Tanaka 1998; Ogbonna et al. 2001). This is especially useful for the cultivation of some microalgae that are not truly mixotrophs, yet can switch between phototrophic and heterotrophic metabolisms, depending on environmental conditions (Kaplan et al. 1986). However, the technical challenge of minimizing contamination risk through the selection and control of the organic carbon source added during the night remains.

4.5 Sequential Heterotrophic–Photoautotrophic Cultures

As previously discussed, mixotrophic cultures have very high potentials for oil production by many strains of photosynthetic organisms. However, optimization of mixotrophic cultures is difficult as the conditions that favor photoautotrophic metabolic activities may not favor heterotrophic metabolic activities. The relative contribution of these two metabolic activities depends on such factors as light intensity, the nature, and concentration of the organic carbon source, and carbon dioxide concentration (Ogbonna et al. 2002a, b). For instance, it has been observed that the assimilation of glucose in a certain strain of *Chlorella* is suppressed by light, even at low light intensities (Haass and Tanner 1974; Kamiya and Kovallik 1987); therefore, only photoautotrophic metabolic activities are observed under mixotrophic conditions. Sequential heterotrophic–photoautotrophic culture system can be used to overcome such limitations. In this system, the cells are first cultivated to high densities in heterotrophic cultures, and the condition is then changed to photoautotrophic. This system ensures that the advantages of heterotrophic cultures (such as high cell densities) and those of the photoautotrophic cultures

(such as light-induced synthesis of metabolites) are realized. The effectiveness of this culture system has been demonstrated for *Chlorella* (Ogbonna et al. 1997), *Euglena* (Ogbonna et al. 1999), and *Haematococcus* (Hata et al. 2001). Sequential heterotrophic–mixotrophic cultivation, in which light illumination is started before the organic carbon source is completely utilized, has also been investigated for biodiesel oil production (Mitra et al. 2012).

4.6 Sequential Photoautotrophic–Mixotrophic Cultures

A major disadvantage of mixotrophic cultures is contamination, especially when open systems are used. Many species of bacteria have much higher growth rates than photosynthetic microorganisms. Thus, they can easily outcompete and outgrow the photosynthetic cells. Although many antibacterial substances can be used to inhibit bacteria growth, their effectiveness depends on the nature of the microalgae. To minimize the risks of contamination, the cells can be cultivated photoautotrophically to a relatively high cell concentration before switching to mixotrophic culture by the addition of an organic carbon source. Thus, the high concentration of microalgae before the addition of the organic carbon source will enable the cells to outcompete the initial low concentration of the contaminant. Furthermore, a shorter period of the mixotrophic culture can be applied so that cultivation is terminated as soon as concentration of the contaminants reaches a critical level. There are many reports on sequential photoautotrophic–mixotrophic cultures; for example, Fernandez Sevilla et al. (2004) employed it for cultivation of *P. tricornutum* UTEX 640, using both bubble column and airlift photobioreactors. The photoautotrophic phase lasted until a cell concentration of 3.5 g/L was reached; it was then switched to mixotrophic condition by the addition of glycerol under limited nitrogen, during which the cell concentration increased to 25.4 g/L. Yen and Chang (2013) similarly reported higher biomass concentration in sequential photoautotrophic–mixotrophic cultures; however, the linoleic acid content (18:1) was the same when compared with photoautotrophic cultures. Das et al. (2011) studied intracellular lipid accumulation by *Nannochloropsis* sp. in sequential photoautotrophic–mixotrophic cultures. Photoautotrophic culture was used for 7 days followed by 3 days of mixotrophic culture, using either glycerol, glucose, or sucrose as the organic carbon source, resulting in a 72 % increase in lipid productivity when compared with the photoautotrophic culture.

4.7 Conclusions

Despite efforts to improve the productivity of photoautotrophic cultures, biodiesel oil productivity is still very low because of the low innate light conversation efficiencies of the microalgae. Large-scale photobioreactors with high light supply

efficiencies remain technically challenging from a design perspective, and thus, the cost of microalgae biodiesel remains very high. The photoautotrophic microalgae cultures have several productivity limits, thus making it difficult to further reduce the cost of biodiesel oil production in photoautotrophic cultures. One solution is to exploit the ability of some strains of microalgae to metabolize organic carbon sources both with and without light. Depending on the strain, facilities available, technical know-how, and other culture conditions, the various culture systems discussed in this chapter present a viable alternative to photoautotrophic cultures in biodiesel production. For example, culture systems employing heterotrophic metabolism can be used to improve both the productivity and quality of oils produced by microalgae by increasing the relative composition of triglyceride oils with high oleic acid contents, which are a better substrate for the production of biodiesel.

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Chapter 5
Wastewater as a Source of Nutrients for Microalgae Biomass Production

Koenraad Muylaert, Annelies Beuckels, Orily Depraetere, Imogen Foubert, Giorgos Markou and Dries Vandamme

Abstract Production of microalgal biomass requires large amounts of nitrogen (N) and phosphorus (P). The sustainability and economic viability of microalgae production could be significantly improved if N and P are not supplied by synthetic fertilizers but with wastewater. Microalgae already play an important role in wastewater treatment, yet several challenges remain to optimally convert wastewater nutrients into microalgal biomass. This book chapter aims to give an overview of the potential of using wastewater for microalgae production, as well some challenges that should be taken into account. We also review the benefits of combining microalgal biomass production with wastewater treatment.

5.1 Introduction

Microalgae have a high areal productivity, do not require fertile land, and are seen as a promising new source of biomass that could complement production by conventional agricultural crops. Microalgae have attracted a lot of interest in recent

K. Muylaert (✉) · A. Beuckels · O. Depraetere · D. Vandamme
Laboratory of Aquatic Biology, KU Leuven Kulak—University of Leuven, Etienne Sabbelaan 53, BE8500 Kortrijk, Belgium
e-mail: Koenraad.muylaert@kuleuven-kulak.be

I. Foubert
Research Unit Food and Lipids, Department of Molecular and Microbial Systems Kulak, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium

I. Foubert
Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

G. Markou
Department of Natural Resources Management and Agricultural Engineering, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece

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years as a novel feedstock for biofuels (Schenk et al. 2008). But microalgae also hold a lot of potential for production of food (Draaisma et al. 2013), animal feed (Benemann 2013), or feedstock for the chemical industry (Wijffels et al. 2010). When compared to conventional agricultural crops, microalgae have a high content of proteins and lipids, and a low content of structural carbohydrates such as cellulose (Lam and Lee 2011). This is an attractive property of microalgae, because it implies that most of the biomass can be valorized. On the contrary, with conventional crops, only a small fraction of the biomass is used (e.g., seeds or tubers) and a large proportion of the biomass is left on the field (i.e., the fraction that contains mostly cellulose and lignin). Because of the low content of structural carbohydrates such as lignin or cellulose, microalgal biomass has a high content of nitrogen (N) and phosphorus (P): about 10 % N and 1 % P per unit dry weight. This is almost three times higher than the N and P content of terrestrial plants (Elser et al. 2000). Because of the high content of N and P in microalgal biomass, production of microalgae requires vast quantities of inorganic fertilizer, much more than the production of terrestrial crops (Sialve et al. 2009). This high fertilizer demand is a challenge to the sustainability of microalgae biomass production, and several life cycle analyses studies have shown that the energy required for synthetic fertilizer production contributes significantly the total energy demand for microalgal biofuels (Lardon et al. 2009; Clarens et al. 2010; Benemann et al. 2012). Production of N fertilizers through the Haber-Bosch process is highly energy-intensive and is reliant on fossil fuels (Smil 2002; Pfromm et al. 2011). Extraction and processing of mineral phosphates for production of P fertilizer is also energy-intensive (Johnson et al. 2013). Moreover, mineral phosphates reserves are limited and are rapidly being depleted (Cordell et al. 2011). If microalgae are to be produced on a large scale, e.g., large enough to contribute significantly to fuel demand, consumption of synthetic fertilizers is expected to increase strongly above current levels (Venteris et al. 2014). Microalgae are often promoted as a biomass source that does not compete with agricultural biomass production, and thus avoids the food versus fuel discussion. However, as both microalgae and agricultural crops require mineral fertilizers, microalgae may indirectly compete with agricultural crops and thus indirectly impact food production through increases in fertilizer prices (Pate et al. 2011). Many studies and opinion papers have in recent years suggested to use wastewater rather than synthetic fertilizers as a source of nutrients for microalgae production and showed that this could significantly improve the sustainability and economic feasibility of microalgae production (Lundquist et al. 2010; Clarens et al. 2010; Christenson and Sims 2011; Pittman et al. 2011; Park et al. 2011b; Olguín 2012; Prajapati et al. 2013). Because microalgae are already being used for wastewater treatment, replacing synthetic fertilizer with nutrients from wastewater is feasible. The goal of this book chapter is to discuss both the potential as well as limitations of using wastewater as a nutrient source for microalgae production.

5.2 Current Use of Microalgae in Wastewater Treatment

Microalgae play an important role in many wastewater treatment facilities around the world. In developing economies in tropical and subtropical countries, wastewater is often treated using facultative ponds or oxidation ponds (Duncan 2004; Rahman et al. 2012). These consist of relatively deep and non-mixed ponds that are spontaneously colonized by microalgae. In these ponds, microalgae serve mainly to supply oxygen for the aerobic oxidation of organic matter present in the wastewater. Because these ponds are relatively deep and are poorly mixed, microalgal productivity is relatively low; only about 10 ton of dry biomass ha^{-1} $year^{-1}$. The microalgal biomass is not harvested at the end of the wastewater treatment process, and either settles to the bottom of the pond or is washed out of the ponds. Because the microalgal biomass is not harvested, removal of nutrients from the wastewater by the microalgae is inefficient.

At the end of the 1950s, high-rate algal ponds (HRAPs) were proposed as an alternative to facultative ponds (Oswald and Golueke 1960). HRAPs are raceway-type ponds in which the water is mixed by a paddle wheel. Compared to facultative ponds, HRAPs are much shallower and better mixed and, as a result, have higher microalgal productivity, about 30 ton dry biomass ha^{-1} $year^{-1}$. The productivity of HRAP's can be further increased with CO_2 addition (Craggs et al. 2013). Because microalgal productivity is higher, a larger volume of wastewater can be treated on the same land area when compared to facultative ponds. Akin to facultative ponds, microalgae in HRAPs supply oxygen for the aerobic oxidation of organic matter, and if the microalgal biomass is harvested at the end of the treatment process, the microalgae remove nutrients from the wastewater. Harvesting of the microalgal biomass, however, is costly and many HRAPs today do not harvest the biomass. Although HRAPs are used in wastewater treatment plants around the world, the technology is much less widespread than oxidation ponds or conventional electromechanical wastewater treatment systems (Craggs et al. 2013).

5.3 Wastewater as a Source of Nutrients for Microalgal Biomass Production

Many industries and human activities generate wastewater, and as a consequence, there are many different types of wastewaters, each with a different chemical composition and volumetric production over time. Table 5.1 gives an overview of different types of wastewaters and their content of N and P. The two major sources of wastewater are domestic wastewater and wastewater derived from animal manure. Each person supplies about 3 kg N per year through domestic wastewater, which translates to about 21 million ton of N per year for a global population of 7 billion (similar to the estimate of Smil 2002; Van Harmelen and Oonk 2006). The major livestock animals that produce manure are pigs, cattle, and chickens.

Table 5.1 Concentrations of N and P as well as their molar ratios in different types of wastewaters

Wastewater source	N (mg L^{-1})	P (mg L^{-1})	N:P ratio (molar)
Domestic	20–85	5–20	11–13
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
Anaerobic digestion food waste	1600–1900	300	–
Olive mill	530	182	2.9

Based on Christenson and Sims (2011), Cai et al. (2013)

Chickens produce a relatively dry type of manure that is suitable for application as fertilizer on agricultural land and we do not consider the N supply from chicken manure for algae production. Pigs produce about 16 kg N $animal^{-1}$ $year^{-1}$, cattle 35 kg N $animal^{-1}$ $year^{-1}$, and dairy cattle 75 kg $animal^{-1}$ $year^{-1}$. With global population sizes of 1 billion pigs, 0.9 billion cattle and 0.25 billion dairy cattle, wastewater from pig and cattle manure can theoretically provide 65 million tons of N, or about 3 times more than domestic wastewater (similar to the estimate of Van Harmelen and Oonk 2006). If we assume a "N" content of microalgal biomass of 7 %, the total human, pig, and cattle wastewater N nutrient is enough to produce about 778 million ton dry microalgal biomass per year. This is in the same order of magnitude as the global production of wheat or of corn. Although this is a lot of biomass, it can produce only about 233 million ton of oil (assuming a 30 % lipid content in microalgae). This corresponds to 1800 million barrels of oil, or only about 5 % of the global oil consumption. Thus, wastewater alone cannot supply sufficient nutrients for microalgal biomass to make a large contribution to the world's energy demand. This conclusion is in line with Peccia et al. (2013) or Chisti (2013), who estimated that nutrients from domestic wastewater of a typical large city can only produce enough microalgal biofuel to supply 3 % of the fuel demand of that city. If microalgal biofuels are ever to make a larger contribution to the global fuel demand, it will be essential to recycle the nutrients during conversion of microalgal biomass to biofuels (Venteris et al. 2014). Although animal manure is a potentially significant source of nutrients for microalgae production, it should be noted that a significant proportion of animal manure is already used as a fertilizer in conventional agricultural production (Bouwman and Van Der Hoek 1997). Because synthetic fertilizer prices are increasing, the value of nutrients in animal manure also increases. Therefore, the use of animal manure as fertilizer in conventional

agriculture is likely to increase in the future (Shilton et al. 2012), and microalgae and conventional agriculture may compete for animal manure nutrients. However, the main limitation of the use of raw animal manure in conventional agriculture is the high transport cost resulting from the high water content of animal manure. In areas where livestock numbers are high and agricultural crop production is nutrient-limited, it will unlikely be economically feasible to transport animal manure to the field, and microalgae may become a more attractive option to process large volumes of manure on a relatively small land area.

In addition to manure, there are many other sources of wastewater that could be used for microalgae biomass production, such as wastewater from olive mills, wineries, breweries, vegetable processing, tanneries, or the paper industry (Cai et al. 2013). Some emerging technologies also generate a lot of wastewater. The use of anaerobic digestion to convert organic waste streams into methane is growing worldwide and generates a nutrient-rich effluent that could be processed with microalgae (Uggetti et al. 2014). Aquaculture is also increasing worldwide and generates a similar nutrient-rich wastewater that may be suitable for treatment using microalgae (e.g., Van Den Hende et al. 2014). The volumes that are produced by these industries are relatively small compared to the volumes of domestic and animal manure wastewater. Nevertheless, microalgae may be a solution to treat some of these wastewaters as conventional water treatment technologies may be too expensive or ineffective.

5.4 Spatial and Temporal Mismatches Between Microalgae Production and Wastewater Availability

Wastewater has a relatively low nutrient content, with usually less than 1 % N and less than 0.5 % P. Because of this low nutrient content, it is not cost-effective to transport wastewater over long distances to microalgal farms. Therefore, microalgal farms should be situated as close as possible to the wastewater sources. It is also costly to store wastewater during periods when microalgae production is low, and the production of microalgal biomass should ideally more or less match the generation of wastewater. Such spatial and temporal mismatches between availability of wastewater and microalgal productivity may limit the potential to convert wastewater nutrients into microalgal biomass. Large cities generate enormous volumes of wastewater that could be used for microalgae biomass production. These cities, however, often lack sufficiently large areas of low-cost land nearby that can be used for microalgae production (Fortier and Sturm 2012). In addition, many of the world's largest cities are situated in temperate climates where microalgal productivity is low in winter and freezing temperatures may even require complete shutdown of microalgal farms for a few months (Chiu and Wu 2013). At high latitudes in winter, it may be impractical to use wastewater for microalgae production (Van Harmelen and Oonk 2006), and at low latitudes, high temperatures may also limit productivity during the warmest months.

Availability of land is probably less problematic when animal manure is used for microalgae production than when domestic wastewater is used. Livestock farms are generally situated away from cities. In general, agricultural land is available that can be converted in microalgae cultivation ponds. In many countries, however, there is an ongoing debate whether microalgae cultivation is allowed on agricultural land or not (Trentacoste et al. 2014). Due to economies of scale, we can assume that the minimum size of a microalgae farm would be about 10 ha (Lundquist et al. 2010). A 10-ha farm that produces 300 ton dry microalgal biomass ha^{-1} $year^{-1}$ consumes 27 ton N $year^{-1}$. This corresponds to the N output of a municipality of 9000 inhabitants, or a farm with at least 7000 pigs or 400 cattle. Wastewater from smaller farms or villages may be collected and transported to a microalgae farm, but this is only possible over relatively small distances due to the high cost for transporting wastewater. Therefore, smaller and isolated sources of wastewater will unlikely be practical for microalgae production.

5.5 Low-Strength Versus High-Strength Wastewater

In open raceway ponds, microalgal biomass concentrations are typically about 0.5 g L^{-1}. The N content of this biomass is about 7 % and the P content 1 %. As a result, the minimal nutrient concentration in the culture medium to achieve a biomass concentration of 0.5 g L^{-1} should be around 5 mg P L^{-1} and 35 mg N L^{-1}. In photobioreactors, microalgal biomass concentrations are higher (up to tenfold), and a higher nutrient concentration is needed in the medium to achieve the maximal biomass concentration. If nutrient concentrations in the wastewater are lower, microalgal biomass will be nutrient-limited and the biomass concentration and productivity will be lower than can be achieved under optimal conditions. If the biomass concentration in the medium is too low, this may in turn result in higher harvesting costs.

Concentrations of N and P vary considerably between different types of wastewaters. Domestic wastewater contains about 15–40 mg N L^{-1} (Rahman et al. 2012; Peccia et al. 2013), which is perhaps just sufficient to achieve the maximal productivity of microalgae in raceway ponds (Olguín 2012), but too low for photobioreactors. If wastewater with a lower concentration of N and P is used as a source of nutrients, the retention time of the microalgae in the system can be increased relative to that of the nutrients. This can be achieved in several ways. One option is to grow microalgae on a fixed support rather than suspended in the culture medium (Hoffmann 1998; Mulbry et al. 2008; Zamalloa et al. 2013; Boelee et al. 2013; Kesaano and Sims 2014). The fixed microalgae can be unicellular or filamentous species and can be grown on a variety of supports. Another option, as summarized in Chap. 2, is to immobilize suspended microalgae in alginate beads or alginate mats (e.g., Mallick 2002; Ruiz-Marin et al. 2010; Eroglu et al. 2012). However, it may be difficult to separate the microalgae from the alginate and use the biomass. Also, membrane photobioreactors can be used that retain microalgae in

the photobioreactors but allow a high throughput of wastewater (Bilad et al. 2014). Wastewater derived from animal manure or industrial effluents are often high-strength wastewaters and can contain up to several grams of N L^{-1}. When nutrient concentrations are higher than the requirements for microalgal production, the microalgae will be light-limited due to self-shading before all nutrients are consumed (Huisman et al. 2002). Wastewater with high "N" and "P" concentration can be diluted with water to match the nutrient supply with the productivity of the microalgae, yet the use of pure water to dilute wastewater is unsustainable (Marcilhac et al. 2014). Concentrated wastewaters can be diluted with seawater to produce a growth medium for marine microalgae (e.g., Craggs et al. 1995; Zhang and Hu 2008; Jiang et al. 2011). Alternatively, low nutrient domestic wastewater could be used to dilute concentrated wastewater. If this is not possible, the culture medium may be repeatedly recycled until all nutrients have been consumed by the microalgae. Few studies, however, have attempted this to date.

5.6 Variation in Nutrient Ratios

An advantage of most wastewaters is that it contains N as well as P; the two most important nutrients required by microalgae. Microalgae, however, consume N and P in a more or less fixed ratio: the Redfield ratio; a molar ratio of N:P of 16:1. The ratio of N to P in wastewater is highly variable between wastewaters and even within a specific type of wastewater. If the N:P ratio in the wastewater exceeds 16:1, P will be limiting the growth of the microalgae, and vice versa. High or low N:P ratios may result in incomplete removal of N or P from the wastewater (Li and Brett 2013). However, studies on the stoichiometric composition of microalgae in the past two decades have indicated that the N:P Redfield ratio of 16 is not as fixed as it was once believed (Sterner and Elser 2002). When both the nutrient supply rate and microalgal growth rates are high, the N:P ratio in algae varies from 5 to 19. Conversely, when microalgal growth rates are limited by nutrients, this range is extended from less than 5 to more than 100; a strong deviation from the Redfield ratio (Geider and Roche 2002). Nutrient limitation influences the biochemical composition of the microalgae. Without nutrient limitation, microalgae tend to have a high content of proteins. When nutrients are limiting, the microalgae will accumulate carbohydrates or lipids (Smith et al. 2010; Markou et al. 2012a; González-Fernández and Ballesteros 2013). Whether carbohydrates or lipids are accumulated under nutrient starvation depends on the species of microalgae and the degree of nutrient limitation. Changes in the biochemical composition of the biomass as a result of variations in the N:P ratio of the wastewater may have important consequences for the valorization of the microalgal biomass. If N and P are balanced, the biomass will have a high protein content and will be attractive for animal feed production. When N or P are limiting, lipids or carbohydrates are accumulated and the biomass can be used for biodiesel, bioethanol, or biomethane production. When P concentrations in the medium are high, some microalgae can accumulate excess

P as polyphosphate granules, a phenomenon known as luxury uptake. Luxury uptake occurs when P-limited microalgae are suddenly supplied with P. This luxury uptake appears to be a transient phenomenon, as microalgae quickly convert their internal P-reserves into growth (Powell et al. 2009). The N:P ratio of microalgae is not only influenced by the N:P ratio of the culture medium, but it also differs between different species or groups of microalgae. P is mainly used in the microalgal cells to produce rRNA, which is part of the ribosomes. Fast-growing microalgae species have a high content of rRNA to produce new proteins and tend to have a high P content (Klausmeier et al. 2004). Cyanobacteria, on the contrary, have a high protein content and therefore have a higher N:P ratio than other microalgae (Becker 2007; López et al. 2010).

5.7 Bioavailability of Nutrients

When nutrient concentrations are reported for wastewater, total concentrations of nutrients are often reported. Dissolved inorganic nutrients, such nitrate and ammonium N and phosphate P are directly available to microalgae. Part of the total N and P in wastewater, however, may be associated with organic molecules (dissolved organic nutrients) or with particulate matter (either organic or inorganic). These nutrients are not necessarily available to microalgae. Relatively few studies have investigated the bioavailability of nutrients to microalgae in different types of wastewater.

The bioavailability of P is highly variable and may vary between 3.4 and 81 % in different types of wastewaters or surface waters (Ekholm and Krogerus 2003). The bioavailability of total P depends on the dominant P-forms that are present (Van Moorleghem et al. 2013; e.g., Li and Brett 2013). Polyphosphates and organic phosphate monoesters, for instance, have a high bioavailability. Microalgae are capable of producing phosphatase enzymes to dissociate phosphate ester bonds (e.g., Huang and Hong 1999). Phytic acid, on the contrary, has a very low bioavailability and is a major phosphate reserve in plant seeds. As it cannot be digested by livestock, it is present in high concentrations in manure from grain-fed livestock (Jongbloed and Lenis 1998). To increase the bioavailability of P from phytic acid to livestock, phytase enzymes are sometimes added to the animal feed, and this may result in a higher bioavailability of P in the manure. If the wastewater contains a lot of sediments, phosphate may be chemically bound to iron (Fe) or aluminum (Al), and this phosphate has a low bioavailability. Phosphate that is precipitated with calcium (Ca) as apatite minerals also has a low bioavailability to microalgae, despite being often detected by colorimetric phosphate analyses. Wastewater from animal manure has a high content of humic substances and these can also bind phosphate. It is assumed that phosphate is bound to oxidized Al or Fe ions that are stabilized by humic acid complexes (Gerke 2010). These humic acid–metal phosphate complexes also have a low bioavailability to microalgae.

Less information is available about the bioavailability of organic N forms. About half of the N in animal manure, wastewater may be present as organic N (Cai et al. 2013). Free amino acids, nucleotides, as well as urea are organic N forms that are highly bioavailable to microalgae. Peptides or proteins have a slightly lower bioavailability, and humic substances contain N and exhibit even less bioavailability (Bronk et al. 2007). Humic substances have a C:N ratio of 18–30:1 for humic acids and 45–55:1 for fulvic acids (See and Bronk 2005). Humic substances can absorb amino acids and ammonium ions, which can represent about half of N in humic substances. Part of the organic N present in wastewater is slowly made available by bacteria that live in symbiosis with microalgae (Pehlivanoglu and Sedlak 2004).

5.8 Losses of Nutrients from Wastewater

The unique environmental conditions in microalgal cultures may result in significant losses of nutrients from wastewater. In microalgal cultures, pH is high due to photosynthetic depletion of carbon dioxide (CO_2) from the culture medium, and this may result in volatilization of ammonia or precipitation of P. In concentrated wastewaters such as animal manure, N is often present as ammonium. When pH is high, ammonium is converted to free ammonia and escaped as a gas from the culture medium through volatilization. Volatilization of ammonia can be significant in open algal ponds used for wastewater treatment, particularly when water temperatures are high (Garcia et al. 2000); this not only results in losses of N, but also causes eutrophication in the surrounding landscape through N deposition. However, maintaining the pH of the culture medium at 8 by addition of CO_2 is effective to prevent ammonia volatilization (Park et al. 2011b). At a high pH, phosphate can also precipitate as calcium phosphates (when Ca concentrations are high; Beuckels et al. (2013) or as struvite when ammonium and magnesium (Mg) concentrations are high. Phosphate precipitation can result in significant losses of P from the wastewater (e.g., Lodi et al. 2003), causing additional turbidity in medium and reducing microalgal production (Belay 1997).

5.9 Growth-Inhibiting Substances in Wastewater

Wastewater not only contains nutrients such as N and P, but also a range of other contaminants that may interfere with microalgal growth. The presence of growth-inhibiting substances probably explains why microalgal growth rates in real wastewaters are often slightly lower than in synthetic wastewaters. Contaminants not only pose a problem because they inhibit microalgal growth, but they can also accumulate in the microalgal biomass and limit the valorization of the microalgal biomass, or fractions thereof. Wastewater can contain a wide range of toxic chemicals such as heavy metals, persistent organic pollutants, and surfactants.

This is particularly the case in industrial wastewaters, although domestic wastewater or animal manure may also contain substantial quantities of pollutants such as heavy metals (Nicholson et al. 1999). Even chemicals with a low toxicity (such as those used in personal care products), may have an inhibitory effect on microalgal growth rates (e.g., Wilson et al. 2003).

Animal manure wastewaters often contain very high concentrations of N present in reduced form as ammonium. Ammonium is converted to free ammonia at high pH and is toxic to many microalgae, with some microalgae inhibited by concentrations as low as 20 mg L^{-1} (Azov and Goldman 1982). High concentrations of ammonium may result in ammonia toxicity even when pH is low (Peccia et al. 2013). Some substances do not inhibit microalgal growth, but accumulate in the microalgal biomass and limit valorization. The cell wall of microalgae is often rich in carboxyl, amino, hydroxyl, or sulfate groups. These groups are anionic and can bind metals through ion exchange (Wang and Chen 2009). Microalgae are efficient absorbers of heavy metals and even low concentrations of heavy metals present in wastewater can be absorbed into microalgal biomass. This is not necessarily a problem when the biomass is converted into biofuels using chemical or physical methods. It may be a problem when biological methods are used to convert the biomass into fuel (e.g., anaerobic digestion, fermentation). It may also be a problem when the protein-rich residue of the biomass remaining after extraction of lipids for biodiesel production is to be used as animal feed, as is proposed in the microalgal biorefinery context (Wijffels et al. 2010). Rwehumbiza et al. (2012) showed that metals used for flocculating microalgae remain in the protein-rich residue after extraction of lipids. However, microalgal absorbtion of heavy metals from wastewater can also be an advantage, and numerous studies have demonstrated that microalgae can be applied to remove heavy metals from a variety of wastewaters (see for instance Wilde and Benemann 1993; Gadd 2009). Wastewater may also contain microbial contaminants such as cysts of parasites, infectious bacteria, or viruses. These may also interfere with the use of microalgal biomass fractions as animal feed. However, due to the high pH, high oxygen concentrations, and exposure to light, many harmful microorganisms tend to be inactivated in microalgal cultures (Davies-Colley et al. 1999).

Many wastewaters of agricultural origin such as piggery waste or anaerobic digestion wastewater often have a dark color. This dark color is predominantly due to the presence of humic substances derived from incomplete breakdown of lignin in plant material (Brezonik and Arnold 2011). This dark color limits the light penetration in the water and reduces microalgal productivity (Martin et al. 1985); potentially a 20–30 % lower productivity when compared to growth rates in a non-colored culture medium (De Pauw et al. 1980). In many laboratory studies on production of microalgae in animal manure wastewater, the wastewater is diluted prior to the experiments and inhibition of microalgal productivity by the dark coloring is barely noticed. However, in large-scale systems, dilution of wastewater with pure water will be unsustainable due to the high water demand. To prevent inhibition of growth by colored substances, the wastewater can be pre-treated with oxidizing agents such as sodium hypochlorite, ozone or hydrogen peroxide, or by coagulants,

flocculants, or adsorbents (Markou et al. 2012b; Depraetere et al. 2013). Alternatively, nutrients can be separated from the wastewater containing colored substances and then added to the microalgae culture medium. This can be carried out, for instance, using dialysis membranes (Blais et al. 1984). Also nutrients can be sorbed onto zeolites and released from the zeolites in fresh medium (Markou et al. 2014).

5.10 Biological Contamination of Microalgal Cultures

Contamination of cultures by "weed" microalgae or predators is emerging as a major issue in large-scale cultivation of microalgae (Kazamia et al. 2012; Shurin et al. 2013). Although little is known about dispersal strategies of microorganisms, it is likely that wastewater contains spores of weed microalgae and predators. Contamination will therefore be more difficult to avoid when wastewater is used as a source of nutrients than when a pure culture medium is used. The risk of contamination can be limited by sterilizing the wastewater by micro- or ultrafiltration or by chemical disinfection. The cost of disinfection, however, will probably be too high when microalgae are produced for low-value products such as fuel or animal feed (Wang et al. 2013). Wastewater will likely contain spores or dispersal stages of herbivores of microalgae such as microcrustaceans, rotifers, or ciliates. The microcrustacean *Daphnia* or the water flea is often an important herbivore in microalgae-based wastewater treatment systems. If *Daphnia* invades the system, it can reduce microalgal biomass by two orders of magnitude within a few days (Cauchie et al. 2000). Due to its large size, *Daphnia* can be relatively easily removed by simple screening using a nylon mesh (Borowitzka et al. 1985). Smaller herbivores like rotifers or ciliates can also decimate microalgal biomass within a few days once they have invaded the culture (Schlüter et al. 1987; Moreno-Garrido and Cañavate 2001). However, these smaller herbivores cannot be so easily controlled by simple screening.

Some authors have proposed to use a wild consortium of microalgae rather than monospecific cultures for biofuel production. Today, most microalgae-based wastewater treatment systems use such wild consortia rather than monospecific microalgal cultures. Consortia of microalgae may be more resistant to the impact of small herbivores than monospecific microalgal cultures. If a small herbivore invades the culture, small microalgae will be consumed by large microalgal species that cannot be ingested by the herbivore may take over the culture and maintain a high productivity (Shurin et al. 2013). Consortia of microalgae are not only more resistant to herbivores, but may also be more efficient converting nutrients into biomass than monospecific cultures (Ptacnik et al. 2008; Kazamia et al. 2012; Shurin et al. 2013). However, the use of consortia rather than pure algal cultures may pose a problem for the valorization of the resultant biomass. First, if the consortia contain toxic species such as cyanobacteria, the biomass cannot be used for food or animal feed. Second, it is more difficult to control the biochemical composition of the biomass in mixed consortia than in pure cultures. Some species in the consortium may produce carbohydrates, for instance, while others produce

lipids. The consortia that occur in wastewater treatment systems are often dominated by a few freshwater microalgal species, very often chlorophytes (*Chlorella*, *Scenedesmus*, *Micractinium*, *Pediastrum*) (Pittman et al. 2011). Some control over community composition is possible by recycling part of the harvested biomass. In a long-term study in a HRAP, recycling of the harvested biomass resulted in a 90 % dominance of the community by the large species *Pediastrum*, which improved the harvestability of the biomass (Park et al. 2011b).

5.11 Harvesting Microalgae Produced Using Wastewater

Due to the low biomass concentration of microalgae (about 0.5 g L^{-1} in open ponds) and the small size of microalgal cells (usually 5–50 μm), harvesting microalgal biomass is a major challenge (Uduman et al. 2010). Centrifugation is an efficient method for harvesting microalgae; however, this is too energy-intensive for most low-value applications (i.e., biofuels). Options such as flocculation are a promising approach to reduce harvesting costs (Vandamme et al. 2013). During flocculation, individual cells form larger aggregates that can easily be separated from the culture medium by gravity sedimentation, flotation, or enhanced settling in an inclined lamella separator. Using flocculation, the biomass can be concentrated from a dilute culture with a dry matter content of about 0.05 % to a sludge with a dry matter content of 0.5–5 %. Mechanical techniques such as centrifugation or a filter press are required to remove the remaining extracellular water and to obtain a thick paste with a dry matter content of 20 %.

Most HRAPs used for wastewater treatment today contain mixed consortia of microalgae rather than pure cultures. Usually, these communities are dominated relatively large, colony-forming chlorophytes such as *Pediastrum*, *Microctinium*, *Scenedesmus*, *Dictyosphaerium*, and *Coelastrum* (Benemann et al. 1980; Park et al. 2013). Possibly, these species are favored by the flow regime generated by the paddle wheel in high-rate algal ponds. These relatively large colonial microalgae often flocculate spontaneously, a process that is referred to as bioflocculation (Park et al. 2011a). Bioflocculating microalgae may form aggregates with other non-bioflocculating species (Salim et al. 2011), and bioflocculated microalgae have high settling rates and can be relatively easily concentrated to a slurry of 1–3 % dry matter by simple gravity sedimentation (Sheehan et al. 1998). By recycling part of the harvested biomass, the dominance of these bioflocculating microalgae can be maintained (Benemann et al. 1980; Park et al. 2011b, 2013). Bacteria present in the wastewater may also play a role in bioflocculation (Su et al. 2011). Bacteria grow on organic matter present in wastewater, and research by Lee et al. (2008) and Lee et al. (2012) showed that the presence of bacteria in cultures of *Chrysotila* and *Chlorella* resulted in flocculation of the microalgal cells. In both studies, it appeared that extracellular polymeric substances produced by the microalgae were involved in the flocculation process. Van den Hende et al. (2011) showed that a sufficient supply of organic matter is important to sustain mixed algal–bacterial flocs.

The high pH that is typical of microalgal cultures can induce precipitation of Ca or Mg salts and can also induce flocculation of microalgal cells; a process that is referred to as autoflocculation. Ca phosphates precipitate at a relatively low pH of about 8.5–9 and can induce flocculation of microalgae. Such pH levels are regularly encountered in outdoor microalgal cultures when irradiance levels and temperatures are high. Flocculation by Ca phosphate precipitation requires relatively high Ca and phosphate concentrations in the wastewater and is therefore only applicable in hard waters with excess phosphate levels (Sukenik and Shelef 1984; Sukenik et al. 1985). While autoflocculation by Ca phosphate works well in laboratory conditions, it often fails in large-scale systems, even when Ca and phosphate concentrations are sufficiently high (Nurdogan and Oswald 1995). This may be due to autoflocculation by Ca phosphate is inhibited by the presence of organic matter in microalgal cultures (Beuckels et al. 2013). Bioflocculation and autoflocculation have been studied in the past 30 years in laboratory conditions and pilot systems. It appears that their performance depends strongly on species and cultivation conditions, yet, the reliability of these methods remains to be proven in long-term and large-scale operations (Benemann et al. 2012). More details on the recent developments on harvesting and dewatering can be found in Chaps. 12–14.

5.12 Environmental Benefits of Microalgae-Based Over Conventional Wastewater Treatment

As outlined above, using wastewater nutrients rather than synthetic nutrients improves the sustainability of microalgae biomass production. On the other hand, using microalgae rather than conventional wastewater treatment technology may also result in a more sustainable method for treating wastewaters. In conventional wastewater treatment, N and P are removed from the wastewater without being reused: N is removed primarily by denitrification and is lost to the atmosphere as N_2, while P is removed from wastewater by precipitation with metal salts and disposed of in landfills. When wastewater is treated using microalgae, N and P are not only removed from the wastewater, but can also be reused to produce extra biomass. As N and P are extremely valuable resources to our society, initiatives are increasingly being taken to not only remove but also reuse N and P from wastewater (Dawson and Hilton 2011; Cordell et al. 2011; Elser 2012). Combining wastewater treatment with microalgae biomass production can achieve parts of this goal.

Eutrophication of lakes, rivers, wetlands, and coastal waters is a major environmental issue. To reduce eutrophication, regulations for discharge of effluents from wastewater treatment plants are becoming stricter. In the EU, for instance, discharge limits for wastewater have recently been decreased to 1 mg L^{-1} for P and to 10 mg L^{-1} for N (Oliveira and Machado 2013). Conventional technologies have difficulties in removing N and P from wastewater down to these levels. Residual concentrations of N and P in effluent from conventional wastewater treatment plants

are often quite high, high enough to cause eutrophication in receiving natural ecosystems. Microalgae have half-saturation constants for uptake of N and P that are well below the strictest limits. Therefore, the use of microalgae to remove nutrients from wastewater will certainly lead to lower N and P concentrations in the effluent, and less eutrophication of aquatic ecosystems.

Wastewater contains large amounts of organic matter, and it is important that it is oxidized before the effluent is discharged into the environment. In modern wastewater treatment plants, electromechanical air blowers supply oxygen that allows bacterial oxidation. This process consumes a lot of energy, and it is the major contributor to the capital and operational costs of modern wastewater treatment plants. If a proper cultivation design is developed, microalgae can produce sufficient oxygen for bacterial oxidation of organic matter. Microalgae-based systems are equally effective as electrical air blowers for oxidation of organic matter, but have a much lower cost (Owen 1982; Craggs et al. 2013). The CO_2 that is produced during degradation of organic matter can also be used as a carbon source in microalgal photosynthesis. Some microalgae are mixotrophic and can contribute to the degradation of organic matter from wastewater. This mixotrophic growth based on organic matter present in wastewater can even boost microalgal biomass production (Bhatnagar et al. 2011).

It is clear that combining microalgae production with wastewater treatment not only improves the sustainability of microalgae production but also that of wastewater treatment (Sturm and Lamer 2011). Beal et al. (2012) and Menger-Krug et al. (2012) showed that combined wastewater treatment and microalgae production has a much better energy balance than both processes operating separately. Combining microalgae biomass production with wastewater treatment would also make microalgae biofuel production economically more attractive, as additional income can be generated from the treatment of wastewater (Lundquist et al. 2010; Pittman et al. 2011). Combining microalgae production with wastewater treatment, however, is also a challenge because both processes need to be optimized simultaneously. On the one hand, the productivity and biochemical composition (e.g., lipid content) of the microalgae should be optimal. On the other hand, the quality of the wastewater effluent should comply with national water treatment standards (e.g., biological oxygen demand removal, N and P removal).

5.13 Conclusions

The high demand of microalgae for N and P poses an important environmental burden on microalgal biofuels. This environmental impact can be avoided by replacing synthetic fertilizer with N and P from wastewater. It is feasible to use wastewater as a source of N and P because microalgae have been used for many years in wastewater treatment (in facultative ponds or HRAPs). The resource base provided by wastewater nutrients is theoretically large enough to produce a similar amount of biomass as the global production of rice or wheat, yet it is not large enough to produce enough microalgal biomass to replace fossil fuels. Climatic and

geographical factors limit the potential to use wastewater for microalgae production. Using wastewater as a source of nutrients rather than synthetic fertilizer poses several challenges. The N and P demand of microalgae should be matched with the variable supply of these nutrients by wastewater. Wastewater contains many types of contaminants that can interfere with the production of microalgal biomass and/or with the valorization of certain microalgal biomass fractions. The high pH that is typical of microalgal cultures may result in nutrient losses (precipitation of P and volatilization of N). Further research is needed to overcome some of these challenges. Combining microalgae production with wastewater treatment not only improves the sustainability of microalgal biofuels but also increases efficiency of wastewater treatment because microalgae-based wastewater treatment has a lower energy demand, can result in a better effluent quality, and is a way to recycle valuable nutrients from the wastewater.

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Chapter 6
Algae-Based Wastewater Treatment for Biofuel Production: Processes, Species, and Extraction Methods

Stephen R. Lyon, Hossein Ahmadzadeh and Marcia A. Murry

Abstract This chapter develops the principles and rationale for an algae-based biofuel production coupled to bioremediation of municipal and agricultural wastewaters. A synergistic model for algal wastewater treatment is proposed, which addresses several economic bottlenecks to earlier algal systems and promotes value-added products, including a high-quality effluent in addition to biodiesel to improve the economic feasibility of algal biofuels. Finally, we review candidate species for full-scale algae production ponds based on algal structure, physiology and ecology, and methods for extraction of algal oils for biodiesel production and coproducts. The dominant strains of algae that are commonly found in wastewater ponds, including *Euglenia*, *Scenedesmus*, *Selenastrum*, *Chlorella,* and *Actinastrum*, are suggested as candidates for large-scale culturing based on their ability to strip nutrients and organic matter from wastewater, grow rapidly, and produce a significant level of algal oil. Oil extraction by supercritical fluid extraction (SFE) is discussed as an efficient means of isolating algal oil and other commercially important high-value compounds from algal biomass. Together with water and CO_2 reclamation, such products may shift the economics of algal biomass production to allow production of low-value commodities including biodiesel and biogas.

S.R. Lyon
AlgaXperts, LLC, Milwaukee, WI, USA

H. Ahmadzadeh (✉)
Department of Chemistry, Ferdowsi University of Mashhad, Mashhad, Iran
e-mail: h.ahmadzadeh@um.ac.ir

M.A. Murry
California State Polytechnic University, Pomona, CA, USA

M.A. Murry
Sinai Technology Corporation, Los Angeles, MI, USA

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6.1 Big Picture

The global biofuels market has made the transition from concept to multibillion-dollar reality in the past twenty years. The first-generation biofuels included ethanol and biodiesel. Ethanol is produced from the fermentation of sugar or starch-rich crops such as sugar cane and corn and distilled to yield pure ethanol. More recently, a variety of novel cellulase and xylanase enzymes have been identified from symbiotic and free-living microbes for use in the saccharification process allowing a greater fraction of the plant biomass used for ethanol production (Gladden et al. 2011). Biodiesel is primarily derived from the extraction and transesterification of triacylglycerols (TAGs) or triglycerides from various oil-bearing plants such as canola or jatropha or from the extraction of oil from soy, corn, and oil palm. Traditionally, biodiesel is made by transesterification of extracted TAGs producing fatty acid methyl esters (FAMEs) that can be used in diesel engines without modification and glycerol as a co-product.

Global ethanol production has grown to 22 billion gallons (US) in 2011 with the USA and Brazil contributing more than 65 % of total production (Renewable Fuels Association 2012). Biodiesel production is also growing rapidly, albeit from a smaller starting point. In 2008, the global biodiesel market was worth $8.6 billion (US) and is expected to grow to $12.6 billion (US) in 2014 (Davis et al. 2013). Although these first-generation biofuels represent a rather small contribution to the transportation fuel industry, they have come under criticism for competing with food production for arable land, nutrients, and water. Early biofuel programs had a negative impact on global food supplies with regard to soy, corn, and other grains or the destruction of tropical rain forests with regard to sugar cane and oil palm. In addition, terrestrial crops require months or in the case of oil palm years of growth before they can produce a harvestable crop. In response to the difficulties associated with the first-generation biofuels, interest in algae-based biofuels has been renewed and wide-spread efforts have gone into solving some of the technical problems associated with cost-efficient large-scale algal biomass production.

Algae are defined as a group of photosynthetic organisms, ranging from unicellular to multicellular forms, which lack true roots, stems, and leaves characteristic of terrestrial plants. Photosynthetic microorganisms, generally referred to as microalgae, represent a complex and diverse array of life forms that vary greatly in their metabolic capabilities, environmental adaptations, and morphology. The four common characteristics that are of significance with regard to this chapter are that they are small, autotrophic (i.e., they take up carbon dioxide to produce their own carbon compounds for metabolic purposes in sunlight), some are mixotrophic (i.e., they can assimilate a variety of carbon compounds in the absence of sunlight), and they produce varying amounts of oil (lipid) in the form of diglycerides and triglycerides. Algal oil is similar in structure and molecular weight to the oils extracted from the terrestrial plants described above for the production of biodiesel or as a feedstock for industrial chemicals that have a higher market value.

Lipid production in microalgae is species specific and influenced by environmental conditions. Oil content in pure cultures of microalgae can range from 1 % to

over 50 % of the dry weight. In addition, some algae can double their biomass in as little as 3.5 h in the laboratory and 24 h in outdoor ponds. Oil seed plants require an entire season for maturation of oil-rich seeds, which, in turn, comprise only a relatively small fraction of plant biomass. Algae lack non-photosynthetic structures (i.e., roots and stems), and since microalgae are unicellular and float in the water column, they have no need for the massive amounts of structural cellulose found in land plants. Furthermore, the photosynthetic efficiency of microalgae can theoretically reach up to 12 % (Oswald 1963; Zelitch 1971; Weissman and Goebel 1987), while terrestrial plants at mid-latitudes convert less than 0.5 % of solar energy into biomass (Li et al. 2008). Thus, productivity of microalgae per unit of land use can yield 7–20 times greater biomass than soy or corn and many strains can grow in saline or wastewaters. Taking these factors into consideration, it is easy to see why the potential difference in biomass/oil production between plant-based and algae-based biodiesel is so great. However, while it is true that some algae can accumulate biomass faster than terrestrial crops and tend to store excess carbon as lipids rather than structural carbohydrates, this frequently cited point distracts attention from the proper metric, which is total cost of oil production.

Macroalgae (i.e., seaweed) have been commercially produced for centuries. About 1.8×10^6 t of seaweed is produced commercially throughout the world. Until recently, the annual production of microalgae amounted to roughly one-hundredth the amount of commercially produced seaweed on an annual basis (Neori 2008). While many commercial microalgae production operations have been established in the last 40 years to produce high-value phytochemicals (e.g., beta-carotene, astaxanthin, and zeaxanthin), pharmaceuticals, feed for mariculture applications, and health food supplements (see Spolaore et al. 2006, for a review), the economic feasibility of producing algae biomass for low-value commodities including biofuels remains uncertain. Significant improvements in several key technologies, including strain selection, best cultivation practices, maintaining selected species in ponding operations, harvesting, and oil extraction, are needed to advance the economics of algae-based biofuel production. Considerable progress has been made over the past six years to develop and commercialize missing elements in the algae biofuels production chain. Innovative algae dewatering technology (AlgaeVenture Systems, Inc., Marysville, Ohio) and wet extraction and oil conversion technologies (SRS Energy Solutions, Inc., Dexter Mich.; Genifuels, Inc., Salt Lake City, Utah) are in demonstration phase.

While continued research and development of these technologies will improve the economics of algae biofuels, major economic limitations could be overcome in the short term by integrating biofuel production with wastewater treatment, to provide additional economic and environmental benefits. In synergy with biofuel production, algae-based wastewater treatment is a lost-cost, simple process compared to conventional wastewater systems, and algae systems have about 50 % lower energy consumption compared to conventional mechanical treatment technologies (Downing et al. 2002; Lundquist et al. 2010; Craggs et al. 2012).

6.2 Algae-Based Wastewater Treatment

For microalgae biofuel production, a major cost factor is the provision of water and nutrients (Davis and Aden 2011; Borowitzka and Moheimani 2013), which can both be provided by wastewater. Microalgae ponding systems were developed in the 1950s for municipal sewage treatment (see Oswald 2003 for a review of the early work), and this approach continues to serve as a starting point for the development of cost-efficient algae biomass for fuels production. At least 70 % of the cost of wastewater treatment can be attributed to secondary and tertiary treatment. Much of this is due to the energy costs of oxygen transfer in biological secondary treatment and chemical requirements in tertiary treatment.

Dr. William Oswald at the University of California-Berkeley and his colleagues over the following 50 years (Oswald 2003) developed the fundamental engineering design parameters and described the basic biological processes in bioremediation in high-rate ponds. Microscopic algae convert about 2 % of total solar energy to algal biomass. The photosynthetically generated oxygen is consumed by bacterial populations that decompose organic wastes to simple nutrients including CO_2. Although algae-based secondary and tertiary treatment is economically feasible, at least in warm regions with ample land, few municipal algae ponds attempt to control species composition or even harvest the algal biomass (Benemann and Oswald 1996). Two of the persistent problems noted in the early years of investigation were maintaining a stable algal population in the treatment ponds and harvesting the algae in an efficient and economic manner. Open pond cultures are subject to all the variations that occur in natural ponds and lakes. At any given time, there can be a major shift in algal dominance such as a transition from green algae to cyanobacteria (potentially toxic) or there can be a sudden collapse of the community structure due to algae bloom crashes and predation by zooplankton. The sudden death of phytoplankton communities, or algal blooms, is thought to result from several factors including insufficient light for photosynthesis, limiting nutrients, phycoviruses (Brussaard 2004), the aging of the blooms, and perhaps by photoinhibition in gas-vaculate cyanobacteria.

Successful open-air algal monoculture is currently limited to a small number of species that can tolerate extremes of pH or salinity that preclude invasion by competing microbes and undesired predators conquering unenclosed outdoor ponds. Enclosed bioreactors mitigate some of the problems of maintaining monocultures and predation issues, but capital and labor costs limit their use to production of high-value products. Other major hurdles to economically feasible algae-based biofuels include the use of high oil strains adapted to the local environment, development of resource-specific production and management systems, and, at least in the short term, coupling algae culture with mitigation of environmental problems and co-production of high-value compounds.

Minimal nutritional requirements for algal growth can be estimated from the approximate molecular formula of algal biomass: $C_{(0.48)}$ $H_{(1.83)}$ $N_{(0.11)}$ $P_{(0.01)}$ (Grobbelaar 2004). The chemical composition of municipal and dairy wastewaters

typically has less N than P relative to algal biomass (Fulton 2009). Although CO_2 limits algal growth in high-rate oxidation ponds (Benemann and Oswald 1996), when CO_2 is supplemented, N typically limits algal growth on municipal (Benemann et al. 1977) and agricultural (Lundquist et al. 2011) wastewaters. N limitation has long been known to be a trigger for lipid synthesis in some algal species. In a variety of microalgae, as the nitrogen or phosphorus levels drop limiting growth, there is a rapid increase in oil (lipid) content (Takagi et al. 2000; Li et al. 2008). The mechanisms involved in the "N-trigger" have remained elusive. Although recently comparative proteomics and transcriptome analysis of the haptophyte *Tisochrysis lutea* (formerly known as *Isochrysis galbana*) have revealed a wide variety of proteins and transcripts involved in various pathways including lipid, carbohydrate, amino acid, energy, and pigment metabolisms, photosynthesis, protein translation, stress responses, and cell division in strains are subjected to N limitation (Garnier et al. 2014). Most of the oleaginous algae are non-motile. They are thought to produce lipids as a buoyancy compensator to position them in the ideal part of the water column during the light/dark cycle of photosynthesis. As a survival mechanism to counter the depletion of nutrient supplies, they may produce high levels of lipid to bring them to the surface where the wind may blow them to a more nutrient-rich environment. This insight favors the concept of two-phase semi-continuous cultures for wastewater treatment to promote lipid biosynthesis. As the rapidly growing algae take up all the available nitrogen and phosphorus, biosynthetic pathways for growth are nutrient limited and oil content rises.

Figure 6.1 is a flow diagram of a hypothetical algae-based wastewater treatment systems with multiple benefits that take into account the drawbacks described in previous algae-culturing systems. The treatment process is based on a sequence of events designed to yield a high-quality effluent and a consistent supply of algae biomass:

1. Filtered primary wastewater is added to the first of three shallow raceways. There are three raceways: one is filling, one is in the algae growth stage, and the third raceway is in the harvesting stage. A fourth raceway may be needed to account for variations in productivity.
2. The filtered primary effluent is rich in biochemical oxygen demand (BOD), ammonium, and organically bound phosphorus. This effluent is mixed with a high concentration of a pure strain or strains of algae that are grown in a bank of photobioreactors. The algae in the photobioreactors are added to the pond when they are at or near the top of their log-phase growth. The dilution of the concentrated algae into the pond is adjusted to the lower end of early log-phase growth. These ideal strains were selected for their ability to grow rapidly on wastewater, adapted to local waters, high lipid content, and harvestability. In the daylight, the algae produce large quantities of oxygen that facilitate the oxidation of the various organic compounds that contribute to the BOD. During the dark cycle of photosynthesis, the algae can take up a variety of organic compounds including the organic compounds that were degraded during the light cycle of photosynthesis.

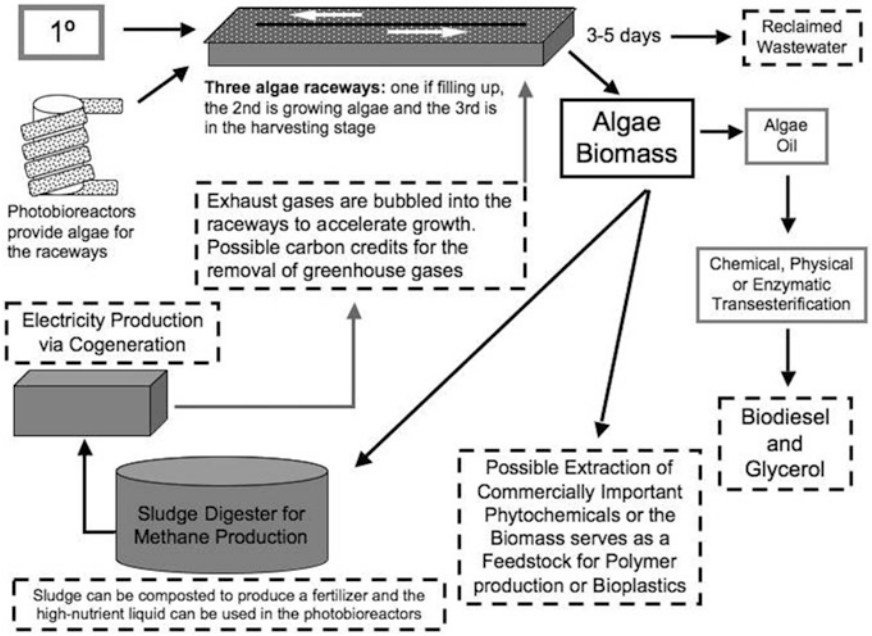

Fig. 6.1 Flow diagram of an algae-based wastewater treatment system. The boxes with the *dashed border* indicate an end product or benefit

3. Due to the log-phase growth, in 72–120 h, the algae biomass has increased fourfold to eightfold; the nutrients have been assimilated by the algae, and the BOD has dropped to the level associated with tertiary treatment. The short residence time in the open ponds lowers the likelihood of contamination by another strain of algae or predation by zooplankton mainly by competitive exclusion (Lang 1974; Hillebrand 2011).
4. The algae can be harvested by one of many physical separation processes (for more information of dewatering, see Chaps. 12–14), and the tertiary effluent can be disinfected and prepared for some form of water reuse. At this point, the algae can be ground up to yield a green crude or lysed in order to separate the lipid content from the aqueous and solid fractions.
5. The green crude can be processed into a variety of fuels in the same way the crude oil is processed. If the cells were lysed and just the lipid fraction was isolated, it would then be converted to biodiesel by one of a variety of physical, chemical, or enzymatic processes.
6. The algae biomass could potentially contain a number of valuable phytochemicals that can be extracted and purified for use in the chemical or personal care products industry. The biomass could also be used as a feedstock for the production of synthetic polymers.

7. If the previous option is not feasible, the cracked algae cells can be put into a digester to produce methane. Unlike activated sludge, this organic matter has a uniform composition, and since the cells are already lysed, the degradation of the organic matter and subsequent production of methane should be more efficient than digesters filled with activated sludge.
8. The energy demands on this process should be considerably less than traditional secondary and tertiary treatment such that the energy produced by cogeneration could be sold on the grid.
9. The CO_2 that is produced by burning the methane is usually released into the air. In order to raise algae in high concentrations, it is necessary to add CO_2 to the water. By bubbling in CO_2 into the rapidly growing algal cultures, it is possible to capture the majority of CO_2 produced by the cogeneration system (for a recent review, see Raeesossadati et al. 2014 and Chap. 7).
10. In the event that carbon "cap and trade" rules are implemented, this process provides a way for substantially shrinking the carbon footprint of a wastewater treatment plant.
11. The solid residue from the digester can be composted to produce a high-quality fertilizer, and the liquid fraction of the digester (mixed liquor) can be sterilized and used as a nutrient source for the bank of photobioreactors.

6.3 Candidate Species for Large-Scale Culturing of Algae

Two criteria that drive strategies for algae-based wastewater treatment for biofuel production are the need to produce a high-quality effluent on a consistent basis and the need to produce biomass with high oil content. While there are proponents for hydrothermal liquefaction (HTL) treatment of algal biomass to develop a "green crude," the goal in the scenario described above is to separate the oil and the biomass. The nitrogen and phosphorus content captured in the biomass is needed in the production of methane in the digester, and later, the biosolids from the digester can be composted with green waste to make a high-quality fertilizer.

To meet both criteria, compromises must be made in the selection of algae. In traditional wastewater oxidation ponds, there are a wide array of different prokaryotic and eukaryotic photosynthetic organisms. These ponds are subject to seasonal shifts in dominant populations as well as changes due to predation by protozoans and zooplankton. The variation in algal population dynamics can be minimized by periodic inoculation of the pond with a desired unialgal strain cultured in photobioreactors. Another consideration is the relationship between bacteria in the wastewater and the algae. In addition, evidence exists for a role for heterotrophic microbes in algae auto-flocculation (Lundquist et al. 2011). A close examination of algae collected from wastewater ponds using a light microscope will

usually show a significant number of heterotrophs associated or attached to the surface of the algae. If the algae are to be co-cultivated in photobioreactors as described above, it would be prudent to include the strains of wastewater bacteria associated with the specific type(s) of algae and which promote flocculation. The microalgae encompass a phylogenetically diverse assemblage of prokaryotic and eukaryotic photosynthetic microorganisms found in a wide range of habitats ranging from terrestrial environs to fresh and marine to hypersaline waters. It follows then that the ecology, morphology, biochemistry, and physiology are also diverse. Although the number of algal species is estimated to range between 30,000 and 300,000 with 7500 species systematically estimated from the literature (Guiry 2012), less than 1 % have been isolated and characterized (Radner and Parker 1994). Thus, the biotechnological potential of these microorganisms is just beginning to be explored for production of high-value and value-added products and biofuels. Microalgae have been used for decades as a source of high-value compounds with pharmaceutical activity including anticancer, antimicrobial, antiviral agents, and pigments including a variety of carotenoids, cosmetics, nutraceuticals, and feed supplements for poultry, livestock, and mariculture (see Walker et al. 2005 for a review). Many groups are now exploring the use of transformable eukaryotic strains to produce heterologous proteins since they are capable of intron-splicing, glycosylation, and multimeric protein assembly (Spolaore et al. 2006).

Several aspects of algae biology and physiology are relevant to their economic potential of microalgae as a feedstock for biodiesel coupled to bioremediation. Of particular interest to the biofuels industry are productivity, biochemical composition, and the influence of environmental and cultural practices on physiological processes, especially lipid metabolism and its regulation, photosynthetic efficiency, cell wall structure, and heterotrophic/mixotrophic capabilities. In addition, techniques to control algal/microbial pond community structure are essential for quality control of biodiesel composition from algae biomass. The ASTM International consensus-based standards group, whose standards are recognized in the United States, have specifications for the quality of biodiesel. The fuel characteristics are strongly influenced by FAME composition including chain length and degree of saturation. FAMEs isolated from algae species range in size from 12 to 38 carbons. The hydrocarbons that comprise petroleum products range in length as follows: 5–12 carbons for gasoline, 10–15 for diesel fuel, and 12–16 for kerosene (the main component of jet fuel). Refineries crack the longer hydrocarbons found in crude oil, then distill and blend the resulting compounds to formulate standard petroleum products. Maintenance of species composition, especially in outdoor ponds and when using wastewaters, is problematic. Cultivation of *Spirulina* in open outdoor ponds has been a success story in commercial algaculture. This strain grows in nearly pure culture in the alkaline, high-salinity waters of Lago de Texcoco. Competition from invading species is minimized due to the inhospitable nature of these waters. Control of species composition is crucial to quality control of

biodiesel production. Lipid profiles are characteristic of some organisms and have even been used as a taxonomic feature. However, microalgae show great inter- and intraspecific variation in fatty acid profiles and these profiles can be affected by culture conditions (Roessler 1990).

6.4 Lipid Productivity

Key parameters determining the economic feasibility of algae biofuels include biomass productivity, lipid content, and lipid productivity. Microalgae produce a variety of lipids, tri- and diglycerides, phospholipids, glycolipids, alkenes, and pigments such as the carotenoids. Reports of total lipid content for specific strains (i.e., compounds soluble in organic solvents per dry weight, as originally described by Bligh and Dyer 1959) vary in the literature (Griffiths and Harrison 2009). This is due, in part, to variations in the sequence and polarity of solvent systems used for extraction (Guckert et al. 1988). Because of the complexity of lipid compounds in algae and that the fractions of each class can vary with environmental conditions, lipid quantification, which is essential to the development of production models for algae biofuels, needs refinement. The biodiesel industry is currently based on transesterification of plant triglycerides forming alkyl esters of the fatty acid moiety. The fate of other cellular lipid compounds in the tranesterification process, potentially a large fraction of lipoidal extracts, will require more attention.

Perhaps a more important consideration of reported variations in lipid content, even within a specific species, is the physiological responses in lipid metabolism due to culture conditions including temperature, salinity, growth phase, nutrient deprivation, and the diurnal light cycle, all of which have a strong influence on lipid content (Roessler 1988, 1990). Unfortunately, biomass productivity is often inversely correlated with overall lipid productivity. High lipid and carotenoid content is usually produced under stress conditions, especially nutrient limitation which prevents cell growth and division resulting in excess photosynthate shunted toward triglyceride accumulation (Griffiths and Harrison 2009; Illman et al. 2000; Jakobsen et al. 2008; Lv et al. 2010; Rodolfi et al. 2009).

Lipid productivity is the product of lipid content and productivity. A survey of the literature on growth rates and lipid content under nutrient-replete and nutrient-deficient conditions showed a stronger correlation between biomass and lipid productivity rather than simply lipid content (Griffiths and Harrison 2009). In continuous ponding operations, selection of fast-growing strains increases yield and decreases the cost of harvesting and extraction (Borowitzka 1997) and reduces competition by invading strains. High productivity is also advantageous in a two-stage process, as described above, with the first stage designed to optimize biomass production and nutrient removal from wastewater followed by a second phase to induce hyper-lipid production.

6.5 Potential Microalgae for Biodiesel Production

6.5.1 Cyanobacteria

Divisions of prokaryotic algae, the Cyanophyta and Prochlorophyta, include the cyanobacteria (blue-green algae) and the Prochlorophyta, which use a unique form of chlorophyll, divinyl-chlorophyll, lack red and blue phycobilin pigments, and have stacked thylakoids. These organisms are oxygen-evolving photosynthetic bacteria and have cell wall structures similar to gram-negative bacteria which include a cell membrane, a layer of peptidoglycan, and an outer membrane. Of all the microorganisms described in this chapter, the cyanobacteria pose the least difficulty in terms of cell lysis for lipid extraction. Although cyanobacteria including *Spirulina* and *Nostoc* have been used for food for centuries, they tend to have high protein concentrations but low levels of lipids (Becker 1994). However, the cyanobacteria may provide some significant advantages in biofuel production including bacterial cell walls that are easily lysed for lipid extraction and the ability to grow in extreme conditions allowing species control in outdoor ponds, and many have a filamentous morphology that enables facile harvest.

The genetics of cyanobacteria are now well developed (Park et al. 2013), and many strains are easily transformed. Another argument for the use of cyanobacteria is the production of compounds important to the chemical and nutraceutical industries whose current market value is far greater than generic algal oil. For example, *Arthrospira platensis* produces significant quantities of phycocyanin and gama linolenic acid (C18:3, ω6, GLA) (Colla et al. 2004).

Various groups have engineered cyanobacteria to overexpress either native alkane biosynthetic genes or regulatory genes (Rosgaard et al. 2012; Wang et al. 2013) and have determined compatibility issues between the host and high levels of alkanes synthesized through engineered pathways.

6.5.2 Eukaryotic Microalgae

The morphology, pigments, metabolic capabilities, and cell wall structure of the eukaryotic microalgae are quite diverse because they represent a multitude of phylogenetically distinct groups of organisms. Recent molecular evidence suggests that the “algae” fit into very different evolutionary lineages including those related to plants, fungi, or animals (Lucentinii 2005) via one or more serial endosymbiotic events (Moestrup 2001a, b). Three classes of primary interest for biofuels production include the golden-brown algae (Chrysophycea), prymnesiophytes (Prymnesiophyceae), and the eustigmatophytes (Eustigmatophyceae) (Sheehan et al. 1987) and for bioremediation, the Chlorophycea.

6.5.2.1 Chlorophyta

The green algae are a large group estimated to have over 13,000 species, (Guiry 2012) from which the higher plants emerged. Green algae, in common with land plants, have chloroplasts that contain chlorophyll a and b, as well as the accessory pigments β-carotene and xanthophylls, and store carbon as starch and lipids. Their distribution is ubiquitous with marine, freshwater, and terrestrial species and groups adapted to extremes including deserts, arctic zones, hypersaline waters, and deep-sea thermal vents (Lewis and Lewis 2005; De Wever et al. 2009). Several strains are known to have rapid growth rates and high lipid content and have been a focus of biofuel research. *Chorella* spp., used since the 1950s as a food supplement, have short doubling times and can be cultured to produce between 30 and 55 % lipid content (Becker 1994; Miaoa and Wu 2006). Species of *Scenedesmus*, *Ettlia*, *Nannochloris*, and *Monoraphidium* grow rapidly (doubling times from 7 to 12 h under nutrient-replete conditions) and have lipid contents ranging from 30 to over 60 % (Griffiths and Harrison 2009). A number of genera in the order of Chlorococcales, including *Actinastrum*, *Scenedesmus*, *Chlorella*, *Closerium,* and *Golenkinia*, tend to dominate algae communities in eutrophic waters (Rawson 1956) and algae wastewater ponds (Martinez et al. 2000; Benemann and Oswald 1996). Many are either heterotrophic or mixotrophic.

6.5.2.2 Haptophyta

The haptophytes, predominately marine phytoplankton, are recognized as a division divided into two classes, Pavlovophyceae and Prymnesiophyceae (Cavalier-Smith 2007). The chloroplast pigments are similar to those of the heterokonts, and in both divisions, chloroplasts are derived from red algal symbionts (Anderson 2004). Several members of the class Prymnesiophyceae including the *Prymnesium* (Becker 1994), *Isochrysis* spp. (Chisti 2007), *and the Coccolithophores* are oleaginous algae. *Pavlova* and *Isochrysis* spp. are widely used in the aquaculture industry because of their favorable lipid content (Walker et al. 2005). While the Haptophyta and Heterokonts are predominantly marine organisms, there are a number of species in both groups that are found in freshwater systems. In addition, certain types of wastewater such as the effluent from food processing plants have a much higher salinity than domestic wastewater.

6.5.2.3 Heterokonts

Heterokont algae are a monophyletic group with chloroplasts containing chlorophyll a and c and the accessory pigment fucoxanthin which gives the group a golden-brown color. Marine, freshwater, and terrestrial heterokonts are known and

range in the form of giant kelp (brown seaweeds), diatoms, Eustigmatophytes, and Chrysophytes. The later three groups have species that are high-lipid producers. *Nannochloropsis* (Eustigmatophytes) are primarily known from marine environments but also occur in fresh and brackish water (Fawley and Fawley 2007). All of the species are small (diameter of about 2–3 μm) non-motile spheres with no distinct morphological features, and many are mixotrophic (Das et al. 2011). *Nannochloropsis* strains contain 30 % lipids under nutrient-replete growth conditions and over 60 % lipid content after nitrogen deprivation (Rodolfi et al. 2009; Huerlimann and de Nys 2010). The Chrysophyceae and Xanthophyceae are predominately freshwater organisms, although a substantial number of xanthophytes are terrestrial.

Diatoms, widely studied as a feedstock for biodiesel production, are important members of planktonic and attached biofilm communities in both freshwater and marine environments (Round et al. 1990). Over 100,000 species are known which are estimated to contribute up to 45 % of total primary productivity in open oceans (Yool and Tyrrell 2003). Diatoms are distinguished by a unique silica cell wall composed of two separate valves and yellowish brown chloroplasts, surrounded by four membranes and containing the carotenoid pigment fucoxanthin as a photosynthetic accessory pigment. Other xanthophylls are present as well as β-carotene and chlorophylls a and b. The main storage compounds are lipids (TAGs) and a β (1 → 3-linked carbohydrate chrysolaminarin (Horner 2002). Several genera include species known for high lipid content including *Nitzschia*, *Navicula*, *Amphiprora*, *Amphora*, and *Phaedodactylum* (Griffiths and Harrison 2009). Diatoms lack flagella (except in sperm of some species), and their dense cell walls cause them to sink in the water column. Planktonic forms rely on turbulence to keep them in the photic zone, and many species regulate their buoyancy using intercellular lipids. Most diatoms are phototrophic, but a few groups are either obligate heterotrophs or are diurnally heterotrophic in the dark when supplied with a carbon source.

Silicon metabolism has relevance in the culture of diatoms as a feed source for biodiesel production. The silicon-laden cell wall is synthesized intercellularly by polymerizing silicic acid monomers (taken up by transporters from the media) in a specialized membranous compartment (Pickett-Heaps et al. 1990). Because silicate is a relatively expensive and an essential nutrient for diatoms, production costs can be raised significantly. However, silicon limitation prevents cell division and triggers rapid lipid biosynthesis which may allow for methods to control oil production in a two-stage production process. Nutrient limitation, including N and P that promote lipid hyper-accumulation in a variety of microalgae, has also been shown to promote lipid accumulation in diatoms (McGinnis et al. 1997). However, several studies suggest that Si deficiency stimulates lipid biosynthesis more rapidly and can result in up to 70 % (dry weight) lipid content (Adams et al. 2013).

6.6 Ecology of Microalgae and Preferred Strains for Use in Wastewater Treatment

The extensive work carried out by the DOE/NREL sponsored Aquatic Species Program to explore large-scale algae production for biodiesel provided a number of recommendations for further work. Key among these was to isolate native, local strains for mass cultivation to ensure adaptation to local seasonal conditions. Currently, there are widespread efforts in the field to screen large numbers of algal strains for lipid production from widely differing natural habitats. Most of these are unlikely to thrive in the specific conditions of algae production ponds (water quality, nutrient sources, mixing, large seasonal light and temperature changes, grazing zooplankton, etc.), as discovered during the Aquatic Species Program. It is well known from limnology studies (McCormick and Cairns 1994) and municipal ponding operations (Benemann et al. 1977; Murry and Benemann 1980) that certain algae strains dominate the population at different seasons and in response to nutrient and other environmental parameters. An ecologically sound approach is to identify seasonally dominant strains in outdoor ponds at a specific site and with regard to local environmental factors and the desired objectives (i.e., high-lipid strains for biodiesel, bioremediation, value-added co-products, etc.).

Algal strains that have been identified for their high oil content and suitability for mass production include the following: *Botryococcus braunii*, *Dunaliella tertiolecta*, *Euglena gracilis*, *Isochrysis albana*, *Nannochloris* sp., *Neochloris oleoabundans*, *Phaeodactylum tricornutum*, *Chrysotila carterae*, *Prymnesium parvum*, *Scenedesmus dimorphus*, *Tetraselmis chui, and Tetraselmis suecica* (Oilgae 2009 and Chap. 1 of this book). Unfortunately, many of these species are adapted to waters with far higher salinity and grow very slowly in freshwater, while others tolerate wide ranges in salinity. Genera commonly found in wastewater ponds include the following: *Chlorella* (Ponnuswamy et al. 2013), *Chlorococcum*, *Microcystis* and *Phormidium* (Mahapatra and Ramachandra 2013) *Chlorella*, *Euglena* and *Selenastrum* (Ojala et al. 2013) and *Scenedesmus*, *Ankistrodesmus*, *Micractinium*, *Oocystis*, *Phytoconis*, *Chlamydomonas*, *Oscilitoria,* and *Synechocystis* (De Pauw and Van Vaerenbergh 1981). In his work on dairy wastewater treatment, Woertz et al. (2009) worked with batch cultures that were dominated by *Scenedesmus*, *Micractinium*, *Chlorella*, and *Actinastrum*. Because these strains thrive in wastewater operations, they are prime candidates for bioremediation–biofuel production.

Three final points of consideration for selecting the ideal algal strains would be the ease of harvesting, the ease of cell lysis, and/or lipid extraction and the ability to grow under autotrophic and heterotrophic conditions. While the cyanobacterium *Microcystis* proliferates in wastewater ponds, its small size (2–5 microns) would make it difficult to harvest. Various strains of *Chlorella* have been studied for their production of oil, but one of the main impediments in dealing with this genus is its

resistance to cell lysis (Gerken et al. 2013; Zheng et al. 2011). Given the considerations of algae's ability to grow in wastewater, be relatively easy to harvest and lyse, and contain a significant amount of oil, the candidate genera would include *Euglenia*, *Scenedesmus*, *Selenastrum*, *Chlamydomonas*, and *Actinastrum*. Species of the genus *Chlorella* can be added to this list with the understanding that future technologies will overcome the challenge of breaking open the cell walls. A consortium of two to five of these types of algae along with epiphytic wastewater bacteria could be the essential components of an algae-based wastewater treatment system that yields a high-quality effluent and a significant amount of triglycerides that can be converted into biodiesel or chemicals of industrial significance.

6.7 Methods of Extraction of Algae Oils: Supercritical Fluid Extraction of Lipids from Algae for Use in Biodiesel Production

One of the areas of microalgae biofuels that must be optimized or re-engineered to make production cost-effective is oil extraction. It is estimated that up to 60 % of the cost of algae biodeisel production involves solvent emulsification and recovery (Molina-Grima et al. 2003). The best studied approach to biodiesel production, first developed for oil seed plants, is the extraction and TAGs or triglycerides into FAMEs. The spent biomass can be used for a variety of applications including biogas, feed, and fertilizers. In the past ten years, a number of alternative forms of catalysis have been developed that use lipases (Ranganathan et al. 2008) or make use of solid bases (hydroxyl groups added to mineral crystals) or catalysts that form biodiesel under high pressure. However, the expense of harvesting, drying, and breaking cell walls remains problematic. In addition, organic solvents for oil extraction are expensive and generate hazardous wastes that must be disposed of at further cost (Williams and Laurens 2010).

HTL has recently been adapted to circumvent many of these problems inherent in lipid extraction processes. HTL is a thermal process that heats a wet slurry of intact algae to 250–350 °C at 1500–3000 psi, converting the biomass to several products including an oil portion ranging from 29 to 52 % yield (See Frank et al. 2013 for a recent review). While TLC produces more oil from algae than lipid extraction, there are several issues with the quality of the oil produced based on the inclusion of other cell components (proteins, nucleic acids, carbohydrates) in the thermal process. A life cycle analysis of TLC of several algal strains (Frank et al. 2012) indicated that the lipid fraction had high levels of N (Williams and Laurens 2010), leaving questions on combustion emissions for fuel use.

Another recent advancement in the field, supercritical fluid extraction (SFE) of algal biomass, may be an efficient means to extract oils that avoids the use of toxic

organic solvents, eliminates the need for the energy-intensive drying of biomass, and avoids high N content in the oil. In addition, SFE allows for the co-extraction of high-value chemicals and leaves a residual biomass that is solvent free and could be marketed as a livestock feed supplement or fertilizer. A carbon dioxide supercritical fluid extraction (CO_2-SFE) apparatus for oil extraction using water as a co-solvent would avoid the high cost of drying algae while extracting triglycerides and the co-extraction of valuable nutraceuticals using wet algal biomass.

SFE technology is well developed for processes such as decaffeination and dry-cleaning and is now widely accepted for extraction, purification, and fractionation operations in many industries, especially in the nutraceutical and other "green" industries. SFE is far more efficient than traditional solvent separation methods and is selective, providing high purity of specific products. Additionally, there are no organic solvent residues in the extract or spent biomass. Extraction is efficient at modest operating temperatures, for example, at less than 50 °C, thus ensuring product stability (Herrero et al. 2010). CO_2-SFE has been shown to be an efficient solvent for the extraction of a valuable nutraceutical docosahexaenoic acid (DHA) (Couto et al. 2010), for which there is a large growing market.

Based on the literature (Patil and Gude 2011; Choi et al. 1987; Couto et al. 2010; Herrero et al. 2010), there are reasonable starting parameters of temperature, chamber, and release pressures for maximum lipid extraction using dry algal biomass. The development of CO_2-SFE lipid extraction from wet algae cultures has been explored but is still in its infancy. Adjustments of parameters must be made to use water and methanol co-solvents that alter the overall behavior of the extraction process. Slight variation in temperature will significantly alter the density of the solvent, and therefore the efficiency of the extraction of specific lipoid compounds. An increase in temperature also reduces yield of specific fractions due to product degradation (Patil and Gude 2011; Choi et al. 1987). Cell disruption is a major factor in lipid yields independent of extraction process. Using SFE, cell disruption of wet algae is based on chamber and release pressures, water content, and temperature treatment to determine the need for cell lysis prior to CO_2-SFE. Halim et al. (2011) have demonstrated the efficiency of SFE extraction of triglyceride fractions from intact wet algal biomass. The extraction efficiency for total lipid extraction including valuable co-products, specifically the marketable nutraceuticals, DHA, and luteins, makes the cost–benefit analysis of the whole process favorable. The fractionated products of CO_2-SFE, ranging in size from free fatty acids, DHA, triglycerides (the feedstock for biodiesel), and carotenoid compounds such as lutein may be fractionated further using liquid chromatography and the triglycerides transesterified to FAMEs. The parameters for extraction of specific lipids from algae and developing methods that balance cost with production of each specific lipid product that can be scaled up for industrial use is of paramount importance. The CO_2 used for extraction can also be recycled to support algal photosynthetic growth. The process outlined here has the potential to be entirely renewable and recyclable as well as cost competitive with liquid fuels.

6.8 Current Technology Limitations: Algae as a Feedstock for Biofuels and Industrial Chemicals

There are a number of technical bottlenecks that need to be addressed. Some of the basic questions yet unanswered include the following:

1. In a full-scale field operation, what are the ideal strains of algae that will yield both a high-quality effluent and a high-quality biofuel?
2. What are the most efficient methods for separating and concentrating the algae? The transition from 300 mg/L in the pond to a slurry that is 20 % solids may take two or three steps.
3. What are the most efficient and cost-effective methods for breaking open algae cells for the production of a green crude or the separation of the lipid, aqueous, and solid fractions of the lysed cells?
4. What are the most efficient and cost-effective methods for converting the green crude or purified lipid into a commercially reliable biofuel? In the past few years, several new methods have been developed on a bench and demonstration scale, but no one process has been made the leap to be an industry standard for full-scale systems.
5. The companies that favor large photobioreactor systems have yet to show how their systems could be implemented on a grand scale. There is roughly an order of magnitude difference in capital and labor costs between photobioreactors and open pond systems. For algae-based biofuel system to be commercially viable, the output would need to be on the order of thousands to millions of liters of biofuel per day. While practitioners from the fields of biotechnology and biochemical engineering have very reliable data from their bench and demonstration-scale bioreactors, the only two fields of engineering that have a long-standing history of working on systems that reliably process liquids on a grand scale are in the chemical/petroleum industry and wastewater treatment.
6. The photobioreactors can provide the initial step of a high-quality starter culture into an open pond system, but when one takes into account the energy needs and capital cost of mega-scale bioreactors, the economic feasibility of hundreds of hectares of photobioreactors fades rapidly.
7. A number of companies are trying to outdo their competition based on the hopes of genetically altered strains of algae. Considering the fact that it takes the approval of several different local and national regulatory agencies just to restore a disturbed habitat with native plant species, the likelihood of a company being allowed to generate 50 tons/day of genetically altered algae in open ponds could face some very tough opposition that would include regulatory agencies and well-organized citizen groups. In addition, the fate of genetically introduced microbes into the environment can be precarious; for example, consider the failure of engineered *Rhizobium* strains introduced into soybean fields to inoculate seedlings but were outcompeted by native strains (Kent and Triplett 2002)

8. What will make or break this industry is the ability to produce biofuels on a mega-scale basis with a high degree of reliability. A serious economic analysis is needed for each step (i.e, culturing, harvesting, dewatering, lysing, and biofuel processing) in the development of algae-based fuels.
9. Most large wastewater treatment plants that process 200–1000 ML/day are located adjacent to dense urban landscapes with little available room for large-scale algae ponds. The ideal candidates for the system proposed in this chapter would be rural wastewater treatment plants that process 2–40 ML/day. Most often, these plants are located at a good distance from populated areas, and there is ample land that could be developed into algae-culturing ponds. In parts of the world where there is a cold or monsoon season, the plant can revert back to its original treatment process and use the ponds for short-term storage of wastewater.

6.9 Conclusion

The ability to culture and harvest algae has improved dramatically over the past five decades. There are numerous treatment options that can be used to make the transition from concept to demonstration to full-scale implementation of algae biofuel programs. This will require the ability to adapt preexisting technologies from several disciplines. Many of the answers are already out there but have yet to put in the proper sequence or combination. There is no one technical solution to make this process commercially viable. As demonstrated in this chapter, it will require contributions from several disciplines to go beyond their technical comfort zones. While this is an emerging field with great promise, it will be built on the fundamental principles of engineering and science.

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Chapter 7
CO_2 Environmental Bioremediation by Microalgae

Mohammad J. Raeesossadati, Hossein Ahmadzadeh, Mark P. McHenry and Navid R. Moheimani

Abstract Various microalgae species have shown a differential ability to bioremediate atmospheric CO_2. This chapter reports biomass concentration, biomass productivity, and CO_2 fixation rates of several microalgae and cyanobacteria species under different CO_2 concentrations and culture conditions. Research indicates that microalgal species of *Scenedesmuss obliquss*, *Duniella tertiolecta*, *Chlorella vulgaris*, *Phormidium* sp., *Amicroscopica negeli*, and *Chlorococcum littorale* are able to bioremediate CO_2 more effectively than other species. Furthermore, coccolithophorid microalgae such as *Chrysotila carterae* were also found to effectively bioremediate CO_2 into organic biomass and generate inorganic $CaCO_3$ as additional means of removing atmospheric CO_2. Important factors to increase the rate of CO_2 bioremediation such as initial cell concentration, input CO_2 concentration, and aeration rate are reviewed and discussed.

7.1 Introduction

In 2012, 34.5 billion tons of CO_2 were emitted through human activities, and in 2013, an unprecedented modern age atmospheric CO_2 concentration of more than 400 ppm was measured (Olivier et al. 2013). Carbon capture and sequestration (CCS) offers an effective solution to mitigate environmental impacts and can be

M.J. Raeesossadati · H. Ahmadzadeh (✉)
Department of Chemistry, Ferdowsi University of Mashhad, Mashhad
1436-91779, Iran
e-mail: h.ahmadzadeh@um.ac.ir

M.P. McHenry
School of Engineering and Information Technology, Murdoch University,
Murdoch, WA 6150, Australia

N.R. Moheimani
Algae R&D Centre, School of Veterinary and Life Sciences, Murdoch University,
Murdoch, WA 6150, Australia

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considered a long-term remediation policy (Yang et al. 2008). There are a number of CO_2 remediation methods that can be classified in three main categories: capture, separation, and fixation.

Power plant CO_2 capture can be divided into several scenarios, such as post-combustion process, pre-combustion, and oxy-combustion (Fig. 7.1) (Figueroa et al. 2008; Yang et al. 2008), and being stored in aquifers, porous geologic depleted oil and reservoirs, and deep ocean floors. In post-combustion processes, CO_2 is separated from other flue gas constituents. In pre-combustion capture, carbon is removed from the fuel before combustion, and in oxy-combustion, the fuel is burned in an oxygen stream that contains little or no nitrogen (Figueroa et al. 2008). Furthermore, other chemical approaches, such as amine absorption, ammonium absorption, molecular sieve adsorbent, and adsorption by activated carbon (Bezerra et al. 2011; Thote et al. 2010), are amenable for CO_2 separation (Yang et al. 2008). The disadvantages of these methods are the use of large amounts of absorbents and solvents which makes the processes generally expensive (Figueroa et al. 2008), in addition to the processes relatively undeveloped and the possible wider impacts of the use of these chemicals which are not well understood (Wang et al. 2008).

In contrast to traditional methods of carbon capture, biological remediation processes via photosynthesis are major contributors to atmospheric CO_2 remediation (approximately 12 billion tons per year) (Bilanovic et al. 2009), with photosynthetic organisms in the oceans responsible for removing over 40 % of annual

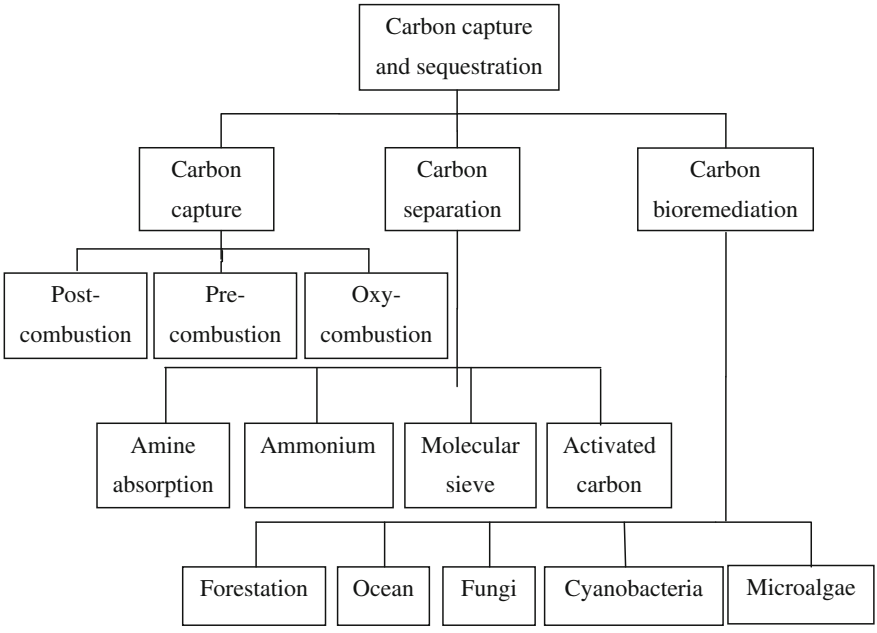

Fig. 7.1 Different methods of carbon capture and sequestration (CCS)

CO_2 emissions (Pires et al. 2012). Bioremediation of CO_2 can be accomplished through forestation, ocean, fungi, cyanobacteria, and algae (Skjånes et al. 2007) (Fig. 7.1). It is estimated that 1.4 ± 0.7 Gt carbon is captured by terrestrial systems from atmosphere via photosynthesis (Yang et al. 2008). The oceans store more CO_2 than terrestrial vegetation (Israelsson et al. 2010), with around 38,000 Gt carbon, and about 1.7 ± 0.5 Gt taken up annually from the atmosphere (Yang et al. 2008). The production of phytoplankton at 50–100 Gt carbon annually is much higher than that of terrestrial vegetation. While part of the carbon is released back into the atmosphere by respiration, a large fraction would descend into the deeper ocean in the form of particulate organic matter either by the death of phytoplankton or after grazing. This sequestration process could be enhanced by ocean fertilization that refers to the practice of increasing limiting nutrients to stimulate the production of phytoplankton (Yang et al. 2008).

Organisms that can convert CO_2 into organic molecules are called autotrophs and include plants, algae, some bacteria, and some archaea. Microalgae are the most promising bioremediation alternative for many sources of CO_2 emissions. They have the capability of removing 10–50 times more CO_2 than terrestrial plants, primarily due to more chlorophyll per unit area (Raeesossadati et al. 2014). Microalgae can also utilize CO_2 from different sources, such as atmospheric CO_2, industrial exhaust gases, or CO_2 in the form of soluble carbonates (e.g., $NaHCO_3$ and Na_2CO_3) (Kumar et al. 2010). Open ponds and closed photobioreactors (PBRs) are commonly used for culturing microalgae to both consume CO_2 and produce useful products (Kumar et al. 2010; Pires et al. 2012). Many microalgal strains can tolerate extreme environments and are able to grow with high production rates in large open ponds (e.g., *Dunaliella*, *Spirulina*, and *Chlorella* sp.), whereas closed PBRs allow better control of cultivation and reduce contamination issues (Eberly and Ely 2012; Pires et al. 2012). Despite many advantages of closed PBRs, large-scale open ponds are usually used for commercial microalgae production due to lower investment and production costs per unit of output (Lee 2001; Posten 2009). The ability of microalgae to bioremediate atmospheric CO_2 is commonly thought of as dependent on freshwater and land availability, and the associated concerns of negatively influencing food security (Borines et al. 2011a, b; Clarens et al. 2010; McHenry 2012; Moheimani et al. 2013). However, marine and hypersaline microalgae (eukaryotic or prokaryotic) can fix CO_2 with almost no need for freshwater (McHenry 2010, 2013; Moheimani et al. 2012a; Sing et al. 2013). As such, microalgae are now one of the most promising alternatives to bioremediate many sources of CO_2 emissions (de Godos et al. 2010). The authors have selected the term "bioremediation" as we are discussing temporary fixation of CO_2 in the microalgal biomass. Furthermore, biomass productivity plays a significant role in any microalgae production system, and the production of many target constituents is dependent on primary biomass productivity (including the production of lipids, hydrocarbons, polysaccharides, and other energy storage compounds). The more biomass productivity in any microalgal system is the results of more photosynthetic fixation of CO_2. Therefore, to produce industry-scale microalgae biomass, there is a need for cheap carbon source and nutrients.

7.2 Carbon Dioxide Bioremediation by Microalgae

Carbon constitutes half of the weight of the biomass, and it is usually supplied as CO_2 (González-López et al. 2012). CO_2 is normally added to the microalgae cultures as either air (generally no more than 1.0 % CO_2) or flue gas (typically 10–20 % CO_2) (Cheng et al. 2006). Environmental CO_2 bioremediation using microalgae has a great potential, and many parameters have been investigated to optimize this process. Table 7.1 shows microalgae production parameters, including initial cell concentration, input CO_2 concentration, aeration rates, temperature, light intensity, and PBR technologies, all normalized for CO_2 fixation rates as g L^{-1} d^{-1}. Using *Tetraselmis* sp. and *Nannochloropsis oculata* to fix CO_2 from the air, it was reported an improved CO_2 fixation rate for *Tetraselmis* sp. (0.0241 g L^{-1} d^{-1}) relative to *N. oculata* (0.017 g L^{-1} d^{-1}) (Chik and Yahya 2012). Similarly, in another work, the CO_2 sequestration rate for *Dunaliella tertiolecta* (0.12 g L^{-1} d^{-1}) and *Chlorella vulgaris* (0.09 g L^{-1} d^{-1}) was studied and it was demonstrated that *D. tertiolecta* fixed CO_2 at a higher rate and greater efficiency than *C. vulgaris* (Hulatt and Thomas 2011). Furthermore, when 0.03 % CO_2 was used for *Chlorella pyrenoidosa SJTU-2* and *Scenedesmus obliquus SJTU*, CO_2 fixation rates of 0.134 and 0.150 g L^{-1} d^{-1} were obtained, respectively, which showed higher fixation rates than those reported in the literature (Hulatt and Thomas 2011). The results presented in Table 7.1 show that a wide range of CO_2 biofixation rates are achieved (from 0.0241 to 0.150 g L^{-1} d^{-1}) from a number of different microalgae strains (*Tetraselmis* sp., *N. oculata*, *C. vulgaris*, *D. tertiolecta*, *C. pyrenoidosa SJTU-2*, *S. obliquus SJTU*, and *Synechoccus* sp.), using different PBRs (aerated flask, bubble column, and light diffusing optical fiber). The major differences in these publications are the use of different algal species and the reported higher fixation rates that could be due to the use of two genetically engineered strains of microalgae (Tang et al. 2011). Determining the major parameters for increased CO_2 fixation rates requires a more in depth study. For example, research on CO_2 mitigation efficiency under 0.03, 0.55, and 1.10 % environmental CO_2 concentrations for cyanobacterium *Synechococcus* sp. using light diffusing optical fibers (LDOF) showed 100, 33, and 15.4 % CO_2 fixation efficiency, respectively (Takano et al. 1992). As the CO_2 fixation efficiency decreased with increased CO_2, the least percentage of input CO_2 (0.03 %) attained the highest CO_2 fixation efficiency (100 %), as supplying less CO_2 allow microalgae cells to consume CO_2 more efficiently. Yet, microalgal cell growth highly depends on supplied CO_2 concentrations, and CO_2 concentrations in air (0.03–1 %) do not commonly yield sufficient microalgal growth in PBRs. As industrial flue gases consist of higher CO_2 concentrations suitable for microalgae production (Wang et al. 2008), using flue gas as feed for microalgae, not only can increase the productivity and cell growth of microalgae, but also remove CO_2 from atmosphere more efficiently (Chen et al. 2012; Chiu et al. 2011). Furthermore, calcified microalgae (i.e., coccolithophorid microalgae) are of additional interests for CO_2 bioremediation as they are able to form $CaCO_3$ together with

Table 7.1 Environmental CO_2 sequestration rates by different microalgae species

PBR		Microalgae	T (°C)	Supplied CO_2 %	Gas flow rate $\frac{L}{\min}$	Daily growth rate (g)	Cell density $(\frac{g}{L})$	Biomass concentration $(\frac{g}{L})$	Biomass productivity $(\frac{g}{L.d})$	Light intensity (Lux)	CO_2 fixation		Ref.
Type	Vol (L)										Rate $(\frac{g}{L.d})$	Efficiency (%)	
Flask	0.5	(1)	26	Air	–	–	–	–	–	600	0.0241	–	Chik and Yahya (2012)
Flask	2	(2)	26	Air	–	–	–	–	–	600	0.0177	–	Chik and Yahya (2012)
Bubble column	1.4	(3)	26	0.04	$0.005\frac{m}{s}$	–	–	0.58	0.1	3400	0.09	35.5	Hulatt and Thomas (2011)
Bubble column	1.4	(4)	26	0.04	$0.005\frac{m}{s}$	–	–	0.72	0.07	3400	0.12	47.9	Hulatt and Thomas (2011)
–	–	(5)	25	0.03	–	0.688	–	0.87	0.065	–	0.134	–	Tang et al. (2011)
–	–	(6)	25	0.03	–	0.507	–	1.05	0.083	–	0.150	–	Tang et al. (2011)
LDOF[a]	2.5	(7)	–	0.03	2	–	0.39	0.56	–	1250	–	100	Takano et al. (1992)
LDOF[a]	2.5	(7)	–	0.55	0.8	–	0.33	0.76	–	1250	–	33	Takano et al. (1992)
LDOF[a]	2.5	(7)	–	1.10	0.8	–	0.45	0.85	–	1250	–	15.4	Takano et al. (1992)

[a]Light diffusing optical fiber. Rates of CO_2 fixation are normalized to g L^{-1} d^{-1}. *Tetraselmis* sp. (1), *Nannochloropsis oculata* (2), *Chlorella vulgaris* (3), *Dunaliella tertiolecta* (4), *Chlorella pyrenoidosa* SJTU-2 (5), *Scenedesmusobliquus* SJTU (6), and *Synechoccus* sp. (7)

photosynthetic carbon fixation (Moheimani et al. 2012b). In this case, carbon is removed by photosynthesis as a part of the carbon cycle, while the $CaCO_3$ can be discharged (precipitated) out of the carbon cycle.

7.3 Biomass Concentration and Productivity, and CO_2 Removals

Various microalgae species accumulate biomass under different CO_2 concentrations. Two experiments on *S. obliquus* supplied with 12 % CO_2 resulted in 1.14 and 1.81 g L^{-1} as a maximum dry weight biomass yield, whereas a higher biomass concentration (3.5 g L^{-1}) was obtained with *Spirulina* sp. when using a higher light intensity at the same 12 % CO_2 input gas (Table 7.2) (De Morais and Costa 2007a, b). As shown in Table 7.2, the highest biomass concentration of *Chlorococcum littorale* in three runs under 20 % CO_2 was 14.4 g L^{-1} in a small PBR (Kurano et al. 1995). There has been several research publications evaluating the biomass productivity of different strains of microalgae under different CO_2 concentrations (De Morais and Costa 2007a; Radmann et al. 2011; Yoo et al. 2010). Table 7.3 shows the biomass productivity of 0.077 g L^{-1} d^{-1} for *Botryococcus braunii* under 5.5 % CO_2 (Yoo et al. 2010). A biomass productivity of 0.09 g L^{-1} d^{-1} for *C. vulgaris* was achieved (under 12 % CO_2) (Radmann et al. 2011). Similarly, *Spirulina* sp. was used under 6 % CO_2 and biomass productivity of 0.18 g L^{-1} d^{-1}, significantly higher than most other reports, was obtained (De Morais and Costa 2007a). This higher biomass productivity might be due to lower CO_2 concentration (6 %) used for *Spirulina* sp. in comparison with 12 and 20 % CO_2 supplied for species in other reports. Similarly, microalgal species have shown differing capabilities for CO_2 fixation at different concentrations of CO_2 (10–20 % is a common range of CO_2 for microalgae production systems with enhanced CO_2 delivery (Ho et al. 2011)). The CO_2 fixation rate of 0.17 g L^{-1} d^{-1} was obtained (Eberly and Ely 2012) for *Thermosynechococcus elongates* at 20 % CO_2. Similar input CO_2 concentration (20 %) (Tang et al. 2011) was supplied for *S. obliquus SJTU-3* and *C. pyrenoidosa SJTU-2*, fixing 0.244 and 0.223 g L^{-1} d^{-1} CO_2, respectively. However, the researchers also cultured the same strains under 10 % CO_2 and obtained higher fixation rates, such as 0.288 g L^{-1} d^{-1} for *S. obliquus SJTU-3* and 0.260 for *C. pyrenoidosa SJTU-2*, when providing high levels of CO_2 into culture mediums that leads to acidification and lowering fixation rates of *S. obliquus SJTU-3* and *C. pyrenoidosa SJTU-2* at 20 % CO_2. Research (Kurano et al. 1995) with *C. littorale* under 20 % CO_2 assessed removing CO_2 by 4, 0.65, and 0.85 g L^{-1} d^{-1} for selected culture volumes of 10 mL, 4 L and 20 L, respectively. The research indicated that *C. littorale* may achieve a better CO_2 fixation rate than *T. elongates*, *S. obliquus SJTU-3*, and *C. pyrenoidosa SJTU-2*, although the differences were not significant. The higher CO_2 fixation rate may be either due to engineering issues in scaling up or due to higher light intensity used for *C. littorale* (15650 lux) compared to other

Table 7.2 Biomass concentration produced by microalgae under different CO_2 concentrations

PBR		Microalgae	T (°C)	Supplied CO_2 %	Flow gas rate $\frac{L}{\text{min}}$	Growth rate (d^{-1})	Cell density ($\frac{g}{L}$)	Biomass concentration ($\frac{g}{L}$)	Light intensity (Lux)	Ref.
Type	Vol (L)									
–	–	(8)	30	12	–	–	–	1.14	2500	De Morais and Costa (2007b)
CPBR	2	(8)	30	12	–	0.22	0.15	1.81	3200	De Morais and Costa (2007a)
CPBR	2	(9)	30	12	–	0.29	0.15	3.5	3200	De Morais and Costa (2007a)
Vessel	20	(10)	25	20	1	1.80	–	4.3	15,625	Kurano et al. (1995)
Vessel	4	(10)	25	20	1	1.87	–	4.9	15,625	Kurano et al. (1995)
Vessel	0.01	(10)	25	20	1	1.87	–	14.4	15,625	Kurano et al. (1995)

Scenedesmus obliquus (8), *Spirulina* sp. (9), *and Chlorococcum littorale* (10)

Table 7.3 Biomass productivity of microalgae under different CO_2 concentrations

PBR		Microalgae	T (°C)	Supplied CO_2 %	Flue gas rate $\frac{L}{min}$	Growth rate (d^{-1})	Cell density ($\frac{g}{L}$)	Biomass productivity ($\frac{g}{L.d}$)	Light intensity (Lux)	Res.
Type	Vol(L)									
–	–	(11)	25	5.5	0.3 vvm	–	–	0.077	9375	Yoo et al. (2010b)
Tubular	2	(12)	30	12	0.540	–	0.15	0.09	3200	Radmann et al. (2011)
Tubular	2	(9)	30	12	0.540	–	0.15	0.08	3200	Radmann et al. (2011)
CPBR	2	(9)	30	6	–	0.29	0.15	0.18	3200	De Morais and Costa (2007a)
		(13)	30	12	–	0.267	–	0.087	2500	De Morais and Costa (2007b)
Tubular	2	(3)	30	12	0.540	–	0.15	0.09	3200	Radmann et al. (2011)
–	–	(3)	25	10	0.3 vvm	–	–	0.1	9375	Yoo et al. (2010b)
Tubular	2	(8)	30	12	0.540	–	0.15	0.06	3200	Radmann et al. (2011)
CPBR	2	(8)	30	10	–	0.22	0.15	0.14	3200	De Morais and Costa (2007a)
–	–	(8)	25	10	0.3 vvm	–	–	0.22	9375	Yoo et al. (2010b)

Botryococcusbraunin (11), *Synechococcusnidulams* (12), *Spirulinasp* (9), *Chlorella kessleri* (13), *Chlorella vulgaris* (3), and *Scenedesmusobliquuss* (8)

Table 7.4 Carbon dioxide removal rates by different microalgae species

PBR		Microalgae	pH	T (°C)	Supplied CO_2 %	Flue gas rate $\frac{L}{\min}$	Daily growth rate (g)	Cell density $(\frac{g}{L})$	Biomass concentration $(\frac{g}{L})$	Biomass productivity $(\frac{g}{L.d})$	Light intensity	CO_2 fixation		Ref.
Type	Vol (L)										(Lux)	Rate $(\frac{g}{L.d})$	Efficiency (%)	
–	–	(14)	7.8	50	20	0.020	0.13	–	–	0.09	(Lux) 11250	0.17	1.36	Eberly and Ely (2012)
–	–	(6)	7	25	20	–	0.780	0.05		1.65	11,250	0.244	–	Tang et al. (2011)
–	–	(5)	7	25	20	–	0.909	0.05		1.22	11,250	0.223	–	(Tang et al. 2011)
–	–	(6)	7	25	10	–	0.887	0.05	1.84	0.155	11,250	0.288	–	Tang et al. (2011)
–	–	(5)	7	25	10	–	0.993	0.05	1.55	0.144	11,250	0.260		Tang et al. (2011)
Vessel	20	(10)	–	25	20	1	1.80	–	4.3	0.53	15,625	0.85	–	Kurano et al. (1995)
Vessel	4	(10)	–	25	20	1	1.87	–	4.9	0.4	15,625	0.65	–	Kurano et al. (1995)
Vessel	0.001	(10)	–	25	20	1	1.87	–	14.4	2.5	15,625	4	–	Kurano et al. (1995)

Rates of CO_2 fixation are normalized to g L^{-1} d^{-1}

Thermosynechococcuselongates (14), *Scenedesmusobliquus SJTU* (6), *Chlorella pyrenoidosa SJTU-2* (5), *Chlorococcum littorale* (10), *Scenedesmusobliquuss* (8), *Dunaliellatertiolecta* (4), *Chlorella vulgaris* (3), *Phormidiumsp* (15), *and A. microscopicaNegeli* (16)

investigations (11,200 lux) (Table 7.4). Light intensity controls photosynthetic growth in any microalgal system, CO_2 removal rates, biomass concentrations, and overall growth rates. While increasing light intensity is usually accompanied by increasing CO_2 removal rates in microalgal systems, any photosynthetic system has a saturation point where further increasing light intensity will either produce no benefit or may decrease productivity.

7.4 Effect of Different Carbon Sources on the Growth of Microalgae

Bicarbonate (HCO_3^-) is the predominant form of dissolved inorganic carbon (DIC) in seawater (pH = 8). At this pH, only 10 μM (less than 1 %) CO_2 is present as DIC, leading to low CO_2 diffusion for microalgal photosynthesis. The growth of microalgae can lead to alkalization of the growth medium and occurs as a result of CO_2 and HCO_3^- uptake and OH^- efflux. With an insufficient supplementation of DIC, microalgal photosynthesis may decrease (Moheimani 2013). To assist higher photosynthetic productivity, most microalgae species have a carbon concentrating mechanism (CCM) allowing them to not only use CO_2, but uptake HCO_3^- as a carbon source (Huertas et al. 2000). For instance, culturing two strains, *Chlorella* sp. and *Tetraselmis suecica* CS-187, in 120 L hanging bag PBRs by Moheimani is an example of using various inorganic carbon sources (industrial CO_2, flue gas, and sodium bicarbonate) to grow algae. The highest biomass and lipid productivities of *T. suecica* (51.45 ± 2.67 mg biomass L^{-1} day^{-1} and 14.8 ± 2.46 mg lipid L^{-1} day^{-1}) and *Chlorella* sp. (60.00 ± 2.4 mg biomass L^{-1} day^{-1} and 13.70 ± 1.35 mg lipid L^{-1} day^{-1}) were achieved using CO_2 as inorganic carbon source. When using pure CO_2 or flue gases as a source of inorganic carbon, the specific growth rate, biomass, and lipid productivities of *T. suecica* were 23, 10, and 22 % higher than those with $NaHCO_3$, respectively. Using pure CO_2 or flue gases as a source of inorganic carbon, the biomass yield and both biomass and lipid productivities of *Chlorella* sp. were 6, 7, and 8 % higher than those with $NaHCO_3$, respectively (Moheimani 2013). Growth rate of *Thermosynechococcus* sp. (TCL-1), under various DIC concentrations at a constant pH of 9.5 and temperature of 50 °C, increased with an increase in initial DIC concentrations (Fig. 7.2) (Hsueh et al. 2009). The effects of DIC concentrations on TCL-1 growth were investigated at four DIC levels (Su et al. 2012). Using steady-state conditions and 28, 57, 85, and 113 mM DIC, the cell mass productivities of 486, 620, 948, and 1056 mg L^{-1} d^{-1} were achieved, respectively. The cell mass productivity was enhanced with increasing DIC, and thus, DIC is considered a limiting production factor. However, as DIC increased from 85 to 113 mM, the increase in cell mass productivity was only from 950 to 1050 mg L^{-1} d^{-1} (Fig. 7.3). Furthermore, the uptake of CO_2 from bicarbonate by photosynthesis will release hydroxyl anion and increase the pH that

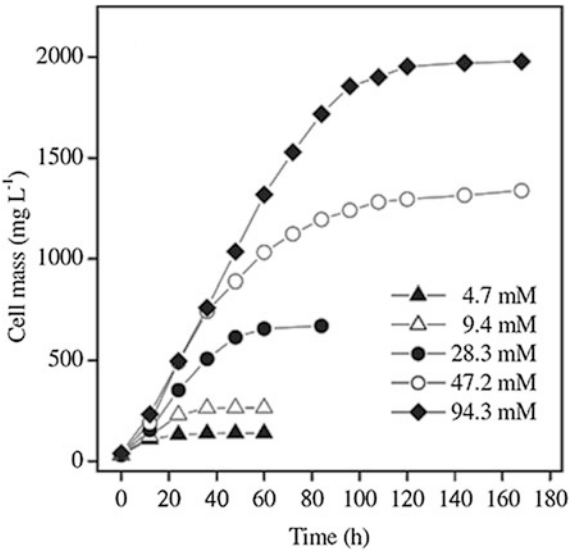

Fig. 7.2 Growth curves of *Thermosynechococcus* sp., under different DIC concentrations. (Copied from Hsueh et al. (2009) with permission)

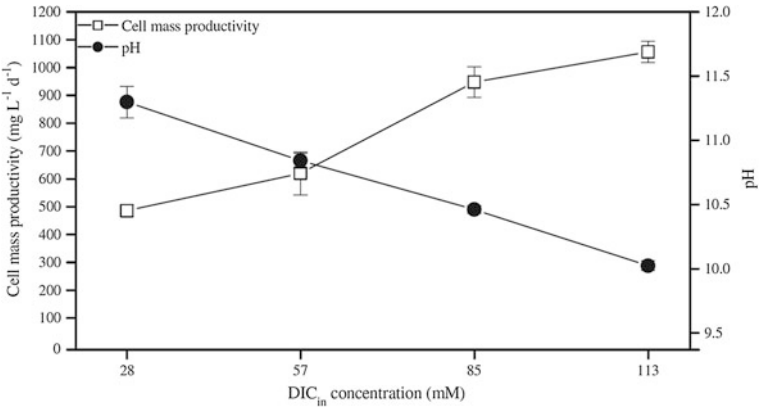

Fig. 7.3 Effects of DIC concentration and pH on the cell mass productivity of *Thermosynechococcus* sp. cultures. (Copied from Su et al. (2012) with permission)

can be used as an indicator to confirm the alkalization process as a result of CO_2 and/or HCO_3^- uptake. The inverse correlation between DIC and pH may be due to the buffer capacities of bicarbonate, as it is achieved under higher DIC levels and lead to smaller changes in pH (Su et al. 2012).

7.5 Effective Factors in Flue Gas Bioremediation

Various parameters such as initial cell concentration, input CO_2 concentration, aeration rate, photobioreactor design, light intensity and temperature should be taken into account for biofixation of CO_2 from flue gases by microalgae. These factors are important when seeking to achieve high-productivity microalgae bioremediation of CO_2 from input gases. In this section, the effect of initial cell concentrations, input CO_2 concentration, aeration rate and data analysis from these factors on microalgal CO_2 bioremediation is discussed.

7.5.1 Initial Cell Concentration

Initial cell concentration covers the range of 1–6.76 g L^{-1} which is summarized in Table 7.5. For example, two mutants of *Chlorella* sp. (*MT-7* and *MT-15*) was investigated at different initial cell concentrations on CO_2 fixation rates from 1 to 3 g L^{-1} (Ong et al. 2010). The CO_2 fixation rate increased from 0.0124 to 0.0168 g L^{-1} d^{-1} and from 0.0109 to 0.0177 g L^{-1} d^{-1} for *Chlorella* sp. *MT-7* and *MT-15,* respectively (Table 7.5), indicating a significantly higher CO_2 fixation rate at higher biomass concentrations (Ong et al. 2010). Furthermore, Table 7.5 shows the effect of initial cell concentration on CO_2 removal and biomass concentration of cyanobacterium *Synechococcus* sp. (Takano et al. 1992). When the initial microalgal cell concentration increased from 1.4 to 6.8 g L^{-1}, the CO_2 fixation rate increased from 1.06 to 2.22 g L^{-1} and biomass concentration increased from 1.92 to 7.76 g L^{-1} (Takano et al. 1992). Additionally, CO_2 retention times will be increased in higher biomass concentration as a result of higher culture medium viscosity, resulting in an enhanced CO_2 removal rate and fixation efficiency (Ong et al. 2010).

7.5.2 Input CO_2 Concentration

Data in Table 7.1 present the effect of CO_2 concentration of the input gas on biomass concentration and CO_2 removal of cyanobacterium *Synechococcus* sp. (Takano et al. 1992). When the CO_2 concentration of the input gas increased from 0.03 to 0.55 %, the biomass concentration rose by 1.5-fold and the CO_2 removal rate more than doubled. However, when the input gas concentration further increased from 0.55 to 1.10 %, only a slight increase in CO_2 removal occurred (Takano et al. 1992). Furthermore, Fig. 7.4 shows the influence of the input CO_2 content on *S. obliquus* WUST4 (Li et al. 2011) in the range of 6–18 %. The highest CO_2 fixation efficiency (67 %) was achieved at 12–14 % CO_2, indicating higher CO_2 concentration is an inhibitory factor to CO_2 fixation, and is an species-dependent variable tolerance.

Table 7.5 Effect of initial cell concentrations on CO_2 removal rates

PBR		Microalgae	T (°C)	Supplied CO_2 %	Gas flow rate $\frac{L}{min}$	Cell density $(\frac{g}{L})$	Biomass concentration $(\frac{g}{L})$	Light intensity (Lux)	CO_2 fixation rate $(\frac{g}{L.d})$	Ref.
Type	Vol (L)									
Vertical bubble column	40	(17)	40	5	10	1	–	1500	0.0124	Ong et al. (2010)
Vertical bubble column	40	(17)	40	5	10	2	–	1500	0.0144	Ong et al. (2010)
Vertical bubble column	40	(17)	40	5	10	3	–	1500	0.0168	Ong et al. (2010)
Vertical bubble column	40	(18)	40	5	10	1	–	1500	0.0109	Ong et al. (2010)
Vertical bubble column	40	(18)	40	5	10	2	–	1500	0.0148	Ong et al. (2010)
Vertical bubble column	40	(18)	40	5	10	3	–	1500	0.0177	Ong et al. (2010)
–	2.5	(7)	–	0.55	800	1.4	1.92	1250	1.06	Takano et al. (1992)
–	2.5	(7)	–	0.55	800	2.4	3	1250	1.52	Takano et al. (1992)
–	2.5	(7)	–	0.55	800	5.5	6.28	1250	1.98	Takano et al. (1992)
–	2.5	(7)	–	0.55	800	6.76	7.76	1250	2.22	Takano et al. (1992)

Chlorella sp. *MT-7* (17), *Chlorella* sp. *MT-15* (18), and *Synechoccus* sp. (7)

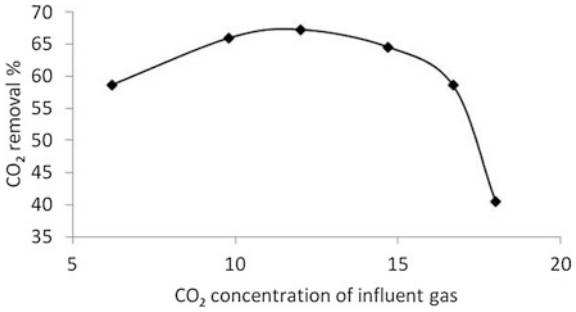

Fig. 7.4 Influence of CO_2 contents of influent gas on the CO_2 removal efficiency. (Reproduced from Li et al. (2011) with permission)

7.5.3 Aeration Rate

Aeration is a key parameter in mass transfer of CO_2. Flue treatment productivity in particular will be decreased in low aeration rates, and adequate CO_2 concentrations will be required. However, simply increasing CO_2 aeration rates does not necessarily lead to a higher CO_2 fixation efficiency (Li et al. 2011). For example, Fig. 7.5 shows that increasing aeration rates from 0.1 vvm (volume of gas per volume of culture per min) to 0.5 vvm in a *S. obliquus* WUST4 culture medium resulted in a decreasing CO_2 removal efficiency from 67 to 20 % (Li et al. 2011). A comparable result was obtained for *C. vulgaris* (Fig. 7.6), with the capability of CO_2 fixation and O_2 evolution decreasing with increasing feed gas flow rates (Fan et al. 2007). Therefore, in low aeration rates, gas retention time increases leading to an increased interface between CO_2 and microalgal cells (Fan et al. 2007). One factor may be the influence of bubble coalescence; as it increases with increased flow rates, larger bubbles rise to the surface at a faster rate than smaller bubbles and the bubble surface area per unit of gas volume declines. This leads to decrease in CO_2 absorption (Chiu et al. 2009). However, this is far from consistent across the literature, as the opposite can result in which increasing aeration rates improves

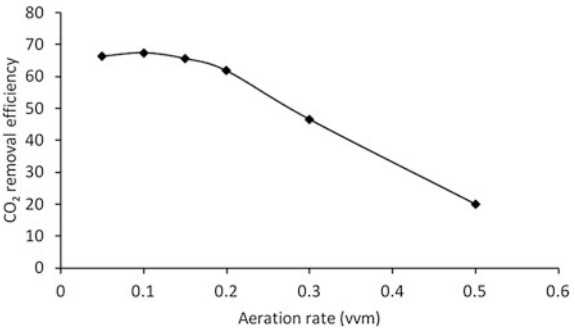

Fig. 7.5 Influence of aeration rates on CO_2 removal efficiency. (Reproduced from Li et al. (2011) with permission)

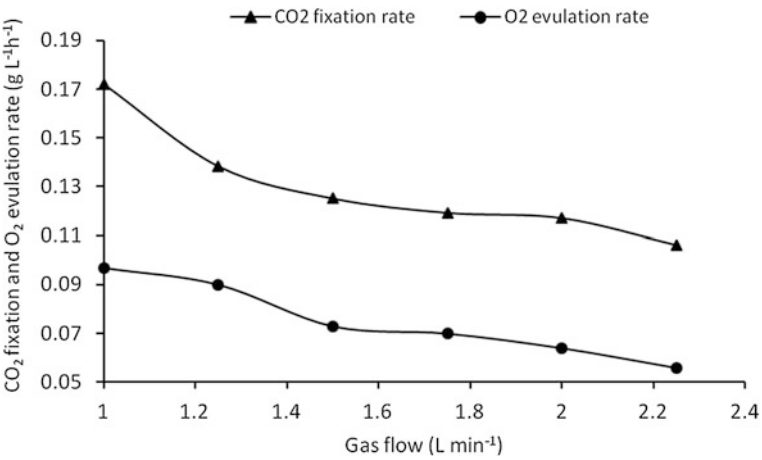

Fig. 7.6 Effect of gas flow rates on CO_2 fixation and O_2 evolution, (T = 25 °C, cell number = 5×10^7 cells mL^{-1}, luminous intensity = 5400 lux, red inner light source, PVDF-1 membrane length = 30 cm, and membrane number = 30). (Reproduced from Fan et al. (2007) with permission)

CO_2 removal rates (Ong et al. 2010). For example, the effect of aeration rates from 0.25 to 0.5 vvm on CO_2 fixation rate of *Chlorella* sp. *MT-7* and *MT-15* is significantly higher than CO_2 fixation rate at 0.5 vvm as compared with 0.25 vvm (Table 7.6) (Ong et al. 2010). Furthermore, the effect of different aeration rates (0.001, 0.002, and 0.005 ms^{-1}) and CO_2 fixation rates (1.5 g d^{-1}) on the dry weight of *C. vulgaris* and *D. tertiolecta* was studied (Hulatt and Thomas 2011). The maximum biomass concentration for *D. tertiolecta* was obtained at the 0.005 ms^{-1} gas flow rate and at 12 % CO_2 (1.5 g d^{-1}), and 0.005 ms^{-1} and at 12 % CO_2 (1.12 g d^{-1}) for *C. vulgaris* (Table 7.6) (Hulatt and Thomas 2011). This indicates that improved mass transfer occurs at higher gas flow rates. Therefore, coming to an overall conclusion on the effect of flow gas rates on CO_2 fixation rates is complicated due to opposite results being reported (see Table 7.6). While it is true that normally decreasing aeration rates lead to CO_2 fixation efficiency increase, the opposite results have been obtained. This might arise as a consequence of the various production parameters [biomass concentration, light regime, nutrients, and types of PBRs (Hulatt and Thomas 2011)], and how the individual microalgae species affect each system. Nonetheless, increasing or decreasing aeration rate effectively determines whether CO_2 fixation rates will increase or decrease in a microalgal system.

Table 7.6 Effect of different aeration rates on CO_2 removal rate

PBR		Microalgae	T (°C)	Supplied CO_2 %	Gas flow rate $\frac{L}{\min}$	Cell density $(\frac{g}{L})$	Biomass concentration $(\frac{g}{L})$	Biomass productivity $(\frac{g}{L})$	CO_2 fixation		Ref.
Type	Vol (L)								Rate $\frac{g}{L.d}$	% Efficiency	
Vertical bubble column	40	(17)	40	5	10	3	–	–	0.0168	–	Ong et al. (2010)
Vertical bubble column	40	(17)	40	5	20	3	–	–	0.0211	–	Ong et al. (2010)
Vertical bubble column	40	(18)	40	5	10	3	–	–	0.0177	–	Ong et al. (2010)
Vertical bubble column	40	(18)	40	5	20	3	–	–	0.0256	–	Ong et al. (2010)
Bubble column	1.4	(3)	26	12	0.001 $\frac{m}{s}$	–	2.78	0.49	0.86	5.8	Hulatt and Thomas (2011)
Bubble column	1.4	(3)	26	12	0.002 $\frac{m}{s}$	–	3.04	0.53	0.95	3.2	Hulatt and Thomas (2011)
Bubble column	1.4	(3)	26	12	0.005 $\frac{m}{s}$	–	3.79	0.60	1.12	1.5	Hulatt and Thomas (2011)
Bubble column	1.4	(4)	26	12	0.001 $\frac{m}{s}$	–	2.85	0.71	1.29	8.8	Hulatt and Thomas (2011)
Bubble column	1.4	(4)	26	12	0.002 $\frac{m}{s}$	–	3.17	0.79	1.40	4.7	Hulatt and Thomas (2011)
–	1.4	(4)	26	12	0.005 $\frac{m}{s}$	–	3.19	0.83	1.51	2.1	Hulatt and Thomas (2011)

Chlorella vulgaris (3),*Dunaliellatertiolecta* (4), *Chlorella* sp. *MT-7* (17), *Chlorella* sp. *MT-15* (18)

7.6 Conclusion

To achieve economical bioremediation of CO_2 emitted from power stations using microalgae requires much research in order to maximize its efficiency and at the same time improve the microalgal biomass productivity at larger scales. Furthermore, various microalgae and cyanobacteria species exhibit very different CO_2 bioremediation rates and potentials for large-scale production. Results presented in this chapter demonstrated that the most attractive species for environmental CO_2 mitigation include *S. obliquss*, *D. tertiolecta*, *C. vulgaris*, *Phormidium* sp., *A. microscopic negeli*, and *C. littorale*. The CO_2 removal rate by the aforementioned species will require customization and optimization to meet each system-specific requirements. This chapter has reported on the initial cell concentrations, initial input CO_2 concentrations, and aeration rates impact on CO_2 bioremediation. In general, increasing initial cell concentrations and decreasing aeration rates lead to increasing CO_2 fixation efficiency. It is to be noted that lowering aeration rates lead to a higher CO_2 biofixation efficiency because of improved CO_2 mass transfer between microalgal cells and the culture medium. Moreover, the input CO_2 concentration influences removal efficiency of CO_2, however, providing high levels of CO_2 into culture mediums leads to acidification. In contrast, the consumption of CO_2 by microalgae through photosynthesis results in pH increase that may impact growth rates of some microalgae species.

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Chapter 8
Prospective Applications of Synthetic Biology for Algal Bioproduct Optimization

Basel Khraiwesh, Kenan Jijakli, Joseph Swift, Amphun Chaiboonchoe, Rasha Abdrabu, Pei-Wen Chao, Laising Yen and Kourosh Salehi-Ashtiani

Abstract Synthetic Biology is an interdisciplinary approach combining biotechnology, evolutionary biology, molecular biology, systems biology and biophysics. While the exact definition of Synthetic Biology might still be debatable, its focus on design and construction of biological devices that perform useful functions is clear and of great utility to engineering algae. This relies on the re-engineering of biological circuits and optimization of certain metabolic pathways to reprogram algae and introduce new functions in them via the use of genetic modules. Genetic editing tools are primary enabling techniques in Synthetic Biology and this chapter discusses common techniques that show promise for algal gene editing. The genetic editing tools discussed in this chapter include RNA interference (RNAi) and artificial microRNAs, RNA scaffolds, transcription activator-like effector nucleases (TALENs), RNA guided Cas9 endonucleases (CRISPR), and multiplex automated genome engineering (MAGE). DNA and whole genome synthesis is another enabling technology in Synthetic Biology and might present an alternative approach to drastically and readily modify algae. Clear and powerful examples of the potential of whole genome synthesis for algal engineering are presented. Also, the development of relevant computational tools, and genetic part registries has stimulated further advancements in the field and their utility in algal research and engineering is described. For now, the majority of synthetic biology efforts are

B. Khraiwesh · A. Chaiboonchoe · R. Abdrabu · K. Salehi-Ashtiani (✉)
Division of Science and Math, and Center for Genomics and Systems Biology (CGSB), New York University Abu Dhabi, 129188, Abu Dhabi, United Arab Emirates
e-mail: ksa3@nyu.edu

K. Jijakli
Division of Engineering, New York University Abu Dhabi, 129188, Abu Dhabi United Arab Emirates

J. Swift
Department of Biology, New York University, 12 Waverly Place, New York 10003, USA

P.-W. Chao · L. Yen
Department of Pathology and Immunology, Department of Molecular and Cellular Biology, Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA

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focused on microbes as many pressing problems, such as sustainability in food and energy production rely on modification of microorganisms. Synthetic modifications of algal strains to enhance desired physiological properties could lead to improvements in their utility.

8.1 Introduction

More basic research is needed before algal biotechnology can reach a capacity to compete with other systems (Stephen and Joshi 2010). Because many physiological, morphological, biochemical, or molecular characteristics of algae are quite different from higher plants or animals, algae can meet several requirements that other systems cannot (Hallmann 2007). The demand for improved systems of production of nutraceuticals and cost-effective protein expression systems (both industrial and pharmaceutical applications) lend themselves to explore the potential useful capacities of algae (Hallmann 2007).

Synthetic biology is the design and construction of biological devices and systems for a specific purpose (Ferry et al. 2012). This is an area of biological research and technology that combines biology and engineering, thus often overlapping with bioengineering and biomedical engineering (Serrano 2007). It encompasses a variety of different approaches, methodologies, and disciplines with a focus on bioengineering and biotechnology. Through the innovative re-engineering of biological circuits and optimization of certain metabolic pathways, biological modules can be designed to reprogram organisms to produce products, or exhibit desired metabolic behaviors (Khalil and Collins 2010). Synthetic biology can enable model transferability to address a multitude of industrial needs and projects (Anderson et al. 2012). Researchers in this field are realistically optimistic that synthetic biology can provide solutions to a multitude of worldwide problems from health to energy (Purnick and Weiss 2009). The success of synthetic biology as a promising approach is demonstrated by a number of successful attempts in constructing microbial strains to lower production cost of pharmaceutical products (Khalil and Collins 2010). Similarly, there are ongoing works to introduce desirable traits into algae, and to re-engineer algal cells (Ferry et al. 2012; Gimpel et al. 2013; Rabinovitch-Deere et al. 2013).

Although sometimes referred to as genetic engineering, synthetic biology differs in terms of scale, scope, techniques of manipulation, and application (Serrano 2007). Genetic engineering focuses on the alteration of a single characteristic of an organism through transgenic hybrids or genetic chimeras that carry altered genes, or genes from other organisms. In contrast, synthetic biology seeks to reconfigure, design, and construct new pathways, whole processes, or novel systems for the purpose of achieving some desired biosynthetic activity or phenotype (Alper and Stephanopoulos 2009; Khalil and Collins 2010).

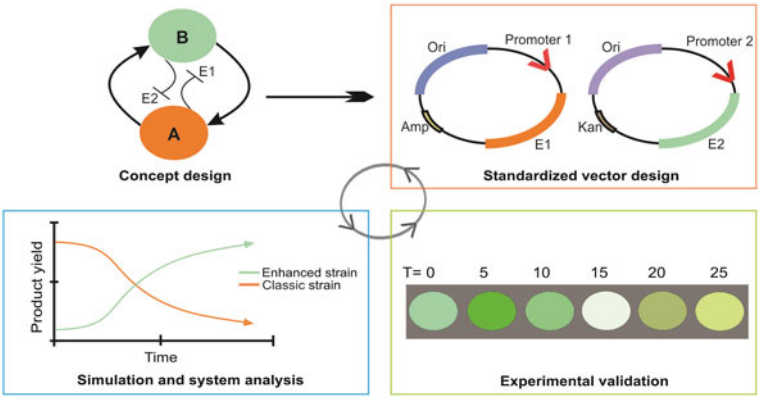

Fig. 8.1 Design of artificial algae genetic circuits based on synthetic biology

Microalgae exhibit enormous biodiversity, and have the potential for producing large quantities of biomass containing high concentrations of lipids (Gimpel et al. 2013). Synthetic, systems, and post-genomics biology are terms that are increasingly encountered in the biofuels and biotechnology research space with all such approaches likely to be deployed to enable algal biofuels to become economically competitive with fossil fuels. In order to create a viable technology, the field of synthetic biology has been moving towards modular genetic systems. Modularity encompasses the reliance on standardized genetic parts and circuitry models—just like the field of electronics and electric circuits relies on standard collections of resistors, transistors, and capacitors (Fig. 8.1).

RNA-mediated silencing and targeted genome editing tools, including artificial microRNA (Khraiwesh et al. 2011), RNA scaffolds (Delebecque et al. 2012), TALENs, and RNA guided Cas9 endonucleases (Gaj et al. 2013), have allowed synthetic biology to develop gene circuits designed to perform specific functions, often by combining components from multiple organisms to generate novel functionality. These new research endeavors will undoubtedly increase our knowledge and usage of these important primary-producing organisms.

8.2 Part Registries

Part registries are lists and collections of standardized biological parts that can be strung together to build advanced and more complicated biological devices. Parts in this context mean DNA constructs that encode a given function. On the other hand, biological devices perform more complicated functions and are made from combining different parts. An example would be that a part encodes a specific enzyme in a pathway, while a device performs a complicated function like biological arsenic detection or bioplastics production. These registries are a list of DNA

constructs (parts) and synthetic circuits (biological devices) that are ready for genetic insertion. The idea behind creating such registries is to list reliable and well tested biological parts that can be manufactured to a given standard and that serve as the building blocks of sophisticatedly engineered biological tools and devices (Canton et al. 2008). This standardization liberates the process of designing and fabricating complex biological devices from the daunting task of designing and fabricating each individual and necessary component. In addition, the fabricators of those devices can rely on specialized manufacturers of those standard parts to provide the components needed for fabricating the advanced biological devices. All in all, this has the effect of opening up the field and providing the impetus for more advanced applications (Baker 2006).

In biological part registries, standardization is of essence. Standardization in the context of biological parts refers to the ability of a part to assimilate into a larger structure without any complications. In other words, the part does not have any restriction sites that interfere with the process of assembling into larger devices. Another important aspect of standardization is with respect to function. Biological parts should serve a given function that is both well-defined and consistent. Perhaps the most widely known and used registry is the Registry for Standard Biological Parts (http://parts.igem.org/Main_Page) (Kahl and Endy 2013). The registry, like most other registries, relies on community contributions to expand its biological part offerings. The community accessible approach of the registry has expanded the registry's offering to include thousands of parts serving numerous functions. The registry's part types include promoters, ribosome binding sites, protein domains, protein coding sequences, translational units, terminators, plasmid backbones, primers and composite parts which are a composition of two or more simpler parts. To ensure openness, efficiency and consistency, parts offered by the registry comply with the BioBricks assembly standard (http://parts.igem.org/Help:Standards/Assembly). The current standard relies on defining a DNA prefix and suffix on a standard plasmid backbone. The part is then inserted into the plasmid backbone specifically between the prefix and suffix. The prefix and suffix also contain specific restriction sites, and this precise definition that is included in the standard also forbids the introduction of restriction sites that interfere with the part assembly and usage process. In general, those features allow the smooth and immediate use of the biological parts.

Algae-specific registries with the ambition of the registry for standard biological parts are yet to be fully developed. However many repositories are available that provide cell lines, several thousand algal strains, DNA constructs, and specific genetic engineering tools. One prominent example is the Chlamydomonas Resource Center, a repository of *Chlamydomonas reinhardtii* strains, plasmids, kits, and cDNA libraries among other things (http://chlamycollection.org/). While most of these registries are not registries of synthetic biological parts specifically, they still offer many tools and products of value to synthetic biology endeavors in algae. Also, the availability of standard and customized algal optimized plasmids prepared and sold by private companies, such as Life Technologies, (a brand of Thermo Fisher Scientific, Carlsbad, CA, USA), is another step forward into easing up synthetic biology applications with microalgae.

While the breadth of registries and repositories up and running is a call for optimism in the field of synthetic biology, certain challenges still permeate the part registry model. For example, characterization of many biological parts and the precise definition of their functions are still lacking. Furthermore, many parts display different behavior in different cells or organisms and in different laboratory conditions; this introduces a major challenge to the field with respect to reproducibility of function. Stability and reliability become even more daunting challenges as the organism's complexity increases. This means that extending biological parts and the registry model to algae, or organisms of higher complexity than simple microbes, becomes additively challenging quite quickly. Still, another challenge is in the long-term behavior of parts and their behavior as components of increasingly complex devices. Cell functions are prone to seemingly random behavior and noise which can complicate the ability of biological parts and complex devices to behave consistently for a significantly long period (Kwok 2010).

8.3 Computational Tools for Synthetic Biology

Computational tools that help to improve synthetic biology have been developed and are currently being expanded. Improvement of algae production to increase biofuel yields through synthetic biology involves many distinct processes that can be aided by different computational tools. Genome-scale metabolic network reconstructions and models are available for a number of algal species. These can be useful for identifying and selecting gene targets for knockout and strain engineering. Some of the available tools and algorithms that are able to perform such tasks include (but are not restricted to) Optknock (Burgard et al. 2003) and Optstrain (Pharkya et al. 2004).

The standard approach for computing metabolic fluxes is flux balance analysis (FBA) through using toolboxes such as COBRA or Pathway Tools (these are discussed in the accompanying Chap. 10, *Towards applications of Genomics and Metabolic Modeling to Improve Biomass Productivity*). FBA allows prediction of optimal flux distribution throughout the network for a given cellular phenotypic state. Through the use of computational tools associated with FBA, consequences of changes introduced in a metabolic network can be predicted. For example, quantitative mapping of intracellular fluxes in relation to single or multiple gene deletions can easily be carried out by FBA. There are many strategies available to predict alterations that result in increased production of a desired metabolite. For instance, one method entails identifying key and relevant pathways that are impacted using simulated gene knockouts (Reed et al. 2010). The accuracy of this method can be enhanced by integrating experimental data; such as metabolite concentration, gene expression data, and uptake and secretion rates.

'Pathway Tools' (Karp et al. 2010) is an integrated reconstruction, analysis, and visualization software created by the Bioinformatics Research Group at SRI International (http://bioinformatics.ai.sri.com/ptools/). Pathway tools can automatically

generate organism-specific metabolic network databases and provides details of genes/proteins, reactions, and compound associations, as well as create pathway databases (called Pathway Genome Database, or PGDBs). To create a PGDB, one of the components of the Pathway Tools called PathoLogic is used. This tool allows users to create PGDBs using the genome annotation of an organism of interest directly from the organism's GenBank annotation file. Users can manually adjust, edit, or add (new) content as needed. There are already many well developed and intensively curated pathway databases or PGDBs including BioCyc, EcoCyc and MetaCyc (Caspi et al. 2014), which aid metabolic analysis and network reconstructions. BioCyc alone has a collection of about 3530 PGDBs, which users can query, visualize, manage and analyze. Among these, algal PGDBs include *Thalassiosira pseudonana*, *Nannochloropsis gaditana*, *Acaryochloris marina*, *Anabaena cylindrica*, *Anabaena variabilis*, *Synechococcus elongatus* and *Chlamydomonas reinhardtii*. Other offered functionalities in Pathway Tools include tools that can be used in downstream analyses to identify the shortest path between two metabolites, identify dead-end metabolites, fill pathway gaps, identify chokepoints (potential drug targets), and infer operons and transport reactions. Many new metabolic reactions have been added to EcoCyc using the dead-end metabolite analysis approach (Mackie et al. 2013). Pathway Tools can aid synthetic biology experiment designs by identifying potential pathways, which may be targets for alterations.

8.4 RNA interference (RNAi)

The RNAi pathway has been studied in the unicellular green alga *C. reinhardtii*, and used as a reverse genetics tool in different algal species. Complex sets of endogenous small RNAs, including candidate microRNAs and small interfering RNAs, have been identified in four algal species, *C. reinhardtii* (Molnar et al. 2007; Zhao et al. 2007), *Porphyra yezoensis* (Liang et al. 2010), *Phaeodactylum tricornutum* (De Riso et al. 2009), and *Ectocarpus siliculosus* (Cock et al. 2010). However, RNAi mechanisms and their applications remain largely uncharacterized in most algae. RNAi against specific genes can be induced by the introduction of exogenously synthesized dsRNAs or siRNAs into cells or whole organisms (Cerutti et al. 2011; Moellering and Benning 2010; Molnar et al. 2009). Within algal species, in vitro-synthesized long dsRNAs have been electroporated into *Euglena gracilis* cells and shown to silence two endogenous genes homologous to the introduced dsRNAs (Iseki et al. 2002; Ishikawa et al. 2008). Recently, an RNAi triple knockdown of the three most abundant LHCII proteins (LHCBM1, 2 and 3) has been reported in *Chlamydomonas* with the aim of increasing the efficiency of photobiological H_2 production (Oey et al. 2013). Artificial microRNA (amiRNA) expression successfully exploits endogenous miRNA precursors to generate small RNAs that direct gene silencing in *C. reinhardtii* (Molnar et al. 2009; Schmollinger et al. 2010; Zhao et al. 2008). Zhao et al. (2009) developed an artificial amiRNA-based strategy to

knock down gene expression in *Chlamydomonas* using an endogenous *Chlamydomonas* miRNA precursor (pre-miR1162) as the backbone. Other studies show that amiRNAs can be used as a highly specific, high-throughput silencing system, and they propose that they will become the system of choice for analysis of gene function in *Chlamydomonas* and related organisms (Molnar et al. 2009; Schmollinger et al. 2010). The synthesized miRNAs provide a convenient tool for reverse genetic studies in *Chlamydomonas*. More recently, epitope-tagged protein-based amiRNA (ETPamir) screens were developed, in which target mRNAs encoding epitope-tagged proteins were constitutively or inducibly co-expressed in protoplasts with amiRNA candidates targeting single or multiple genes (Li et al. 2013). This design allowed parallel quantification of target proteins and mRNAs to define amiRNA efficacy and mechanism of action, circumventing unpredictable amiRNA

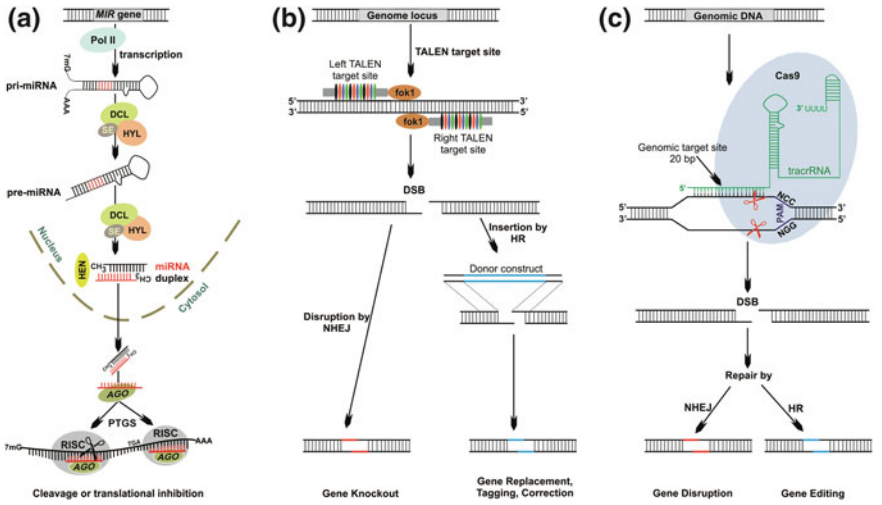

Fig. 8.2 Genome engineering tools. **a** miRNA pathway. *MIR* genes are transcribed by RNA polymerase II into pri-miRNA transcripts that are further processed into pre-miRNAs harboring a characteristic hairpin structure. From the stem of the pre-miRNA the miRNA/miRNA* duplex is excised by DCL1 and can be assisted by HYL and SE proteins. miRNA-guided AGO-containing RNA-induced silencing complex (RISC) directs mRNA cleavage or translation inhibition of the target transcript. **b** Summary of Transcription activator-like effectors (TALEs) nuclease. Custom-designed nucleases introduce double-strand breaks with high precision at predetermined genomic loci. Double-strand breaks are either repaired by error-prone non-homologous end-joining (NHEJ) or high fidelity homologous recombination (HR). NHEJ repair causes random insertions and/or deletions of nucleotides around the target site and some of these mutations will knockout gene function. Gene replacement, tagging, or correction can be achieved by HR-mediated targeted integration of a donor construct that is provided together with a nuclease pair. **c** CRISPR/Cas9 mediated target DNA cleavage. The CRISPR loci include Cas genes, a leader sequence, and several spacer sequences derived from engineered or foreign DNA that are separated by short direct repeat sequences. Cleavage occurs on both strands, 3 bp upstream of the NGG proto-spacer adjacent motif (PAM) sequence on the 3′ end of the target sequence and is followed by DNA repair by the endogenous cellular repair machinery

expression/processing and antibody unavailability. These screens could improve algal biofuel engineering research by making amiRNA a more predictable and manageable genetic and functional genomic technology. From a practical perspective, RNAi is becoming a customary method for directed gene silencing in algae. As the necessary molecular tools are developed, RNAi approaches are expected to contribute to the functional characterization of novel genes, as well as to the strain engineering of algae (Fig. 8.2a). Ultimately, RNAi technology may provide much-needed insights into gene function, metabolic pathways, and regulatory networks allowing us to comprehend the role of algal species in nature, as well as to engineer these organisms for the synthesis of valuable bioproducts.

8.5 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs comprise a powerful class of tools that are redefining the boundaries of biological research. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35 amino acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hypervariable amino acids that are known as the repeat variable di-residues (RVDs). TALEs can be quickly engineered to bind practically any desired DNA sequence (Boch 2011). TALENs can be used to edit genomes by inducing double-strand breaks (DSB) (Fig. 8.2b), which cells respond to with repair mechanisms (Boch 2011). Several methods have been developed that enable rapid assembly of custom TALE arrays such as golden gate cloning, high-throughput solid-phase assembly, and ligation-independent cloning techniques (Gaj et al. 2013).

Site-specific nucleases have enabled the introduction of targeted modifications in several model organisms common to biological research, including zebrafish, rat, mouse, Drosophila, *Caenorhabditis elegans*, and many other non-model species including the monarch butterfly, frogs and livestock (Gaj et al. 2013). In addition to valuable animal models, TALENs have been used to introduce targeted alterations in plants, including Arabidopsis and several crop species (Curtin et al. 2012), allowing the incorporation of valuable traits, such as disease and herbicide resistance.

In algae, studies of TALENs to modify the genome of *Chlamydomonas* have been initiated (Borchers et al. 2012; Spalding and Wright 2011). For instance, TALENs to knockout the *Chlamydomonas* Sta6 and CAH3 genes, which are responsible for starch production and CO_2 uptake, have been designed. These early studies are of interest due to the status of *Chlamydomonas* as a model organism for biofuel production.

The diversity of organisms modified by these site-specific nucleases will undoubtedly continue to grow, expanding the repertoire of model systems for basic research. TALENs will also enhance research efforts in algal biomass production, thus opening new avenues for algal biofuels commercialization.

Custom-designed TALE arrays are commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (brand of Thermo Fisher Scientific, Carlsbad, CA, USA).

8.6 CRISPR/Cas System

The ability to make specific changes to DNA, such as changing, inserting or deleting sequences that encode proteins, enables researchers to engineer cells, tissues and organisms for practical applications. Clustered regularly interspaced short palindromic repeats (CRISPR), a bacterial adaptive immune system effector, has been shown to facilitate RNA-guided site-specific DNA cleavage in bacteria, suggesting a simple alternative strategy for genome engineering (Sorek et al. 2013). The CRISPRs are a diverse family of DNA repeats that all share a common architecture. Each CRISPR locus consists of a series of short repeat sequences (typically 20–50 bp long) separated by unique spacer sequences of a similar length. The CRISPR/Cas systems are phylogenetically and functionally diverse, but each of these systems relies on three common steps: new sequence integration, CRISPR RNA biogenesis, and crRNA-guided target interference (Fig. 8.2c).

The CRISPR/Cas system allows targeted cleavage of genomic DNA guided by a customizable small noncoding RNA, resulting in gene modifications by both non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms. CRISPRs are unevenly distributed between Bacteria and Archaea. Currently, CRISPR loci have been identified in 90 % of the archaeal genomes and 50 % of the bacterial genomes (Sorek et al. 2013). CRISPR-Cas systems have emerged as potent new tools for targeted gene knockout in bacteria, yeast, fruit fly, zebrafish, human cells and plants (Belhaj et al. 2013; Gaj et al. 2013). In August 2012, Jinek et al. (2012) showed that a synthetic RNA chimera (single guide RNA, or sgRNA) created by fusing crRNA with tracrRNA is functional to a similar level as the crRNA and tracrRNA complex. As a result, the number of components in the CRISPR/Cas system was brought down to two, Cas9 and sgRNA (Jinek et al. 2012). For applications in eukaryotic organisms, codon optimized versions of Cas9, which is originally from the bacterium *Streptococcus pyogenes*, have been used. Four of the studies on the application of the CRISPR/Cas technology in plants used a plant codon-optimized version of Cas9, as using the previously described human codon-optimized version was not highly effective (Belhaj et al. 2013; Jiang et al. 2013).

All tested versions of Cas9 appear to work in plants with very high rates. Transgenic plants, generated using the CRISPR/Cas system, have been reported (up to 89 % for Arabidopsis and up to 92 % for rice) with bi-allelic mutation being recovered in the case of both plant species (Jiang et al. 2013). The discussed studies indicate the possibility of introducing functional CRISPR/Cas system in algae to target any sequence of choice, thus offering new opportunity for implementation in algal biotechnology for biomass production.

Together, these technologies promise to expand our ability to explore and alter any genome and constitute a new and promising paradigm to develop new synthetic biology tools for algal biofuels optimization.

8.7 Designer RNA Nanostructures and Pathway Scaffolds

RNA and DNA are highly programmable polymers due to their ability to form specific Watson-Crick base pairing; a property that can be exploited to create well-defined 2D and 3D structures (Rothemund 2006; Seeman 2010). These structures can be thermodynamically stable, and formed via spontaneous self-assembly, a process that requires no catalytic co-factors. In addition to Watson-Crick base pairing, an increasing number of useful tertiary RNA motifs are being discovered in nature. These simple motifs, such as three-way junctions and interacting loops, can serve as diverse building blocks for construction of higher-order structures with increasing complexity.

Single-stranded RNA can be continuously expressed at high levels in live cells, and thus offers the opportunity to program cells to assemble designer nanostructures for specific cellular functions. One potential application of such intracellular nanostructures that is particularly relevant to algal biofuel optimization is the construction of RNA scaffolds (Fig. 8.3). The aim of such RNA scaffolds is to provide a spatially organized docking station where different proteins can 'park' at pre-determined distances and permutations. This higher level of organization can be designed to bring together a specific set of enzymes and organize them at fixed proximity and orientations. The configuration could be used to limit the diffusion of intermediate substrates, hence efficiently channel substrates to final products over several enzymatic steps leading to increased yields from sequential metabolic or cellular reactions (Dueber et al. 2009). An example of this utility in bacteria is demonstrated in a recent study where RNAs are engineered and expressed as 2D scaffolds to spatially organize enzymes that effectively lead to increased reaction output by 48-fold (Delebecque et al. 2011). This points to the possibility that RNA scaffolds could be used to engineer biosynthetic pathways to maximize algal biofuel production.

In general, constructing RNA scaffolds involves the following steps (Delebecque et al. 2012). The first is to design the overall RNA secondary structure with minimal predicted free energy. RNA folding software such as RNA Designer, mfold, and NUPACK can be used to design RNA sequences that fold into desired shapes. Considerations generally include the GC percentage, removal of problematic sequences that may cause alternative folding, and removal of motifs such as splice sites and poly(A) signals that may be processed by cells. The next step is choosing appropriate aptamers that allow the coupling of proteins to the RNA scaffolds. Aptamers are folded RNA structures that function as receptors with specific ligand binding properties. Selective aptamers can be incorporated into the RNA scaffold as the docking sites. Aptamers with high binding affinity and specificity are required to

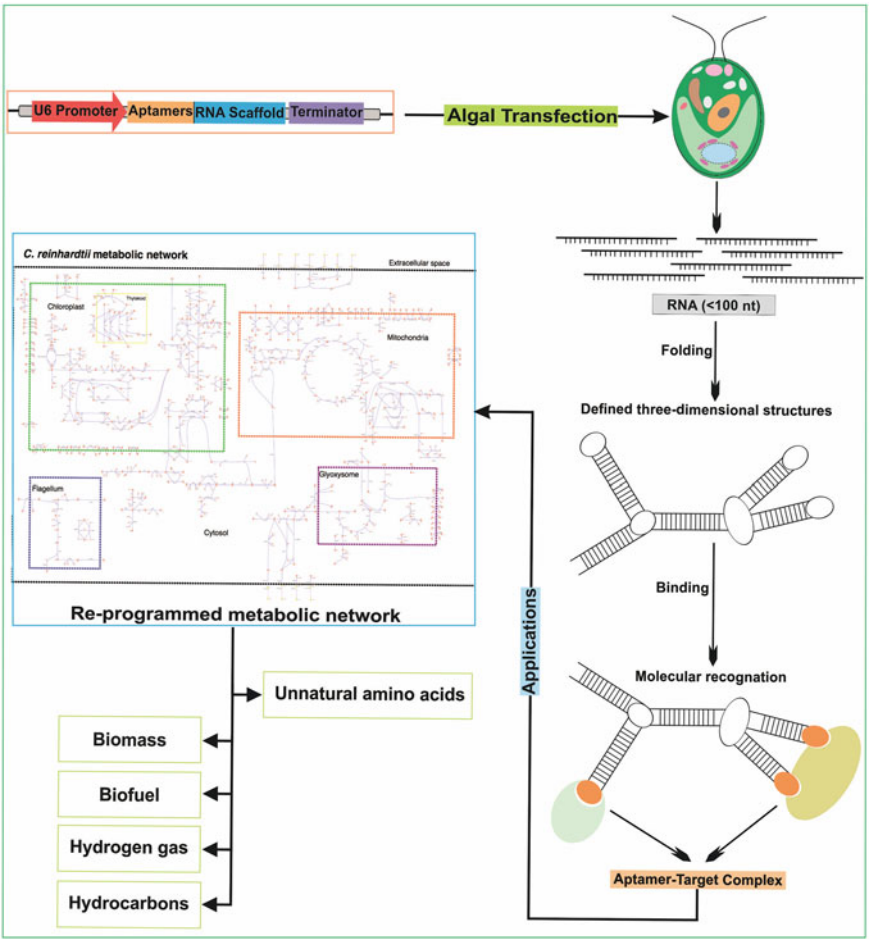

Fig. 8.3 Conceptual illustration of optimizing algal metabolism using RNA scaffolds. RNA scaffold constructs are RNA structures introduced into algae by genetic transformation to form and guide higher order assembly of metabolic enzymes. These composite structures can optimize bioproduction of high value metabolites

achieve good protein docking interactions. RNA sequences can be designed to fold into either discrete self-standing scaffolds, or with additional polymerization between discrete scaffolds to form a more complex structure (Fig. 8.3). In a discrete RNA scaffold, the aptamer binding sites are located within the same RNA molecule separated by spacer sequences. In a polymerizing RNA scaffold, each discrete RNA within the polymer may carry a different aptamer to capture the desired enzyme. The resulting polymer structure is a higher-level one, two or even three-dimensional scaffold with aptamers placed in specific locations. In this case, secondary interactions, kinetic considerations, and non-canonical interactions among the

components of the RNA scaffold must all be considered in the secondary structure design process. Common examples of polymerizing RNA scaffolds are nanotubes and two-dimensional sheets. In such complicated cases, designing the RNA scaffold to be an assembly of modular parts might make the process simpler. Another strategy for simplifying polymerizing RNA scaffold design and assembly is the reliance on palindromic sequences as a design strategy. Palindromic sequences create a symmetrical structure that simplifies scaffold design and minimizes the interactions required to assemble the overall structure. Obtaining a desired RNA scaffold structure usually requires an iterative process that relies on experimental verifications. Finally, a possibly helpful design consideration in RNA scaffold sequences is the incorporation of restriction sites within the different relevant sequences to allow for interchangeability and modifications within the RNA scaffold structure.

The use of RNA scaffolds for the purpose of increasing metabolic reactions hinges on the ability to precisely engineer higher-order scaffolds in the complex cellular environment. There remain several challenges that may limit the general application of RNA nanostructure in the cellular environment. First, thousands of cellular RNAs and proteins that are present in cells may non-specifically interact with individual RNA building blocks and prevent the formation of higher-order structures. Second, RNA scaffolds may cause cellular stress and disrupt cellular functions leading to general toxicity. Additional concerns include the stability and half-life of RNA scaffolds, and whether the scaffolds could be targeted to the appropriate cellular compartment such as cytoplasm vs. nucleus. Nevertheless, the ever-increasing number of useful RNA motifs and knowledge of RNA biology at our disposal may help to solve these issues in the near term.

8.8 Multiplex Automated Genome Engineering

MAGE is a genetic engineering method that relies on recombineering to produce frequent and large scale genetic changes to cells. Simply, it is a cyclical and scalable recombineering system that allows multiple genetic changes in a high throughput manner. A given cycle in MAGE requires cell growth, the introduction of recombineering substrate, and provision of providing synthesized DNA constructs a continuous and interlinked process. Target cells are continuously grown and drawn out of a cell growth chamber into an exchange chamber where the substrates required for recombineering are added under the right conditions. The induced cells are then mixed with the recombinant and synthetic DNA constructs in another chamber and then moved to an electroporator to induce the uptake of the synthetic DNA constructs. Those modified cells are then reintroduced into the growth chamber and grown. This whole process occurs continuously; and movement to each stage is facilitated by microfluidics, or any suitable pumping assembly (Isaacs et al. 2011).

Just like recombineering, MAGE can be used to create a mismatch, deletion or insertional genetic changes. The efficiency of each genetic change is dependent on the size of the homology flanking each introduced DNA construct, the size of the desired change, and the number of cycles. This efficiency is also correlated with the Gibbs free energy from the hybridization that occurs between the DNA strands. Knowledge of the efficiency of each genetic change and the parameters that affect it can be used to tune the MAGE process to produce colonies or strains with desired genetic traits (Isaacs et al. 2011). The MAGE system was initially demonstrated by modifying a 1-Deoxy-D-xylulose 5-phosphate (DXP) biosynthesis pathway in *Escherichia Coli*. The DXP pathway was modified to increase production of isoprenoid lycopene. New genetic modifications were introduced in greater than 30 % of the cell population every 2–2.5 h under optimum conditions. After just five cycles of MAGE, the average genetic change across the entire cell population was 3.1 bp, and increased to 5.6 bp afters 15 cycles. Ultimately, 24 genes were optimized simultaneously and about 15 billion genetic variants were produced at an average rate of 430 million bp changes per MAGE cycle in a total of 35 cycles. This translated to an up to 390 % increase in isoprenoid lycopene production, clearly demonstrating MAGE's potential (Isaacs et al. 2011). Development of a system similar to MAGE in algae is dependent on the design of a suitable genetic engineering method; recombineering strategies are not established in algae.

8.9 DNA Synthesis and Whole Genome Synthesis

In contrast to conventional recombinant DNA techniques, synthetic DNA synthesis can generate nucleotide sequences de novo. In this way, DNA synthesis technology allows for custom design of novel nucleotide sequences. The increased throughput of DNA synthesis now allows entire genetic regions and even small genomes to be derived synthetically (Carr and Church 2009; Gibson et al. 2008). This breakthrough has given rise to the field of 'synthetic genomics'. DNA synthesis techniques hold promising applications in algae biofuel research.

DNA synthesis, coupled with recombinant techniques, can generate over 1 Mb of synthetically derived nucleotide sequence. DNA synthesis technology alone can produce customized sequences of up to 10 kb (known as a cassette) (Gibson et al. 2010a). Assembly of multiple cassettes using in vitro recombination techniques can create large synthetic DNA constructs (up to 150 kb). Larger constructs (>500 kb) can be achieved when in vivo recombination techniques are used (Gibson et al. 2008). Synthesizing small genomes synthetically is now possible. Bacteriophage and viral genomes (5–8 kb) can be created by synthetic oligonucleotides alone (Cello et al. 2002; Liu et al. 2012; Smith et al. 2003). Mitochondrial and chloroplast genomes have also been synthesized and assembled (16 and 242 kb respectively) (Gibson et al. 2010b; O'Neill et al. 2012). The *Mycoplasma mycoides* bacterial genome (1.08 Mb) is currently the largest published assembly. Importantly, the *M. mycoides* synthetic genome was shown to be biologically viable. This synthetic

genome was able to 'boot-up' and co-ordinate normal cell function when it replaced genomic DNA of a *M. capricolum* recipient cell (Gibson et al. 2010a).

Genome synthesis promises unrestricted editing of whole genome sequence. Presently, small autonomous or semi autonomous genetic circuits have been introduced into cells to perform a desired role (Havens et al. 2012; Tigges et al. 2009). Synthetic genomics strives to widen the scale and complexity of circuitry, ultimately delivering new novel phenotypes. Crucially, synthetic genomics must be partnered with CAD tools (such as SynBioSS) to ensure ease of hypothesis testing in silico before embarking on lengthy wet-lab experiments.

Synthetic genomics also enables global editing of cis-regulatory elements. This approach was recently trialed in a biologically active synthetic yeast chromosome. No gross changes to gene circuitry were attempted; rather 98 small elements (loxPsym sites) were introduced throughout the chromosome. When ectopically activated, these sites could initiate chromosomal deletion events (Annaluru et al. 2014). This demonstrated that incorporating small sequence additions by synthetic DNA synthesis can provide unprecedented control of chromosome architecture.

Synthetic genomics is still in its infancy. Technical problems of synthetic assembly exist. The error rate of DNA synthesis, even at 1×10^{-5} bp, is still problematic when synthesizing large nucleotide stretches (Carr et al. 2004). Such base misincorporations have shown to render entire genomic assemblies biologically inactive (Katsnelson 2010). Stability of constructs in host cells during in vivo recombination (e.g. *E. coli* or *Saccharomyces cerevisiae*) significantly limits assembly size. The *Prochlorococcus marinus* genome assembly (1.7 Mb) is currently the largest stably maintained synthetic construct (however it has not been proven biologically active) (Tagwerker et al. 2012). Furthermore small synthetic genomes that have shown to be biologically active were replicates of known genomes. DNA synthesis may support the introduction of novel genetic regions, however building functional gene circuits from the bottom up has been shown problematic (Katsnelson 2010).

8.10 Concluding Remarks

The ongoing progress in sequencing of algal genomes will permit annotation, comprehensive cloning and manipulation of genes, which altogether allow omics approaches to generate large-scale experimental datasets. This advancement will aid in identification of key regulators of metabolism and enables the eventual manipulation of cellular pathways. Synthetic biology combines the use of molecular tools with knowledge gained from systems level analysis of organisms to generate innovative experimental designs. For example, advances in long DNA synthesis make it possible to construct complex genetic circuits designed and informed by metabolic modeling and pathway analyses. With these advances, synthetic biologists have made tremendous progress on the construction of genetic circuits and even entire chromosomes.

The majority of synthetic biology efforts are focused on microbes as many of the most pressing problems, such as sustainability in food and energy production ultimately rely on modification of microorganisms. As such, synthetic modifications of algal strains to enhance desired physiological properties is likely needed to improve their productivity. There has been increasing efforts by synthetic biologists to push for the creation of accessible tools that would improve the potential of algal technology. With synthetic biology, still a young field, the future of this auspicious approach is clearly apparent. While much remains to be achieved to exploit the full potential of algae through various approaches, synthetic biology is likely to play a central role in this process in the coming years.

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Chapter 9
Molecular Genetic Techniques for Algal Bioengineering

Kenan Jijakli, Rasha Abdrabu, Basel Khraiwesh, David R. Nelson, Joseph Koussa and Kourosh Salehi-Ashtiani

Abstract The uniquely diverse metabolism of algae can make this group of organisms a prime target for biotechnological purposes and applications. To fully reap their biotechnological potential, molecular genetic techniques for manipulating algae must gain track and become more reliable. To this end, this chapter describes the currently available molecular genetic techniques and resources, as well as a number of relevant computational tools that can facilitate genetic manipulation of algae. Genetic transformation is perhaps the most elemental of such techniques and has become a well-established approach in algal-based genetic experiments. The utility of genetic transformations and other molecular genetic techniques is guided by phenotypic insights resulting from forward and reverse genetic analysis. As such, genetic transformations can form the building blocks for more complex genic manipulations. Herein, we describe currently available engineered homologous recombination or recombineering approaches, which allow for substitutions, insertions, and deletions of larger DNA segments, as well as manipulation of endogenous DNA. In addition, as reagent resources in the form of cloned open reading frames (ORFs) of transcription factors (TFs) and metabolic enzymes become more readily available, algal genetic manipulations can greatly increase the range of obtainable phenotypes for biotechnological applications. Such resources and a few case studies are highlighted in the context of candidate genes for algal bioengineering. On a final note, tools for computer-aided design (CAD) to prototype molecular genetic techniques and protocols are described. Such tools could greatly increase the reliability and efficiency of genetic molecular techniques for algal bioengineering.

Kenan Jijakli and Rasha Abdrabu contributed equally to this work.

K. Jijakli
Division of Engineering, New York University Abu Dhabi,
P.O. Box 129188, Abu Dhabi, United Arab Emirates

R. Abdrabu · B. Khraiwesh · D.R. Nelson · J. Koussa · K. Salehi-Ashtiani (✉)
Division of Science and Math, Center for Genomics and Systems Biology (CGSB),
New York University Abu Dhabi, P.O. Box 129188, Abu Dhabi, United Arab Emirates
e-mail: ksa3@nyu.edu

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9.1 Introduction

Microalgae have been described as nature's very own power cells and can provide alternatives to petroleum-based fuels without competing with food crops (Dismukes et al. 2008; Singh et al. 2011). The heterogeneity and diversity that algae evolved make the molecular mechanisms that different algae have adopted, along with manipulating those mechanisms of tremendous interest. Currently, research is being conducted to develop methods for genetic modification to introduce desirable traits into algae and to develop synthetic biology approaches to re-engineer algal cells (Ferry et al. 2011; Gimpel et al. 2013; Rabinovitch-Deere et al. 2013). The crux of this research is to advance the molecular biology techniques utilized for algae and to ease the modification of the molecular systems of the species of interest.

One powerful example is the alga *Chlamydomonas reinhardtii*. As a single-celled alga containing a single large chloroplast, *C. reinhardtii* represents typical soil green algae. Moreover, *Chlamydomonas* combines powerful genetics with the availability of unique genetic and genomic resources. All three genomes (nuclear, plastid, and mitochondrial) have been fully sequenced (Merchant et al. 2007); large mutant collections have been established; and all three genomes are amenable to genetic manipulation by transformation (Hippler et al. 1998; Neupert et al. 2009). Most tools required for systematic functional genomics studies are available in *Chlamydomonas*, including high-frequency transformation protocols (Kindle 1990), efficient methods for chemical and insertional mutagenesis (Dent et al. 2005), and workable protocols for RNA interference (RNAi) (Arif et al. 2013; Molnar et al. 2007; Zhao et al. 2007). Overall, this represents a great advance in the molecular techniques and methods, especially with their applications to algae.

In the absence of cell differentiation, some algae such as *Chlamydomonas* can provide a much simpler system for genetic manipulations compared with higher plants. Manipulation of microalgae by metabolic and genetic methods would both permit (1) selection of beneficial pathways redirecting cellular functions toward the synthesis of preferred products and (2) introduction of non-algal genes for the generation of algal recombinant proteins. The selection of favorable pathways may include increased resistance to environmental or stress changes on the culturing and life cycle of the algae, expedited biomass production, and excretion of valuable products. The potential of such system remains to be optimized as an alternative protein expression system.

In light of all these potentials, and particularly during the past two or three decades, algal biotechnology grew steadily into an important global industry with new entrepreneurs realizing the potential of algae. However, creating profitable industries out of microalgae still remains challenging, and perhaps the development of new molecular techniques might expedite microalgae's full industrial development, especially that some microalgal classes have highly complex genetic compositions rendering their modification arduous: Microalgal genome sizes range from 12.6 Mbp in *Ostreococcus tauri,* a Chlorophyta member, to 168 Mbp in the

Haptophyta *Emiliania huxleyi* and up to an impressive 10,000 Mbp for the Dinophyta *Karenia brevis* (Cadoret et al. 2012). Currently, a genome size of 10 Gbp precludes full genome sequencing, and as such, a lesser extent of knowledge would be available rendering the modification of such organisms a highly demanding task.

9.2 Molecular Genetic Techniques

In general, molecular genetic techniques are concerned with manipulating, reproducing, adding, and deleting DNA and RNA molecules in an organism. Manipulations, deletions, and additions are accomplished via genetic transformation and genetic editing, while reproduction is accomplished via cloning. There are also numerous other methods for genetic and genomic editing; however, transformation and cloning remain an integral part of even the most recently developed methods. Novel techniques are required to make those molecular changes easier to manifest, less time consuming, and more permanent in their effect.

9.2.1 Genetic Transformation

Genetic transformation entails introduction of foreign DNA into a cell (Gietz and Woods 2001) (Fig. 9.1). Genetic transformation has been applied to several algal strains, with *C. reinhardtii* obtaining the highest rates of transformation (Kindle 2004). Nuclear transformation of various microalgal species such as *C. reinhardti* is now routine (Walker et al. 2005).

Chloroplast transformation has plastid-specific challenges as compared to nuclear transformation. Nevertheless, chloroplast transformation has been achieved in green (*C. reinhardti*), red (*Porphyridium* sp.), and euglenoid algae (*E. gracilis*) (Wang et al. 2009a). Compared to nuclear transformation, chloroplast transformation has some advantages: primarily, production of high protein levels; the feasibility of expressing multiple proteins from polycistronic mRNAs; and gene containment through the lack of pollen transmission (Wang et al. 2009a). On a final note, attempts in specifically targeting the chloroplast genome of *C. reinhardtti* and achieving a multiple loci modification in vivo have been performed (O'Neill et al. 2012). The assembly of an *ex vivo* chloroplast genome using cloning in yeast cells was done targeting a set of genes involved in the photosynthesis pathway (O'Neill et al. 2012). Subsequently, chloroplast transformation was done to achieve the incorporation of genes altering the photosynthesis pathway, more precisely, photosystem II (Nelson and Ben-Shem 2004; Specht et al. 2010).

C. reinhardtii remains the only algal species in which mitochondrial transformation has been reported (Larosa and Remacle 2013; Remacle and Matagne 2004).

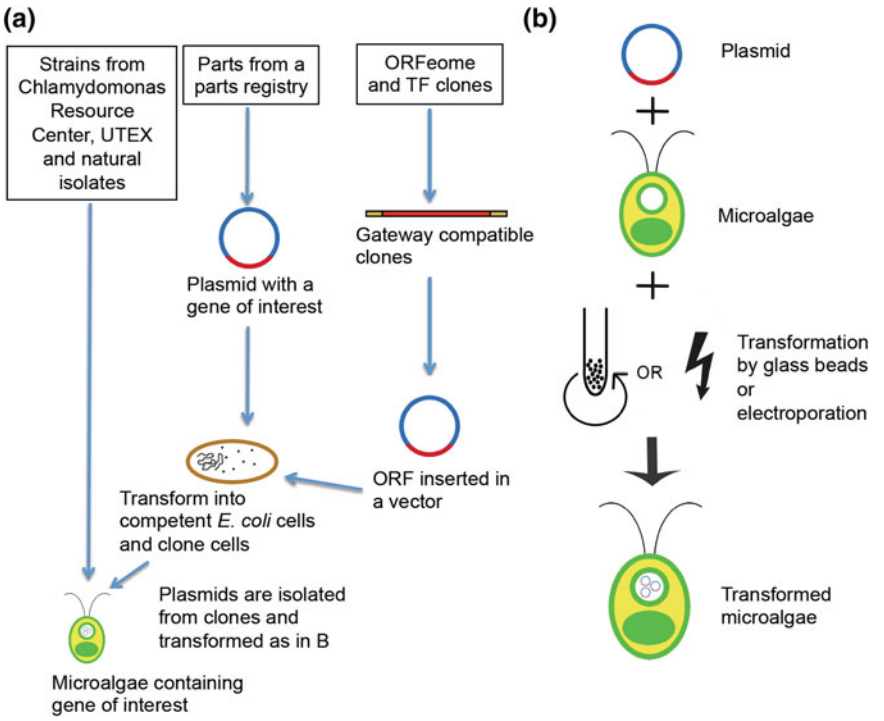

Fig. 9.1 **a** Transformation of microalgae starts with bacterial cloning to replicate the plasmid that is to be transformed into microalgae. The plasmids are then isolated from the cloning organism via DNA isolation techniques. **b** Transformation of microalgae can be performed by either vortexing glass beads in the presence of algal cells and DNA plasmids, or electroporating algal cells in a plasmid containing solution

Mitochondrial transformation is still not as common as nuclear or chloroplast transformations due to the small size of the mitochondria. This small size makes it difficult to deliver DNA into the organelles by methods that are used in other transformations. Another challenge that mitochondrial transformation faces is the absence of a relevant gene reporter. The presence of numerous mitochondria in each cell is also an obstacle for manifesting the transformed genotype at the level of the whole cell (Koulintchenko et al. 2012). Co-transformation with chloroplast or nuclear genes and initial selection for these markers is a possible work-around that facilitates the recovery of mitochondrial transformants (Remacle and Matagne 2004).

As for transformation methods, nuclear gene transfer can be achieved using various methods, including electroporation, agitation with glass beads or silicon carbide whiskers, particle bombardment, and agrobacterium vector infection (Table 9.1) (Guo et al. 2013). Lack of a cell wall in the recipient cells (e.g., *Dunaliella salina*)

Table 9.1 Comparison between different algae transformation methods

Methods	Procedure	Examples	Advantages	Disadvantages
Electroporation	Electrical charge is used to temporarily open pores in the cell membrane to allow foreign DNA to enter (Kindle 1990)	*C. reinhardtti* (Qin et. al. 2012), *Dunaliella salina* and *Chlorella vulgaris* (Guo et al. 2013)	Simple procedure used universally in different genera	Constrained in brown algae, physically destructive to cells
Glass beads	Cells are agitated vigorously with DNA in the presence of polyethylene glycol and glass beads	First reported in the soil alga *C. reinhardtii* (Kindle 2004)	Simple, requires no expensive equipment, hundreds of transformant lines in a single experiment	Constrained in macroalgae, inability to transfer DNA into cells with thick cell walls
Silicon carbon whiskers	Used instead of glass beads during the agitation period	*C. reinhardtii* (Kindle 2004)	No need to remove cell walls prior to treatment, inexpensive	Inhalation hazard
Agrobacterium	Cells are incubated with bacteria carrying the foreign gene (Banta and Montenegro 2008), foreign gene is cloned into a transformation vector (containing T-DNA region)	*C. reinhardtii*, *Dunaliella bardawil* and *Haematococcus pluvialis* (Anila et al. 2011; Kumar et al. 2004)	Highly efficient, simple, and stable	Technically challenging
Particle bombardment	DNA is precipitated or coated onto the surface of small particles; particles are shot into the target cells by a gunpowder charge or high-pressure helium	Used by most laboratories (Walker et al. 2005); used for chloroplast transformation (Wang et al. 2009a)	Exogenous DNA can be introduced into various cells and tissues	Low efficiency, specialized and high-cost equipment

or cell wall deficiency is sometimes necessary to achieve the highest rate of transformation. One way to weaken the cell wall in *Chlamydomonas* is pretreating them with the lytic enzyme autolysin. Autolysin is produced by *Chlamydomonas* itself through pre-incubation of the cells in a nitrogen-free medium to induce autolysin production, followed by collection of the produced enzyme. An alternative to using autolysin is using cell-wall-deficient mutant cells, such as cw15, for transformations (Walker et al. 2005).

9.2.2 Forward and Reverse Genetics

Forward genetics refers to the process of identifying a phenotype and then characterizing the genes that are involved in the phenotype's biological pathway or process through the screening of populations of a modified organism (Lawson and Wolfe 2011). Forward genetics was successfully used to determine genes involved in metabolic and regulatory pathways in *Chlamydomonas*. The availability of a *Chlamydomonas* linkage map and a protocol for genetic crossing, along with the complete genome sequence, has boosted the gene identification process (Gonzalez-Ballester et al. 2011). Modification of an organism for screening purposes can be achieved through random mutagenesis (UV irradiation or chemically induced) to generate mutant populations to be mapped. An alternative approach to obtain a mutant population in various microalgal species is insertional mutagenesis. Insertion of a DNA fragment into the coding region of a particular gene results in the disruption of a protein-coding sequence and the loss of function of that gene (Vuttipongchaikij 2012).

Reverse genetics involves starting with a known gene and then disrupting the function of that gene to produce a phenotype and gain insight into what that gene does. Besides disruption, overexpression of a gene is another option. The gene sequences within a given organism need to be known, and the goal here is to define the sequences' functions. In reverse genetics, mutations are made in specific genes or gene products to determine their effect on the organism, and from that determine the gene's function. The availability of complete genome sequences combined with reverse genetics can allow every gene to be mapped to a function (Ahringer 2006). Using available characterized or annotated genes, improved microalgal strains can be generated through reverse genetics by gene silencing or random mutagenesis or even through gene modification by homologous recombination (Vuttipongchaikij 2012). Gene silencing by RNAi has also been used successfully for reducing gene expression levels in microalgal systems and for controlling gene function (Rohr et al. 2004). RNAi is proving to be a very useful tool for reverse genetics. Still, the most dramatic way to reduce gene activity is to eliminate the gene entirely by deleting it from the genome. This can be achieved through homologous recombination (see Sect. 3.3).

9.2.3 Recombineering

Recombineering is an in vivo genetic engineering method that relies on the ability to induce homologous DNA recombination. Short for recombination-mediated genomic engineering, recombineering relies on bacteriophage homologous recombination proteins that induce and catalyze homologous recombination. These proteins are introduced into the cell by plasmids or bacteriophage vectors, are endogenously expressed, or are expressed in transformed strains. Being based on homologous recombination inducing proteins give recombineering the prime advantage of independence from the use of restriction enzymes to produce changes in DNA. Those changes in DNA can be in the form of insertions, deletions, or alterations through the introduction of a synthesized oligonucleotide substrate that contains the desired change and that replaces the native DNA (Court et al. 2002). To function properly, a recombineering system must have the ability to perform three tasks. First, it must be able to prevent nucleases from degrading the foreign oligonucleotides; second, it must have an exonuclease function to cleave the foreign DNA creating sites for DNA recombination; third, it must promote the annealing of DNA strands to ensure the foreign DNA recombines and is restored into the native DNA (Sharan et al. 2009).

One example of a system that provides all the requirements for recombineering is the λ Red system, commonly used for bacterial recombineering. The λ Red system consists of three bacteriophage recombination proteins from three respective genes: Gam protein produced by the gam gene; Beta protein produced by the bet gene; and Exo protein produced by the exo gene (Sharan et al. 2009). A simple description of the mechanism of the λ Red system is that the Exo binds to ends of the oligonucleotide and cleaves the 5′ DNA ends. This transforms the oligonucleotide to an oligonucleotide with a 3′ overhang. The Beta protein then binds to the overhangs and facilitates annealing with the native DNA, thus completing the recombination process (Fig. 9.2) (Datta et al. 2008).

The design of the produced DNA construct should depend on the ultimate objective of recombineering. Recombineering has been successfully used to insert genetic markers, retrieve DNA fragments, insert non-selectable markers, and produce point mutations (Court et al. 2002).

Homologous recombination has already been shown to be a viable mean of genetically engineering algae, albeit still at a lesser efficiency than bacteria. One such example is the utilization of homologous recombination to knockout nitrate and nitrite reductase genes in *Nannochloropsis* sp. In this case, the required proteins appeared to be expressed endogenously (Kilian et al. 2011). A study on homologous recombination in *C. reinhardtti* showed that recombination occurs readily between overlapping plasmids and requires around 230 homologous base pairs (bp) only, but is lacking when the recombination targets endogenous DNA (Gumpel et al. 1994). The required proteins appear to also be expressed endogenously, but the introduction of exogenous homologous recombination proteins increases the rate of recombination. Homologous recombination has also been demonstrated in

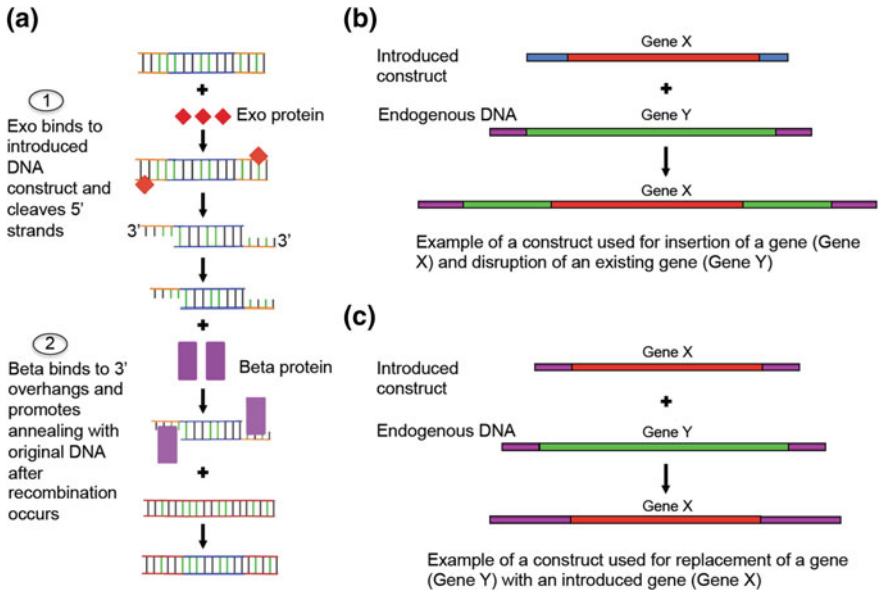

Fig. 9.2 **a** The mechanism of recombineering and exo and beta activity is illustrated. Exo creates overhangs, while beta promotes DNA annealing. **b** An example of a synthesized and/or foreign DNA construct that can be utilized to disrupt a gene and insert another via recombineering. **c** An example of another DNA construct used to replace a gene with another via recombineering

the multicellular green algae *Volvox carteri* (Hallmann et al. 1997) and in the red alga *Cyanidioschyzon merolae* (Minoda et al. 2004). Although those examples of homologous recombination in algae clearly show the complexity that arises from the significant difference in the mechanism of homologous recombination and its efficiency in different species, they also clearly demonstrate the viability of engineering a homologous recombination system (recombineering) in algae.

9.2.4 Clones and Other Biological Resources for Algal Modifications

The ability to alter gene expression, and as such, metabolic pathways within green microalgae, is of a crucial importance in optimizing those algae for biofuel production. Achieving such alterations and rational modifications of expression relies primarily on the identification of transcription factors (TFs), genes, and enzymes implicated in metabolic pathways driving the synthesis of oil and hydrogenated metabolites that can potentially be used as biofuels. Post-identification, via structural and functional annotations of genomes, the establishment of a library of cloned, ready to transform, open reading frames (ORFs) and TFs makes the

optimization process one step closer (Fig. 9.3). Attempts to clone the metabolic ORFeome of model green algae, *C. reinhardtti*, have been made and published by Ghamsari et al. (2011) and Chang et al. (2011). The structural annotation of the ORFs was based on models generated by the Joint Genome Institute (JGI) (Merchant et al. 2007) and AUGUSTUS gene prediction algorithm (http://augustus.gobics.de/predictions/chlamydomonas/). The outcome leads to the generation of Gateway-compatible clones that can easily be moved to a plethora of available expression vectors.

Another attempt, as part of an ongoing work, is the genome-wide cloning of TFs in *C. reinhardtti* to add a regulatory dimension to the available clone resources (unpublished data). The completion of such an endeavor will result in a library of TF clones that are Gateway compatible and readily available for transformation to

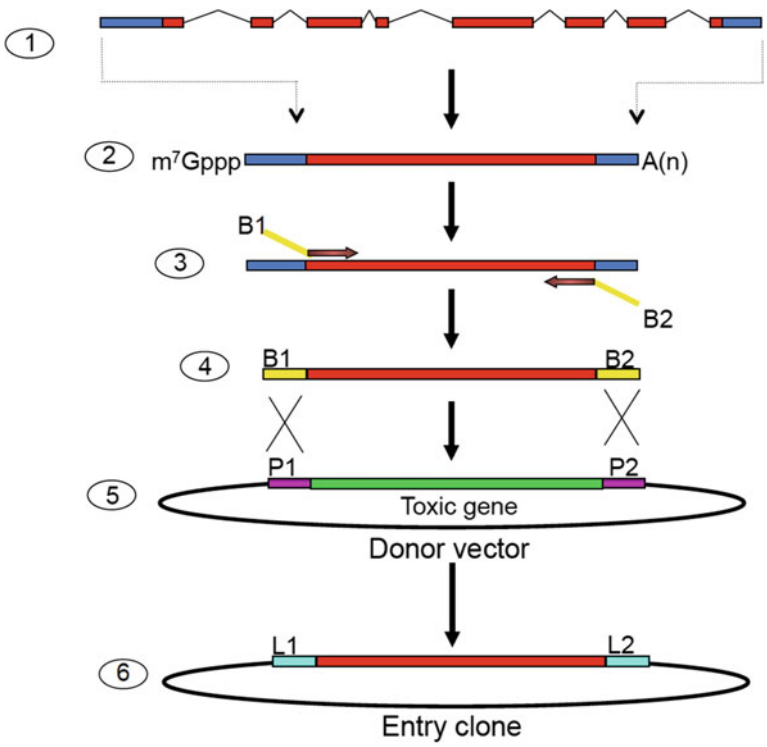

Fig. 9.3 Gateway recombinational cloning of ORFeome from mRNA (Ghamsari et al. 2011). Cloning from cellular mRNAs, which can be carried out in high-throughput formats, follows these steps: Introns from pre-mRNA are spliced out to generate mature mRNA by cellular mRNA processing machinery (*1*); mature mRNA with untranslated regions (UTR) and poly(A) tail is isolated (*2*); following reverse transcription, open reading frame (ORF) is PCR amplified from cDNA, adding Gateway tails and removing UTRs (*3*); generated ORF contains Gateway tails for recombinational cloning (*4*); Gateway recombinational reaction replaces vector's toxic gene with ORF (*5*); generated "entry clone" can donate ORF to "destination" vector with new functionalities (*6*)

alter and modify the expression of target genes. Clones can also be obtained from the *Chlamydomonas* Resource Center (http://www.chlamy.org/). The center hosts a multitude of *C. reinhardtti* strains, plasmids, molecular kits, and cDNA libraries.

The importance and relevance of cloning has been underlined as a major initial step in the process of algal optimization for biofuel production by making available a library of metabolic and transcription factor clones that can be used to modify the genetic information and expression patterns and, subsequently, directionally alter the metabolism in algal cells toward a higher yield of hydrogenated metabolites suitable for biofuel use.

9.2.5 Case Studies and Candidate Genes for Bioengineering

In order to increase productivity of biomass or a desired product, target genes have been selected and manipulated with success using transgenic approaches. These genes may comprise the metabolic pathways under consideration or be part of unrelated pathways that indirectly contribute to higher productivity. Highlighted below are some notable examples of target genes used for genetic manipulation along with examples of potential targets yet to be explored.

Much work has been done to genetically manipulate plants to produce more biomass or more of a specific tissue or compound. The Viridiplantae (green plants) include land plants and two lineages of algae, the chlorophytes and the charophytes (Finet et al. 2010). We therefore can look at gene targets in plants for possible research avenues when considering algae. A number of gene targets have already been picked and utilized with great success from previous knowledge of metabolic pathways. Fruit yield was increased in tomato (*Solanum lycopersicum* L.) when RNAi was used to decrease ascorbate oxidase activity (Garchery et al. 2013). Basically, the stress response to water or environment was down-regulated thus allowing more energy to be allocated for fruit production even in unfavorable conditions. Likewise, stress response genes in algae, such as those involved in oxidative stress (Perez-Martin et al. 2014) and light-related stress (Kukuczka et al. 2014), might be down-regulated to allow for more energy to be allocated to biomass production. One strategy to increase biomass is to manipulate photosynthetic light capture. Internal shading is a limiting factor in biomass production in algae, so reducing the size of the light-harvesting antennae may increase biomass production. However, pigment mutants of *Cyclotella* sp., a diatom, failed to outperform their wild-type counterparts in biomass productivity (Huesemann et al. 2009). The authors note that the mutagenesis procedures (chemical and UV) may have affected other metabolic processes that contributed to the *Cyclotella* sp.'s unremarkable growth and found no difference in growth of wild and mutated strains of this alga when grown parallel in raceway ponds. Green algae (e.g., *C. reinhardtii*) have evolved genetic strategies to assemble large light-harvesting antenna complexes (LHC) to maximize light capture under low-light conditions, with the downside that

under high solar irradiance, most of the absorbed photons are wasted as fluorescence and heat generated by photoprotective mechanisms.

An insertional mutant was obtained with a disrupted gene for the antennae protein TLA1 (Polle et al. 2003). Biochemical analyses showed the *TLA1* strain to be chlorophyll deficient, with a functional chlorophyll antenna size of photosystem I and II being about 50 and 65 % of that of the wild type, respectively. The *TLA1* strain showed greater solar conversion efficiencies and a higher photosynthetic productivity than the wild type under mass culture conditions (Polle et al. 2003). Other researchers have used RNAi to knock down the entire family of LHCs (Mussgnug et al. 2007). The resultant mutant, Stm3LR3, exhibited reduced levels of fluorescence, a higher photosynthetic quantum yield and a reduced sensitivity to photoinhibition. Cultures with these mutants have higher light penetrance, which may lead to more efficient biomass production. Another strategy to increase biomass production is to focus on increasing the rate at which the alga assimilates CO_2 from the atmosphere or through supplied gas, or to increase the efficiency of the carbon capture mechanism (CCM) (Stephenson et al. 2011). Genes that are involved in the CCM and are possible targets for genetic manipulation include an ABC transporter (Hla3/Mrp1 Cre02.g097800), two low-CO_2-inducible chloroplast envelope proteins (Ccp1 Cre04.g223300), an anion transporter (LciA/Nar1.2 Cre06.g309000), and ten different carbonic anhydrases (Winck et al. 2013). When the desired outcome is increased production of a specific product, increased biomass yield may or may not be desirable. For example, in engineering algal cells to increase lipid yield, biomass productivity is only important so far as it increases total lipid yield. A variety of genes have been manipulated with this aim with varying degrees of success (Stephenson et al. 2011). One of the most remarkable achievements in lipid production from *Chlamydomonas* occurred when researchers showed that *Chlamydomonas* deprived of a nitrogen source accumulates a high degree of lipids (Wang et al. 2009b). This effect is much more pronounced in mutants lacking the small subunit of a heterotetrameric ADP-glucose pyrophosphorylase (Zabawinski et al. 2001). The normal response of *Chlamydomonas* under nitrogen deprivation is to accumulate starch; however, the mutants, unable to accumulate starch, instead store energy as lipids (Wang et al. 2009b).

A recent study showed a 12 % increase in the total lipid content of the microalgae *D. salina* by transforming it with a bioengineered plasmid comprising specific parts, genes, and inducible promoters, driving the cellular carbon flux into the fatty acid biosynthesis pathway (Talebi et al. 2014).

In addition to lipid production, hydrogen production is also seen as a possible route to biofuel production in *Chlamydomonas* (Lehr et al. 2012). Increasing hydrogen production does not necessarily follow an increase in biomass; instead, researchers usually aim to increase photosynthetic efficiency or force the cells to more readily assume an anaerobic state.

RNAi knockdown of light-harvesting proteins was found to increase H_2 production in the high-H_2-producing *C. reinhardtii* mutant Stm6Glc4 (Oey et al. 2013). Oey et al. (2013) also stated that the overall improved photon-to-H_2 conversion efficiency is due to (1) reduced loss of absorbed energy by non-photochemical

quenching (fluorescence and heat losses) near the photobioreactor surface, (2) improved light distribution in the reactor, (3) reduced photoinhibition, (4) early onset of HYDA expression, and (5) reduction of O_2-induced inhibition of HYDA (Oey et al. 2013). Rubisco has also been used as a target for genetic manipulation to increase hydrogen production. The Rubisco mutant Y67A accounted for 10- to 15-fold higher hydrogen production than the wild type under the same conditions (Pinto et al. 2013). In conclusion, a variety of gene targets are available in algae that when manipulated may increase biomass and biofuel productivity.

9.3 Computer-Aided Design (CAD)

The advent of computer-aided design in biology holds the promise of greatly increasing the efficiency of biological manipulation and experimentation while easing the process of design and optimization. The hope is that CAD will allow the design and analysis of plasmids, vectors, protocols, and synthetic pathways with a minimal need for laboratory experiments.

Classically, tools used in genetic engineering perform singular specific tasks such as codon optimization, primer design, and ribosome binding site (RBS) design to optimize DNA constructs. A more inclusive set of computational tools combines multiple DNA components' design and optimization operations via a single toolset. One example of such a tool is ApE (http://biologylabs.utah.edu/jorgensen/wayned/ape/). ApE is a plasmid editor that, among many things, clearly highlights the different relevant features of a plasmid (restriction sites, ORF, Dam/Dcm methylation sites, etc.), shows the protein translation, creates plasmid graphics and maps, performs a virtual restriction digest, selects sites matching a given criteria, and performs primer design based on an inputted criteria. Other inclusive tools include Gene Designer 2.0 (https://www.dna20.com/resources/genedesigner), which performs gene, operon, vector, and primer design along with codon optimization. It also performs protein translation and restriction site modification using a graphical-based interface. A similar toolkit to Gene Designer 2.0 is Gene Design. Gene Design is, however, available via a Web interface (http://54.235.254.95/gd/). Finally, Gene Composer is yet another interesting tool worth mentioning (http://www.genecomposer.net). Gene Composer is a CAD-based software that allows alignment generation and constructs design, gene optimization, and assembly (Medema et al. 2012; Zabawinski et al. 2001).

As for molecular and synthetic biology centric computational tools, there are already a few software suits and CAD packages that include tools to aid in biological design. One such tool is Genome Compiler (http://www.genomecompiler.com), an advanced genomic design software package. Besides facilitating, viewing, and editing DNA as constructs, protein translation, and circular views, Genome Compiler allows viewing and editing a DNA construct on a functional basis. Additionally, searching and importing DNA constructs via the NCBI database is supported.

A similar CAD toolkit to Gene Compiler is GenoCAD (http://www.genocad.org). GenoCAD, however, has the added benefit of DNA construct simulation. This allows for quick performance testing of designed constructs. Other CAD tools that are focused on DNA constructs and biological parts design include TeselaGen (https://www.teselagen.com), Clotho (http://www.clothocad.org), and SynBioSS (http://synbioss.sourceforge.net). Both TeselaGen and Clotho offer genetic function centric DNA editing and construction tools. They also aid in the creation of DNA constructs and parts' databases and the utilization of existing databases. Likewise, SynBioSS allows for the design and simulation of DNA constructs and biological parts, but does that through reconstructing reaction networks from a series of genetic parts that are user defined and strung together. Another CAD toolkit that allows for DNA construct simulation, in addition to DNA design and editing, is TinkerCell (http://www.tinkercell.com). TinkerCell is built with the ambition of having optimized CAD-based biological designs feed into laboratory automation tools, and thus easing the design, experimentation, and synthesis aspects of molecular and synthetic biology. Currently, TinkerCell's analysis capability includes a plethora of deterministic and stochastic simulation and analysis options (Medema et al. 2012).

The CAD tools discussed thus far offer powerful design capabilities, but the designer must keep in mind the limitations inherent with each tool. Biological interactions are not yet fully understood, and the models and simulations of such interactions, as provided by the CAD tools, are limited by the assumptions rooted in those tools.

9.4 Conclusion

Genetic molecular techniques have allowed for tremendous progress in the fields of biology and bioengineering. The ability to manipulate, replicate, and modify DNA, RNA, proteins, and organisms via transformation techniques, cloning, and gene-editing tools has allowed for powerful biological insights and applications development. As is already apparent, the future of molecular techniques lies in developing more robust editing tools, simplifying high-throughput techniques and adopting more automatable techniques. Also, the development of multiplexing techniques, techniques that are able to perform multiple manipulations at once, will allow for great progress in discovery.

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Chapter 10
Toward Applications of Genomics and Metabolic Modeling to Improve Algal Biomass Productivity

Kourosh Salehi-Ashtiani, Joseph Koussa, Bushra Saeed Dohai, Amphun Chaiboonchoe, Hong Cai, Kelly A.D. Dougherty, David R. Nelson, Kenan Jijakli and Basel Khraiwesh

Abstract Genomic sequencing is the first step in a systems level study of an algal species, and sequencing studies have grown steadily in recent years. Completed sequences can be tied to algal phenotypes at a systems level through constructing genome-scale metabolic network models. Those models allow the prediction of algal phenotypes and genetic or metabolic modifications, and are constructed by tying the genes to reactions using enzyme databases, then representing those reactions in a concise mathematical form by means of stoichiometric matrices. This is followed by experimental validation using gene deletion or proteomics and metabolomics studies that may result in adding reactions to the model and filling phenotypic gaps. In this chapter, we offer a summary of completed and ongoing algal genomic projects before proceeding to holistically describing the process of constructing genome-scale metabolic models. Relevant examples of algal metabolic models are presented and discussed. The analysis of an alga's emergent properties from metabolic models is also demonstrated using flux balance analysis (FBA) and related constraint-based approaches to optimize a given metabolic phenotype, or sets of phenotypes such as algal biomass. We also summarize readily available optimization tools rooted in constraint-based modeling that allow for optimizing bioproduction and algal strains. Examples include tools used to develop knockout strategies, identify optimal bioproduction strains, analyze gene deletions, and

Joseph Koussa and Bushra Saeed Dohai contributed equally to this work.

K. Salehi-Ashtiani (✉) · J. Koussa · B.S. Dohai · A. Chaiboonchoe · H. Cai
K.A.D. Dougherty · D.R. Nelson · B. Khraiwesh
Division of Science and Math, and Center for Genomics and Systems Biology (CGSB), New York University Abu Dhabi, P.O. Box 129188, Abu Dhabi, United Arab Emirates
e-mail: ksa3@nyu.edu

K. Jijakli
Division of Engineering, New York University Abu Dhabi, P.O. Box 129188, Abu Dhabi, United Arab Emirates

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explore functional relationships within sets in a metabolic model. All in all, this systems level approach can lead to a better understanding and prediction of algal metabolism leading to more robust and cheaper applications.

10.1 Introduction

Algal research gained its first round of momentum, beyond the scientific community, in 1978 with the launch of the Aquatic Species Program to explore alternative transport fuel sources (Sheehan et al. 1998). As many laboratories started focusing their investigations entirely on algal systems, the amount of data available increased at a near exponential pace. In particular, the development of next-generation sequencing platforms has rapidly and dramatically advanced and increased the amount of available data on algal genomes. New high-throughput phenotypic platforms have made metabolic characterizations broader and more rapid. As in other fields of biological research, integration of disparate data types, as well as contextualization of data remains a central challenge that is addressed by the field of systems biology. Systems level understanding of metabolism is needed for prediction of biomass and bioproduct optimization strategies, for algae, or for any other organism because of the wide expanse and the high degree of interconnectivity in metabolic networks.

Evolutionarily, the term algae describe a polyphyletic group of organisms and is contentious in definite meaning (Pröschold and Leliaert 2007). Currently, in some classifications, it includes superphyla in several separate lineages: stramenopiles which include brown, golden, and yellow algae and diatoms; rhodophyta or the red algae; photosynthetic alveolates, such as dinoflagelates; and the viridiplantae which include green algae (Barton et al. 2007). The phylogenetic classification *green algae* describe the presumed plant predecessors with photosynthetic capabilities, and a characteristic green color (Besche et al. 2009; Harris 2001). Both green algae and diatoms have shown great potentials as sustainable sources of biofuel, biomass, and bioproducts; however, *Chlamydomonas reinhardtii*, due to its position as a well studied representative green alga (Harris 2001), has received special interest as a model organism for genomic and metabolic studies.

The interest in algal exploration has steadily increased as commercial and large-scale production of lipid-producing algae has provided a practical importance for the research, with particular demand on more integrated goals, i.e., optimizing algae for biofuel production, optimizing growth of strains of interest and achieving economical viability all at the same time (Koussa et al. 2014). The metabolic optimization of algal organisms, which requires extensive characterization of algal metabolic circuitry, calls for an integrative "systems level" approach. This begins with genome sequencing, which then provides the "parts list" for reconstruction of a metabolic network, and finally ends with the ability to make model-based predictions (Fig. 10.1). In this chapter, we first review microalgal sequencing efforts

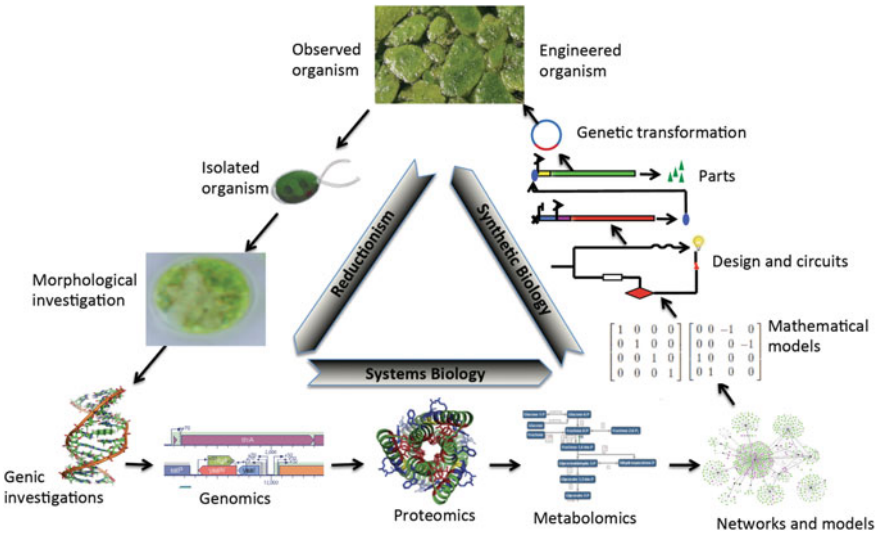

Fig. 10.1 Schematic diagram representing the relationships between the reductionist, systems, and synthetic biology approaches. Through reductionism, a "top-down" approach leads to characterization of few or individual target genes. The collective knowledge gained through individual studies provides the framework for developing large-scale methodologies, building knowledgebases, and executing "bottom-up" omics studies, with the results integrated to describe the emergent properties of the system. Investigations of the models, constraints, and simulations provide predictions, which are implemented through an engineering approach, using biological parts or devices, for synthesis of a desired biological outcome

that are in progress or have been completed, we then outline metabolic network reconstruction and constraint-based analyses, and to conclude, we briefly describe some computational tools that are used in metabolic modeling, which can aid the design of engineering experiments and optimization of cellular metabolic outputs.

10.2 Microalgal Genome Projects

The nuclear genome of *C. reinhardtii* was published in 2007 (Merchant et al. 2007) after the first wave of next-generation sequencing (NGS) became commercially available in 2005 (Margulies et al. 2005; Shendure et al. 2005). Nevertheless, the *Chlamydomonas* genome was sequenced through a conventional shotgun sequencing and assembly pipeline with 13X coverage (Merchant et al. 2007). Following the completion of *Chlamydomonas* genome sequencing, *Thalassiosira pseudonana*, a diatom, was the first eukaryotic marine alga that was sequenced (Bowler et al. 2008). A draft genome sequence of *Nannochloropis gaditana* was also made available in 2012 (Radakovits et al. 2012).

The continued development of NGS platforms, among them Illumina, and Ion Torrent semiconductor sequencing in the main stream, have brought down the time, effort, and cost of genome sequencing well beyond the exponential drop predicted by Moore's law (Moore 1998). This enabled the sequencing of many new algal genomes (Fig. 10.2). The main limitation of NGS has been that the relatively short read length (50–500 bp) introduces inaccuracy in the assembly of sequences (Zhang et al. 2011). Furthermore, the high demand on bioinformatics analysis due to the increased data volume by several orders of magnitudes (Morey et al. 2013) introduces challenges in the use of NGS, particularly when the investigators do not have access to high performance computing infrastructure and appropriate bioinformatics support. Third-generation sequencing (TGS) technologies are being developed to address these problems. For instance, single molecule real-time (SMRT) sequencing makes the whole genome sequencing of single cells from uncultivable organisms possible (Schadt et al. 2010). Many more TGS technologies are expected to be on the way. Moreover, user-friendly software such as the CLC Genomics Workbench (CLC bio, a QIAGEN Company, Denmark) is enabling investigators to carry out genome assembly without the need of high performance computers or dedicated informatics specialists.

While advances in technology will ultimately lead to the generation and in-depth analysis of sequenced genomes, this would still be an initial step to be

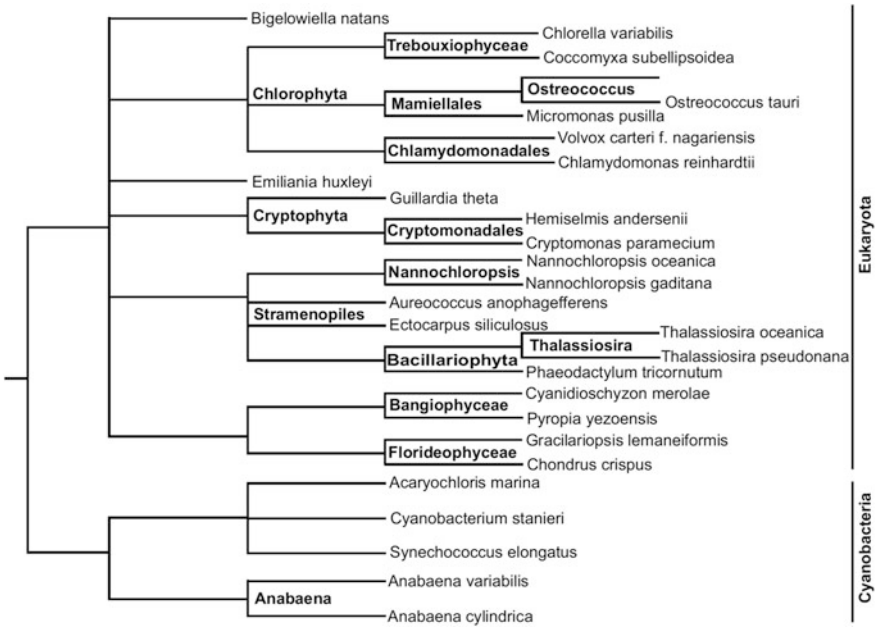

Fig. 10.2 Phylogenetic tree representing algal species with available genome sequences or ongoing genome sequencing projects. Data presented are available at the NCBI genome database (http://www.ncbi.nlm.nih.gov/) and the AlgaeBASE website (http://www.algaebase.org/)

complemented by transcriptomic, proteomic, and metabolic analysis in order to reach a better understanding of the system per se. The integration of all of these levels of analyses, compiling them into a predictive model, and describing the interactions between their respective components, is in fact the main feat of systems biology. In such endeavors, metabolic network models occupy a central and key position in advancing bioproduct optimization.

10.3 Metabolic Network Models

Metabolic models build a characteristic description of the cell's phenotypic state and give insights into systems' emergent properties with respect to metabolic functions, adaptability, robustness, and optimality. Moreover, a metabolic model serves as a basis to investigate questions of major biotechnological importance, such as the effects of directed modifications of enzymatic activities to improve a desired property of cellular systems (Alper et al. 2005). Reconstruction of genome-scale metabolic models has led to a better systems level understanding of microbial metabolism, bridging the genotype-phenotype gap. The steady increase in the number of new genome-scale metabolic models over the past decade is clear evidence of their utility in investigating biological systems for many applications, including those with applicability for pharmaceutical, chemical, and environmental industries (Feist and Palsson 2008).

Soon after the release of *Chlamydomonas*'s genome sequence in 2007, a number of groups began the reconstruction of metabolic network models for this alga, resulting in the reconstruction of its central metabolic network in 2009 (Boyle and Morgan 2009; Manichaikul et al. 2009). Two years later, genome-scale reconstructed networks of *Chlamydomonas* were released by two groups independently. Chang et al. (2011) published a genome-scale metabolic network model for *C. reinhardtii*, *i*RC1080, describing and accounting for $\sim$2000 reactions, $\sim$1000 metabolites and over 1000 associated gene products. Dal'Molin et al. (2011) described a slightly smaller genome-scale reconstruction (AlgaGEM), which encompassed $\sim$1700 reactions, $\sim$900 genes, and $\sim$1900 metabolites. Both are constraint-based models that can predict genome-scale reaction fluxes under steady state growth conditions, as well as a wide range of other metabolic outcomes (see Sects. 10.4 and 10.5 for more details on steady state models).

The iRC1080 model allows for quantitative growth prediction for a given light source. This was accomplished by setting up new reactions that treat light as metabolites. More precisely, reactions for the absorption of light by photosystem I and II (as well as other light driven reactions such as vitamin D3 synthesis and photoisomerase) were defined with their wavelength specificities and stoichiometries. The introduced light reactions can accept different values corresponding to different light intensities the cell is exposed to. In summary, the absorption of photons drives photosynthesis and other reactions according to specified absorption coefficient, stoichiometry of the absorbed photon, and wavelengths. The described

"light reactions" of the model were experimentally validated by photobioreactor growth studies under different light sources and intensities, i.e., photon fluxes, demonstrating the general agreement of actual biomass and oxygen yields with those predicted by the model (Chang et al. 2011).

The aforementioned network models (i.e., iRC1080 and AlgaGEM) can greatly facilitate future developments of network reconstructions for other species of green algae by providing a framework that can be modified according to the alga's species-specific metabolic properties. We note that nongreen algal groups, such as diatoms which are evolutionarily distant to green algae, are likely to have distinct metabolic processes relative to green algae, differing in metabolic wiring and presence or absence of various subsystems in the network. The reconstruction of metabolic networks for these organisms is likely to require a significant adjustment of the existing green algae models, if these were to be used as the framework.

10.4 Reconstruction of Genome-scale Metabolic Network Models

How are metabolic network models reconstructed? A metabolic network consists of metabolites, biochemical reactions, and the relevant genomic evidence for the described enzymatic reactions, or gene-protein-reactions (GPRs) associations . The structural framework of a genome-scale metabolic model begins with compilation of relevant gene annotations and ends with refinement of the reconstructed metabolic network. This reconstruction process passes through four blocks (Fig. 10.3) (Orth et al. 2010; Thiele and Palsson 2010) as described in the sections below.

10.4.1 Draft Reconstruction

In this step (Fig. 10.3a), stoichiometric reactions that can describe cellular metabolism using various sources of information are compiled. These data may be collected from different knowledgebases, including BiGG (http://bigg.ucsd.edu) (Schellenberger et al. 2010), KEGG (http://www.genome.jp/kegg/), MetaCyc (http://www.metacyc.org), and peer reviewed literature. Through genomic and bioinformatics approaches, the functional annotations of open reading frames (ORFs) provide genomic evidence for the presence of specific biochemical reactions, associating genes, or multiple genes with specific reactions in the network, for generating the gene-protein-reaction associations. Gene products are associated with specific reactions through assigned enzyme commission (EC) numbers. This process may include sequence-based searches of the ORFs against well-curated databases such as UniProt (http://www.uniprot.org) (Apweiler et al. 2004) or through profile-based scans (e.g., InterPro, http://www.ebi.ac.uk/interpro/) (Jones et al. 2014) to assign enzymatic function and EC numbers to the ORFs.

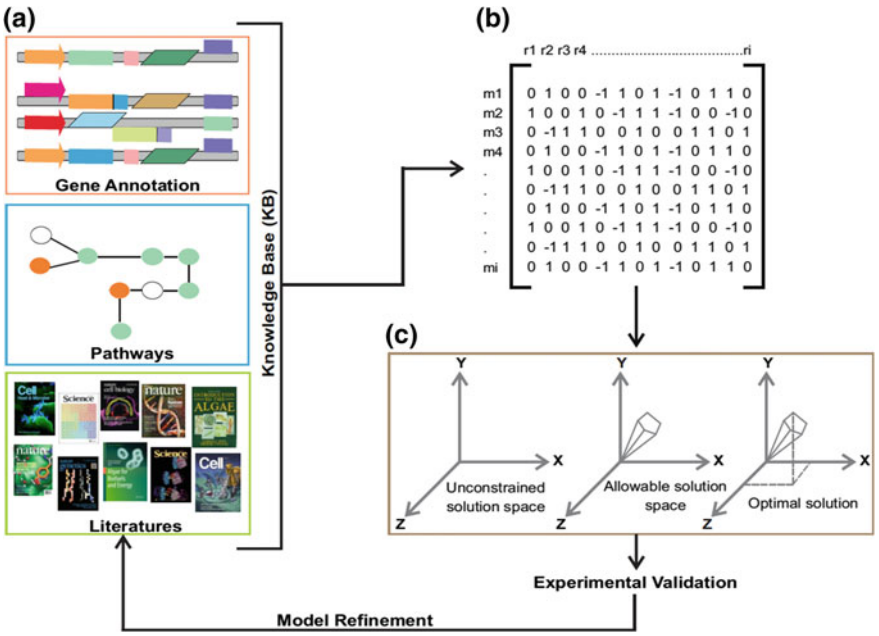

Fig. 10.3 Metabolic model reconstruction and refinement. **a** Information from one or more knowledgebases is extracted to define reactions and pathways to reconstruct a draft model; **b** the draft network model is transformed into a stoichiometric matrix that maps the metabolites and the associated reactions; **c** the obtained mathematical representation of metabolism is constrained with key flux parameters and can be optimized for an objective function. The obtained optimal solution is then validated by experimental data; differences between the two are reconciled by refining the initial model through filling gaps, adding and removing metabolites, obtaining additional experimental evidence

With enzymatic functions assigned, metabolic reactions can be defined which in turn allows reconstruction of a draft metabolic network. The draft network also accounts for metabolites that contribute to biochemical reactions inside the cell.

10.4.2 Mathematical Representation

Once the draft network is ready, a mathematical formulation of the network is made through forming a stoichiometric matrix (**S**). In the matrix (Fig. 10.3b), rows correspond to metabolites, and columns assign reactions. The entries in the matrix correspond to the stoichiometric coefficients of each individual metabolite that contributes to each reaction. A negative coefficient indicates the consumption of a metabolite in a reaction. A positive coefficient indicates the production of a metabolite in a reaction. A zero coefficient is for metabolites that have no contribution to the reaction.

10.4.3 Model Validation with Experimental Data

Many methods can be used to validate the model's proposed predictions; however, comparison of in silico predictions with in vivo experiments is a key method for validation. Measuring experimental growth phenotypes at specific conditions can validate the predicted growth under the same conditions. An alternative validation approach is to carry out in silico and in vivo gene deletion experiments (or to compare in silico results with available gene deletion literature) to check whether there is an agreement between model predictions and the actual deletion mutant phenotype. Moreover, omics data from transcriptomics, metabolomics, and proteomics experiments can be used to check the consistency of the model's predicted results. Available simulation debugging tools may be used if the model has poor agreement with experimental data. Further refinement of the model can be done as described in the next section.

10.4.4 Network Refinement and Gap Filling

Network refinement (Fig. 10.3c) can be viewed as reconciliation between the content of the model and the available biochemical and genomic data, with the end result of enhancing the reconstructed network. This reconciliation is done based on agreements of model simulations and updated genomics, physiological, and biochemical knowledge. A crucial step in the reconstruction of genome-scale metabolic models is filling the gaps to decrease the number of dead-end metabolites and improve network connectivity. Metabolic network gaps are filled by the addition of reactions that are missing in the network yet have corroborating evidence for their existence in the system. These may include spontaneous reactions that are not associated with gene products as well as extracellular and intracellular transport reactions and exchange reactions.

Models may not predict the production of biological compounds with existing biochemical evidence if the prerequisite genes have not been added to the model. Manichaikul et al. (2009), using *Chlamydomonas* as a model, described how genomic data can be used to fill gaps in metabolic models. In their approach, not only genomics and other experimental evidence contributed to the refinement of the network, but also the model itself *informed* "genomics," of the presence of missing annotations, justifying the use of more sensitive sequence search and annotation tools to recover the missing genes. One example that can illustrate this is lactate dehydrogenase (LDH), which initially was absent from the *Chlamydomonas* gene annotation, yet the model reconstruction showed the need for the LDH enzyme in the *Chlamydomonas* pyruvate metabolism pathway. A PSI-BLAST analysis was carried out to identify the gene encoding LDH; the gene was subsequently added to the model. Additionally, orphan genes, or those biochemically characterized

metabolic enzymes lacking sequence data, can be assigned GPRs by reviewing metagenomic sequence data to provide sequences for the missing enzymes. This approach has been experimentally validated (Yamada et al. 2012).

10.5 Constraint-based Models and Flux Balance Analysis

Edward and Palsson developed the first genome-scale metabolic reconstruction in 1999 for *Haemophilus influenza* (Edwards and Palsson 1999) and described its emerging systems properties through flux balance analysis (FBA). In FBA, fluxes of metabolites through biochemical reactions are constrained by four parameters: mass conservation, thermodynamics (reaction reversibility), steady state assumption for internal metabolite concentrations, and nutrient availability. All of these constraints define the boundary conditions required to solve systems of linear equations in which a biologically motivated objective function (e.g., biomass production) is optimized.

The solution of an FBA problem is the optimal distribution of fluxes through the metabolic network, comprising a metabolic phenotype or functional state of the network (Orth et al. 2010). Assumptions and boundary flux parameters can reduce the size of the feasible solution space. An objective function, which is a set of reactions that the cell is assumed to be optimizing for a given mode of growth, can be mathematically (through linear optimization) maximized (or minimized) to derive flux values that support optimal solution(s) for the metabolic state under investigation. The solution is of course sensitive to growth and boundary parameters for a given model. As an example, iRC1080 reconstruction of *C. reinhardtii* metabolic network (Chang et al. 2011) was optimized for biomass production under two different conditions of growth, with light and acetate, and dark with acetate. As the alga grows in the dark, it relies solely on acetate as a source of energy and carbon, while photosynthesis provides both under light growth. Consequently, major flux redistributions can be expected system-wide and are observed between simulated growths under these two conditions (Fig. 10.4).

Metabolic models can make use of high-throughput data such as gene expression data (including mRNA and protein expression data), and ^{13}C flux data by directly imposing additional constraints on the metabolic model based on the values obtained from wet-bench experiments. For example, if obtained experimental data show that glycolytic enzymes are highly active under certain conditions, flux can be pointed through glycolysis by constraining the relevant fluxes in silico, thus driving flux through the activated reactions and allowing estimation of changes in global flux distributions (Shlomi et al. 2005). Phenotypic data, such as Biolog data (Bochner 2003, 2009), which describe cellular metabolic profiles, can also be used to validate in silico phenotypes (Oberhardt et al. 2008). Notebaart and his colleagues used *Saccharomyces cerevisiae* as a model organism for this type of analysis. In their work, they carried out a comparison of in silico metabolic fluxes versus microarray gene expression data in *E. coli* and *S. cerevisiae*. Their results revealed that metabolic genes whose fluxes are directionally coupled generally

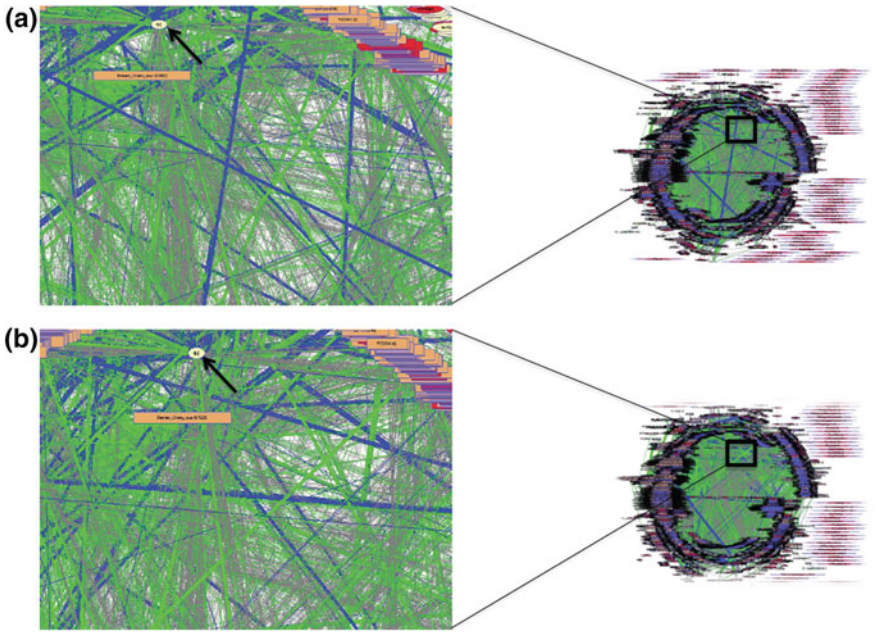

Fig. 10.4 Simulated flux distribution in *Chlamydomonas* metabolic network (iRC1080) under two different aerobic conditions. Major flux changes are shown as growth condition is shifted from light with acetate (**a**), to dark with acetate (**b**). The *left panels* in both **a** and **b** show a region of the network that includes cytosolic proton fluxes in biomass production reactions; *right panels* show the entire network. *Arrows* designate cytosolic protons, *blue lines* represent reverse fluxes, *green lines* represent forward fluxes, *gray lines* represent no fluxes. Visualizations were done using Paint4Net tool (Kostromins and Stalidzans 2012)

show similar expression patterns, share transcriptional regulators, and reside in the same operon (Notebaart et al. 2008).

Flux variability analysis (FVA), which is a variant of FBA, determines the maximum and minimum values of all the fluxes that will satisfy the constraints and allow for the same optimal objective value (Gudmundsson and Thiele 2010). For example, FVA can be applied to predict the range of possible by-product production rates under maximal biomass production, which can be linked to gene expression data. Variations of FVA can also be used to determine blocked or nonessential reactions.

10.6 Common Optimization Tools for Constraint-Based Models

Several integrated toolsets are available for constraint-based analysis. These include Pathway Tools, COBRA Toolbox, SNA, and MetaFluxNet (Table 10.1). The most widely used tools are Pathway Tools and COBRA Toolbox, with COBRA offering

Table 10.1 Software tools available to carry out constraint-based analysis

Software/tool	URL	Description/uses
Pathway tools	http://bioinformatics.ai.sri.com/ptools/	Software includes tools including: model organism databases, flux balance analysis, visualization of metabolic network and omics datasets, and model refinement including dead-end metabolites
COBRA toolbox	http://opencobra.sourceforge.net/openCOBRA/Welcome.html	MATLAB software package for extensive analysis of networks using; FBA, FVA, gene deletion, Monto Carlo sampling, metabolic network visualizations, and gap filling
MetaFluxNet	http://metafluxnet.kaist.ac.kr/Default.aspx	Program package that allows users to interpret and examine metabolic behavior in response to genetic or environmental modifications
CellNetAnalyzer	http://www2.mpi-magdeburg.mpg.de/projects/cna/cna.html	MATLAB software package for structural and functional analysis of cellular networks
SNA	http://www.bioinformatics.org/project/?group_id=546	Mathematica toolbox for analyzing fluxes of metabolic network at steady state by linear programing
PathwayAnalyser	http://openwetware.org/wiki/Chandra:Software_and_Databases:PathwayAnalyser	Software for flux balance analyses and simulations on SBML models

the largest number of tools. A few of these tools (mostly available in COBRA) are described below (Fig. 10.5).

10.6.1 Strain and Bioproduct Optimization Tools

In order to optimize strains for higher production yields of desired products or compounds, computational tools have been developed and employed to guide the design of knockout-strains with modified metabolic pathways leading to an increase in the bioproduct production. Two major examples of such tools are OptKnock (Burgard et al. 2003) and OptStrain (Pharkya et al. 2004). The OptKnock tool, for example, was used to identify gene knockout strategies leading to the optimization of bacterial strains for higher yields of lactate, succinate, and 1, 3-propanediol productions. In a similar approach, the Optknock tool has also allowed the

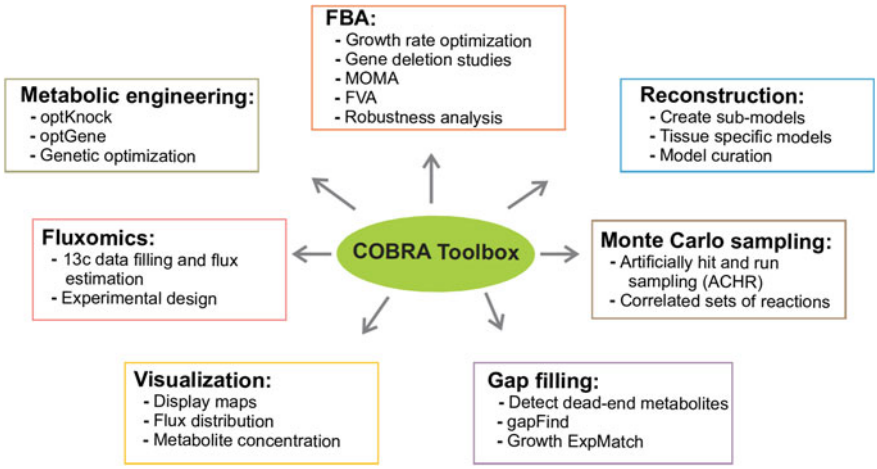

Fig. 10.5 Schematic representation of the constraint-based reconstruction analysis (*COBRA*) tools. The software was developed for systems biology researchers interested in cellular metabolic analysis. COBRA toolbox implements methods of constraint-based modeling of genome-scale models and is considered as a standard framework for constraint-based modeling of metabolism. There are seven categories of COBRA methods including FBA, visualization, reconstruction, gab filling, Monte Carlo sampling, fluxomics, and metabolic engineering as illustrated

optimization of *E. coli* strains to achieve a higher production of several amino acids and has set in place a set of knockout strategies to achieve this goal (Pharkya et al. 2003). Pharkya et al. have established several different gene knockout combinations with each resulting in an increased production of a specific amino acid. For example, a knockout of three genes coding for three enzymes, namely pyruvate dehydrogenase, pyruvate formate lyase, and an ATPase, has allowed the cells to achieve a production of 14.95 mmol/g DW h of alanine. Alternatively, another strategy involving the knockout of a fourth enzyme, phosphofructokinase, on top of the previously mentioned genes, has increased the alanine production to 18.53 mmol/g DW h. Although the actual amino acid production has increased, the growth rate of the organism has sharply decreased and the choice of which strategy to employ remains subject to the investigator's preferences and judgment, and is limited by the experimental setup limitations.

Lastly, OptStrain is a tool that can help identify the optimal strains producing the desired metabolite by determining the best substrate choices and genes that need to be deleted or over-expressed in order to achieve the increase in metabolite production. This strategy ensures high growth rates while optimizing the production of the desired bio-product.

A different approach, metabolic transformation algorithm (or MTA) (Yizhak et al. 2013), can potentially be used to shift the phenotypic state of algae from, e.g., growth with low lipid production, to a state at which lipids are produced at high rates with or without nutritional stress. Yizhak and colleagues introduced this

algorithm that was used to predict gene knockouts that can shift metabolism from a given "source" state to a desired "target" state. The approach uses gene expression profiles of the two states in predicting gene deletions that forces changes in the flux distribution of source state to match the desired target state. While the authors used the yeast system to validate their algorithm, the approach is not system-, or state-specific, and is applicable to any organism, and many different phenotypic states. This algorithm presents a potentially exciting method for altering algal metabolism from a nonproductive to a productive state.

10.6.2 Gene Deletion Analysis Tools

A gene deletion experiment and its effect on cellular behavior can be simulated in a manner similar to the linear optimization of growth (or for any other objective function). The results can be used to guide the design of metabolic engineering strategies. Gene–reaction associations in the model describe the relationship between genes and their corresponding reactions; therefore, reactions can be removed from the model on the basis of individual gene deletions. The possible results from a simulation of a single gene deletion are unchanged maximal growth (nonessential), reduced maximal growth, or no growth ("sick" or lethal effect). The gene deletion analyses can be carried out genome-wide with the outcome tabulated to provide a comprehensive overview of gene essentiality for the system.

In certain cases, mutations in two genes produce a phenotype that is surprising in the light of each mutation's individual effect. This phenomenon, which defines genetic interaction, can reveal functional relationships between genes and pathways. For example, double mutants with surprisingly slow growth define synergistic interactions that can identify compensatory pathways or protein complexes (Harcombe et al. 2013). Like single gene deletions, double gene deletions can be simulated to encompass all possible double gene deletions in the network. This provides a powerful tool to simulate otherwise prohibitively difficult wet-bench genetic interaction experiments.

10.6.3 Sampling of the Metabolic Solution Space

Genome-scale metabolic networks are often computationally explored to characterize functional relationships between their reactions. For instance, identification of correlated reaction sets (cosets), i.e., reactions that are always "on" or "off" concurrently (Papin et al. 2004) can define functional relationships between reactions that are not necessarily in the same pathway or obvious. The significance of these reactions is evident from the observation that mutations in correlated reactions may lead to a manifestation of the same aberrant (or disease) phenotype (Jamshidi and Palsson 2006). Because, the solution space of genome-scale networks can be

enormous, uniform sampling of the space is often carried out using the Monte Carlo method to identify the cosets and the overall shape and size of the steady state flux space (Becker et al. 2007). This sampling method, which is implemented in COBRA, identifies a set of randomly distributed solutions to serve as a proxy for the entire space. In Monte Carlo sampling, points are picked randomly from the space and the fraction inside the defined constraints is counted. This sampling method allows a uniform exploration of the metabolic network space while reducing the computational power demand required for the analysis.

10.7 Concluding Remarks

While the Aquatic Species Program saw its official close in 1996, the afterglow of the program continues to spark interest in algal research. With the increased computational power and the decreased cost of sequencing and network modeling, a significant surge in the availability of organism-specific metabolic models has been noted across species. Algae have remained on the lower end of this advancing front yet have been gaining momentum rapidly. Promising research endeavors are now set in place to assume successful modeling of metabolic networks in various algal species. The genomics era is far from over for algae; nevertheless, systems level metabolic studies are assuming a central role in sustainability research. For example, Genomatica (http://www.genomatica.com) uses an integrated biotechnology platform to produce a variety of target chemicals from sustainable feedstocks. Genomatica's platform combines process engineering with predictive computational modeling to develop more efficient manufacturing processes. Aastrom Biosciences (www.aastrom.com) makes use of predictive computational models to develop patient-specific expanded cellular therapies.

Other companies are applying modeling approaches more specifically to algal systems. Synthetic Genomics (www.syntheticgenomics.com) uses models to inform the design of metabolic pathways in transgenic organisms for the production of next-generation biofuels. Sapphire Energy (http://www.sapphireenergy.com), a company producing biofuel from algae, has partnered with the Institute of Systems Biology (http://www.systemsbiology.org) to make use of biofuel production models to inform engineering strategies as well. Other algal biofuel companies including Algenol (http://www.algenol.com) have also started modeling approaches to inform strain optimization and are actively hiring experts in the field to contribute to their respective modeling programs. These examples demonstrate the wide range of practical utilities that metabolic modeling can offer to guide the optimization process for biofuel, bioproducts, and beyond.

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Chapter 11
Genetic Engineering for Microalgae Strain Improvement in Relation to Biocrude Production Systems

Evan Stephens, Juliane Wolf, Melanie Oey, Eugene Zhang, Ben Hankamer and Ian L. Ross

Abstract An advanced understanding of the genetics of microalgae and the availability of molecular biology tools are both critical to the development of advanced strains, which offer efficiency advantages for primary production, and more specifically in the context of production for biocrude and renewable energy. Consequently, we outline the current state of the art in microalgal molecular biology including the available genome sequences, molecular techniques and toolkits, amenable strains for transformation of nuclear and plastid genomes, and the control of transgenes at both transcriptional and translational levels. We also examine some strategies for improvement of expression and regulation. We suggest the primary strategies in strain improvement that are most relevant to biocrude applications; briefly illustrate the process of photosynthesis to enable identification of targets for improvement of net photosynthetic conversion efficiency in mass cultivation; and further discuss how improvement of metabolic systems may also be achieved and benefit production models. Finally, we acknowledge the aspects of prudent risk assessment and consequent regulation that are developing and how our knowledge of natural algae in existing ecosystems, and GM work in conventional agriculture both contribute lessons to these discussions. We conclude that if properly managed, these developments provide significant potential for increasing global capacity for renewable fuel production from microalgae and that these developments could also have benefits for other applications.

E. Stephens · J. Wolf · M. Oey · E. Zhang · B. Hankamer · I.L. Ross (✉)
Institute for Molecular Bioscience, The University of Queensland, Queensland, Australia
e-mail: i.ross@imb.uq.edu.au

E. Stephens · J. Wolf · B. Hankamer · I.L. Ross
Solar Biofuels Research Centre, The University of Queensland, Queensland, Australia

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11.1 Introduction

The Need for Strain Improvement While microalgae are a proven and promising platform for the production of high-value products, their greatest potential arguably lies in their ability to capture solar energy and convert it to chemical energy in the form of high energy density fuel feedstocks with low net carbon emissions. The importance of this is highlighted by the fact that ~80 % of global energy demand is supplied in the form of fuels, while only ~20 % is utilised as electricity (BP 2014; Stephens et al. 2013b). Consequently, there is a great need for renewable fuel production systems that have an economic and energetically positive return on investment (ROI), and microalgae are one of the very few options for making this a reality at scale.

Thermochemical processing of whole biomass to biocrude is a promising area of research and current commercialisation. At first glance this processing strategy does appear to promise increased yields, since in addition to lipids, other organic molecules such as proteins, starch and cellulose can be converted. It may also address some of the conventional cost/energy bottlenecks, particularly as complete dewatering and cell disruption are not needed. But it must also be considered that, in contrast to the extraction of a relatively homogenous product such as TAGs and neutral lipids, the resultant output product from hydrothermal liquefaction (HTL) can vary in quality from a type I kerogen (a complex carbonaceous organic compound) (Speight 2006) to a higher grade biocrude, equivalent to the best petroleum crudes. The quality of the output depends upon the efficiency of the HTL process as well as the composition of the initial biomass. While the upgrading of kerogen to biocrude can be a much simpler process than lipid extraction from microalgae biomass, it remains an economic and energetic cost in the process. Thus, the technology can be streamlined partly by the development of microalgal production strains that have a more desirable composition for HTL processing and consequently improve the quality of biocrude output. The marketability of the biocrude product to fuel producers depends upon specific quality criteria including high carbon and hydrogen content and low oxygen, nitrogen and sulphur levels. Other qualitative considerations include acyl chain lengths and saturation, as well as finer points related to fuel standards. To achieve such standards and ultimately obtain a biocrude product that is comparable to conventional petroleum crude, HTL kerogens and oils can require fractionation to a higher grade product. This additional process step results in material losses and increased energy costs which offset some of the anticipated benefit of this production strategy. This poses a significant operational loss unless the residual fraction can be efficiently recycled back to production (e.g. through strategies such as anaerobic digestion or gasification) or otherwise contributes to cost recovery and energy balance.

Strain development through the use of molecular biology has greater flexibility than conventional breeding and strain development techniques. This may translate to increases in overall productivity (greater volumes to process) and greater carbon density (higher grade biocrude output) and so is of importance for advancing this

production strategy. Knowledge of algal genetics is not yet as sophisticated as other model systems. The ability to engineer algal biology is correspondingly limited at present, but is growing rapidly. Here, we discuss the ongoing development of molecular research for greater understanding of microalgae systems. In particular, we summarise the increasing set of available molecular techniques (Sect. 11.2), their application to microalgae technologies (Sect. 11.3) and the establishment of prudent regulatory systems to ensure these systems become environmentally responsible and socially accepted (Sect. 11.4).

11.2 Molecular Biology Capacity in Microalgae

11.2.1 Genomics and Molecular Biology of Microalgae

Microalgae-based biocrude production is an established technology, but compared to conventional fossil fuel extraction, it is energetically unattractive and the chemistry poorly understood. Improvements in process chemistry are necessary for microalgae biocrude to compete successfully with fossil fuels and non-algal biofuel technologies and to reach its full potential. While conventional strategies for strain development can yield significant improvements, genetic modification (GM) has the potential to improve aspects of biocrude production more rapidly and potentially to greater effect. As the primary aim of HTL is to generate more biocrude product per unit biomass with reduced energy costs, the manipulation of the initial biomass quality and yield, as well as aspects of the HTL chemistry (e.g. N & S content), may be amenable to GM strategies. The first step is to determine what traits would be helpful for HTL processing; the second is to identify how manipulating algal genetics can produce those traits.

Genetic Research in Microalgae To engineer beneficial traits into production strains, sufficient knowledge of algal biology is required to conduct targeted optimisation. This is embodied primarily in both the understanding of the most appropriate effects to target and of the methods to enable their engineering. Just as bacterial engineering rests upon a deep knowledge of bacterial biochemistry and genetics, algal GM biotechnology needs to rest upon a firm foundation of fundamental research into the way that algal genomes work. Despite the commonality of fundamental genetic mechanisms across the span of life on Earth, great variety is also present, and a consistent lesson is that ‘the devil is in the detail’ with respect to individual organisms. Consequently, the specifics matter greatly. Further, much biological variability will have accumulated from the ancient origins of algal phyla and their early divergence from plants and animals. Much specific knowledge of algal gene regulation will therefore be required before skilful, efficient and routine genetic manipulation will be possible. The recently expanded library of available algal genomes is a welcome advance but is of limited utility until these genomes are systematically mapped, curated, annotated and understood, a much more time-

consuming task than the actual sequencing. Systematic approaches such as the generation of knockout mutants of all *Chlamydomonas* genes at Stanford University (Zhang et al. 2014) and the transcriptomic (FANTOM) approaches pioneered at RIKEN in Japan (Forrest et al. 2014) are needed to provide the ability to quickly and with certainty assign biological functions to specific genes and curate algal genomes similarly to those of mammals. While microalgal genomes are undoubtedly simpler than the human genome, the resources allocated to studying them are miniscule by comparison, and the molecular toolkit is sparse, especially the lack of specific antibodies.

Advancements in Genomics Genome sequencing and sequence analysis is an important first step in deepening our understanding of microalgal systems and ultimately developing improved engineering processes. Only a very small number of genomes are available particularly when considered against the huge microalgal species diversity; however, the number of genome sequencing programs is steadily increasing (see Table 11.1). The National Centre for Biotechnology Information (NCBI) now contains 25 green algae genomes either in full, as scaffolds, or for which sequencing is currently underway (www.ncbi.nlm.nih.gov/genomes). Furthermore, there are novel bioinformatic tools (e.g. KEGG assignments accessible at www.genome.jp/kegg), and as BioModels databases accessible at www.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-main) become available online, they will enable researchers to predict and characterise gene regulatory pathways, forecast outcomes of metabolic shifts and functionally annotate de novo genomes of diverse algal species.

Genetic Mechanisms The existence of functional microRNAs in *Chlamydomonas* (Molnar et al. 2007) demonstrates that much of the convoluted genomic biology being revealed in mammals can also be expected in these simple organisms. The general schema of molecular pattern receptors, signal transduction mechanisms, and complex transcription factor-mediated feedback control of nuclear genes is to be expected, and many of the protein motifs will be familiar (e.g. helix-loop-helix transcription factors). However, given the evolutionary distance between different algal clades and between algae and land plants, it is to be expected that apart from highly conserved central mechanisms (core metabolism, cell replication, and mitochondrial and photosynthetic machinery), many baroque variations remain to be discovered. Algal genetics lags far behind algal physiology, much of which is common to plants in specific detail as well as general principles. To fill this gap, high-throughput gene analysis and bioinformatics will be critical for rapid mapping of the overall territory, even if painstaking molecular analysis is still needed for final validation of proposed biochemical and information pathways.

The algal genes that have so far been studied in detail illustrate this need. Significant changes to cell status, such as nutrient limitation (sulphate, nitrogen, iron, copper), lead not to up-regulation of a few receptors or import proteins, but to coordinated changes of thousands of genes, which resemble those waves of altered

Table 11.1 Update on available algal genome sequences, ongoing and future genome sequencing projects

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Chlorophytes (green algae)	*Chlamydomonas reinhardtii*	CC-503	Genome	121	15,143	Merchant et al. (2007) and Proschold et al. (2005)
	Chlamydomonas incerta		EST	ND		http://tbestdb.bcm.umontreal.ca/searches/login.php
	Volvox carteri	UTEX2908	Genome	138	14,437	Prochnik et al. (2010)
	Dunaliella salina	CCAP19/18	Genome			Joint Genome Institute (JGI)
	Chlorella variabilis (former: *Chorella vulgaris)*	NC64A	Genome	46	9791	Blanc et al. (2010)
	Haematococcus pluvialis					Grossman (2007)
	Scenedesmus obliquus					Grossman (2007)
	Oedogonium cardiacum		Chloroplast genome			Grossman (2007)
	Pseudendoclonium akinetum		Chloroplast genome			Pombert et al. (2005)
	Coccomyxa subellipsoidea	C-169	Genome	49	9915	Blanc et al. (2012)
	Botryococcus braunii		Genome			Joint Genome Institute
	Mesostigma viride		EST			http://tbestdb.bcm.umontreal.ca/searches/login.php
	Nephroselmis olivacea		EST			http://tbestdb.bcm.umontreal.ca/searches/login.php
	Ulva linza	–	EST	–	6519	Zhang et al. (2012)
	Leptosira terrestris		Chloroplast genome			de Cambiaire et al. (2007)
	Pedinomonas minor		Plastid genomes			Grossman (2007), project to be
	Monoraphidium neglectum	SAG 48.87	Genome		16,761	Bogen et al. (2013)

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Eustigmatophyta	*Nannochloropsis gaditana*		Genome	34	3558	Qingdao Inst. Bioe. Biop. Tech.
Prasinophytes	*Ostreococcus tauri*	OTH95	Genome	13	7892	Derelle et al. (2006)
	Ostreococcus lucimarinus	CCE9901	Genome	13	7651	Palenik et al. (2007)
	Ostreococcus sp.	RCC809	Genome	12		Joint Genome Institute
	Micromonas pusilla	CCMP1545	Genome	22	10,575	Worden et al. (2009)
	Micromonas sp.	RCC299	Genome	21	10,056	Worden et al. (2009)
	M. pusilla ?	RCC809	Genome	21		Worden et al. (2009)
	Bathycoccus prasinos	BBAN7	Genome	18		Joint Genome Institute
Rhodophytes	*Cyanidioschyzon merolae*	10D	Genome	17	6170	Matsuzaki et al. (2004)
	Galdieria sulphuraria		Genome	14	6723	Schonknecht et al. (2013)
	Porphyta yezoensis		EST	43	10,327	Kasuza DNA Research Institute
	Chondrus crispus		Genome	105	9843	Collen et al. (2013)
	Porphyridium purpureum		Genome	20	8355	Bhattacharya et al. (2013)
Glaucophytes	*Cyanophora paradoxa*		Genome	70	27,921	Price et al. (2012)
	Glaucocystis nostochinearum		EST			Uni Montreal

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Stramenopiles (diatoms)	*Thalassiosira pseudonana*	CCMP1335	Genome	32	13,025	Armbrust et al. (2004)
	Thalassiosira oceanica		Genome	92	34,684	Lommer et al. (2012)
	Phaeodactylum tricornutum	CCP1055/1	Genome	27	10,398	Bowler et al. (2008)
	Fragilariopsis cylindrus	CCMP1102	Genome	81		Joint Genome Institute
	Pseudo-Nitzschia mutiseries	CLN-47	Genome			Joint Genome Institute
	Amphora sp.	CCMP2378	Genome			Raymond and Kim (2012)
	Attheya sp.	CCMP212	Genome			Raymond and Kim (2012)
	Fragilariopsis kerguelensis					T. Mock, U. East Anglia, USA
	Ectocarpus siliculosus	Ec32	Genome	214	16,256	Cock et al. (2010)
	Aureococcus anophagefferens	CCMP1984	Genome	57	11,522	Gobler et al. (2011)
Haptophytes	*Emiliania huxleyi*	CCMP1516	Genome	168	38,549	Read et al. (2013)
	E. huxleyi	RCC1217	Genome			The Genome Analysis Centre (TGAC), UK
	E. huxleyi	CCMP371	EST			University of Iowa, USA
	Phaeocystis antarctica					Joint Genome Institute
	Phaeocystis globosa					Joint Genome Institute
	Pavlova lutheri		EST			University Montreal
	Isochrysis galbana	CCMP1323	EST			University Montreal

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Cryptophytes	*Guillardia theta*	CCMP2712	Genome	87	24,840	Curtis et al. (2012)
	Guillardia theta		Genome	350	302	Douglas et al. (2001)
	Hemiselmis andersenii		Nucleomorph genome	0.572		NCBI
	Gonomiomonas sp.	ATCC 50108	EST			University Montreal
	Gonomiomonas sp.		EST			University of Iowa, USA
	Chroomonas mesostigmatica	CCMP1168				Moore et al. (2012)
Chlorarachniophytes	*Bigelowiella natans*	CCMP2755		94.7		Joint Genome Institute
Alveolates (Dinoflagellates)						

gene expression seen in multicellular organisms. Only high-throughput mapping can provide the necessary background to support the efficient dissection of these biological responses. Apart from nutrient limitation, the kinds of coordinated responses which might be expected include photoacclimation, responses to predators and pathogens, differentiation-like developmental programs and adaptions to environmental niches. Fortunately, many of the tools developed for the study of other organisms can readily be adapted for algal biology. These include powerful genome-editing platforms either developed [zinc finger nucleases, TALENs (Gao et al. 2014; Sizova et al. 2013)] or under-development [CRISPR/Cas (Sander and Joung 2014)]. Although not yet routine, the ability to conduct precise genome engineering will greatly advance the speed and scope of algal GM production.

Case Studies A number of genetic responses in algae have been described, mainly in response to key physiological processes such as photosynthesis, nutrient limitation and circadian rhythm. These include the analysis of the transcriptional responses of the light-harvesting complex (LHC) genes to light and circadian signals, the carbon concentrating mechanism (CCM) in response to CO_2 limitation, and responses to iron, copper and sulphur limitation.

Few of the estimated 234 transcription factors and regulators initially identified bioinformatically in the *Chlamydomonas* genome (Riano-Pachon et al. 2008) have even tentative roles assigned to them. Although no promoters have been comprehensively analysed, several have been cloned and their behaviours studied and utilised for experimental systems. The best examples are the light-harvesting antenna genes which are regulated both by light and by circadian mechanisms. In addition to promoter regulation, post-transcriptional regulation has been demonstrated by an mRNA binding protein CHLAMY1, composed of two subunits (C1 and C3). In turn, an E-box-like promoter element has been shown to be involved in the regulation of the circadian rhythm protein C3 (Seitz et al. 2010) and some binding factors isolated. Regulatory factors controlling the CCM have been identified [CCMl (Fang et al. 2012; Fukuzawa et al. 2001); LCR1 (Ohnishi et al. 2010; Yoshioka et al. 2004)]. Iron-responsive elements have been identified in several genes [Fox1 (Allen et al. 2007; Fei et al. 2009), Atx1, Fbp1, Fld, Fea1], while the copper response regulator CRR1 has been shown to mediate copper and zinc responses (Malasarn et al. 2013; Sommer et al. 2010) and anaerobiosis [HydA1 (Lambertz et al. 2010; Pape et al. 2012) and Fdx5]; other nutrient uptake regulatory genes include those for sulphur SAC1 (Davies et al. 1996; Moseley et al. 2009) and phosphate PSR1 (Moseley et al. 2009; Wykoff et al. 1999). Although this represents a beginning, it pales in comparison with the extensive analyses of animal genomes, and when contrasted to the ~15,000 genes of *Chlamydomonas*, it is unlikely that this subset will provide an adequate basis for modelling promoter function in algae in general.

Some analysis has started in species other than *Chlamydomonas* including *Dunaliella* (Jia et al. 2012; Lu et al. 2011; Park et al. 2013), and some crossover is expected from plant gene analysis especially in *Arabidopsis.* A start has also been

made in understanding the role of mRNA regulation (Schulze et al. 2010; Wobbe et al. 2009) and chromatin remodelling in *Chlamydomonas* (Strenkert et al. 2011, 2013). While miRNA regulation has been demonstrated (Molnar et al. 2007; Yamasaki et al. 2013), little detail is available, nor is epigenetics well understood. In summary, the detail and breadth of examples typical of the regulation of mammalian promoters and their resultant mRNAs is sorely lacking for algal genomes. Consequently, close study of a set of promoter control mechanisms as models is badly needed and will greatly advance the level of understanding in this area, enabling much more sophisticated photosynthetic engineering, including the discovery of useful inducible/repressible promoters, and the ability to manipulate metabolic pathways and cellular strategies which are normally tightly regulated by photosynthesis. Lipid and starch accumulation, photoprotection and cellular replication, for example, are all cellular functions which are desirable to control for biotechnology applications. Abundant proteins including rubisco and LHC proteins represent substantial cellular resources. Some LHC adaptive functions are important to retain or enhance; others are potentially dispensable under bioreactor conditions or can even reduce biomass growth if allowed to operate naturally. Resource allocation within a cell is complex (Pahlow and Oschlies 2013) and only partly within our control as over- or under-production of specific metabolites can be detrimental to the fitness of the organism and feedback regulation in algae is incompletely understood. Therefore, opportunities exist for the development of the excretion of the end product (e.g. H_2 produced from water via the photosynthetic machinery; volatile metabolic intermediates (Melis 2013); specific secretion mechanisms for proteins and lipids).

The study of gene regulation has traditionally proceeded through intensive analysis of specific cases. As broad understanding evolves of the kinds of mechanisms that are present in biological systems, the emphasis has shifted to high-throughput analyses starting with microarrays and mass mutant libraries, and it is to be expected that this will quickly generate large amounts of data once algal genomics matures. Nonetheless, there are very few case studies of algal genetic mechanisms, and the study of particular cases will still be vital to anchor, interpret and calibrate the results of mass data acquisition.

11.2.2 Techniques for Genetic Modification of Algae

Characteristics such as high photon conversion efficiency, fast growth rate, high growth density, high oil/carbon content, ease of harvesting and high pathogen/predator resistance all represent aspects of importance for the development of high efficiency microalgal production strains. So far, however, there have not been any reports of a single species that is able to meet each of these criteria. The importance of microalgae bioprospecting and breeding, apart from establishing a solid basis for high efficiency strain development, lies in the identification of novel biological mechanisms and algal systems which can be exploited by genetic engineering.

Ideally, these will form libraries of traits, which in combination with tools to conduct species-specific engineering will enable strain customisation. In this context, it is of note that algae possess three genetic systems: the nuclear, the mitochondrial and the plastid genome, each of which may be genetically manipulated.

The green alga *Chlamydomonas reinhardtii* is arguably the most widely used model alga, at least in terms of fundamental biology; its physiology is well described, multiple mutants exist, all three of its genomes have been sequenced (Maul et al. 2002; Merchant et al. 2007; Popescu and Lee 2007), and a range of molecular tools have been developed to facilitate its genetic engineering. It contains one chloroplast with a 203 kb circular genome encoding about 100 genes (Maul et al. 2002). The chloroplast genome of *C. reinhardtii* is AT-rich and highly polyploid with a copy number of about 50–80 copies per chloroplast (Maul et al. 2002). With this high genome copy number, the chloroplast has been the choice of heterologous protein production since protein levels of over 40 % of total soluble protein (TSP) can be achieved (Surzycki et al. 2009). The nuclear genome of *C. reinhardtii* has a high GC content and frequent repeat regions. Protein accumulation is often lower compared to expression in the chloroplast, and transgenes can often be silenced. However, transgene expression from the nucleus offers several advantages such as inducible expression, post-translational modifications and heterologous protein-targeting to various compartments within the cell and secretion. The mitochondrial genome of *C. reinhardtii* is also polyploid with around 50–100 genome copies organised in about 20–30 nucleoids (Hiramatsu et al. 2006). It consists of a 15.8 kb linear DNA molecule which has been fully sequenced, and telomeres corresponding to inverted repeats of ~500 bp are located at each end, with 40-bp single-stranded extensions (Vahrenholz et al. 1993). Compared with the mitochondrial genome typically found in vascular plants, it is extremely compact with 14 genes encoding in total only eight proteins (Remacle et al. 2006) and three tRNAs. The low tRNA content suggests that cytosolic tRNAs are imported, a process known to take place in plant and human mitochondria, making it an interesting potential model system for more detailed process analysis (Remacle et al. 2012).

11.2.3 Molecular Toolkits—Genetic Engineering of the Different Compartments

Nuclear Transformation There are a variety of established transformation methods to integrate heterologous DNA into the nuclear genome including particle bombardment (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990), agitation of cell-wall-deficient strains with glass beads (Kindle 1990), electroporation (Shimogawara et al. 1998), agitation with silicon carbide whiskers (Dunahay 1993) and biologically mediated gene transfer by *Agrobacterium tumefaciens* (Kumar et al. 2004). In *C. reinhardtii*, transformation of the nuclear genome occurs

by random insertion through non-homologous end joining (Tam and Lefebvre 1993) or by using linear DNA that promotes the insertion of multiple copies in one locus (Cerutti et al. 1997b). Phenotypic and genetic screening of transformants can minimise undesirable non-target effects of the random insertion of a transgene that can lead to disrupted genes or regulatory elements. In return, this effect is used to study genes of unknown function using high-throughput insertional mutagenesis (Dent et al. 2005). In *C. reinhardtii,* targeted gene integration through homologous recombination (HR) using single-stranded transforming DNA (Zorin et al. 2005, 2009) is possible, but only at low frequencies to date. However, high rates of homologous recombination have been reported in another green algal species *Nannochloropsis* (Kilian et al. 2011), showing promise for reverse genetics and targeted gene knockouts.

Random and Insertional Mutagenesis The creation of mutants of an organism by the use of irradiation or chemical mutagenesis is the classic approach pioneered by Morgan in *Drosophila* and used for a century to study the effect of significant alteration in the behaviour of a gene, typically by partial or total deletion. These methods produce base pair changes leading to a range of disturbances including altered amino acid sequence, small deletions, truncations, frameshifts and splicing defects; the resultant mutants include temperature-sensitive and dominant mutants as well as functional knockouts sometimes giving rise to complex phenotypes. In the last 3 decades, the insertion of foreign transgenes (usually carrying a marker to allow selection and identification) has also been well established, as described above. Insertion mutants usually have knocked out or disabled genes, though insertion into promoters can lead to alterations in expression levels and splicing. Gene inactivation is largely on a random basis (Zhang et al. 2014) across the genome (at least where the chromatin structure is sufficiently open) which allows the unbiased identification of genes relevant to biological processes and has been very useful for research into biological mechanisms and physiology in *Chlamydomonas* and other algae. The difficulty is usually that a specific phenotypic screen is needed to identify relevant genes. Lethal mutations will not be identified, nor will mutants in genes which are redundant or which show no overt phenotype under the conditions of the screen. Of the estimated ~15,000 genes in the *Chlamydomonas* genome, only a few hundred have been reported in the literature, and many genes known to be important are not represented in collections of mutants. A good example is the multigene family of LHC genes. An insertion mutant in a single LHC gene will usually not produce any easily measurable phenotype, due to compensation by other LHCs, while the highly specific physiological or genetic tests needed to demonstrate the loss of a specific LHC gene are not suitable for screening assays. Finally, each transformation produces only a few hundred colonies, with very likely a biased set of genes being affected. This makes it a major project to uniquely identify mutants of each gene in an alga. Fortunately, this is being performed at Stanford University, and the resulting collection of mutants will be an invaluable research resource for the *Chlamydomonas* community (Zhang

et al. 2014). However, it is unlikely that this resource will be duplicated for every species of algae of research or commercial interest.

Homologous Recombination (HR) in the Nucleus Homologous recombination, the recombination between homologous DNA sequences, is essential for eukaryotes to repair DNA double-strand breaks and introduce genetic diversity during cell division, and two main pathways ('double-strand break repair' and 'synthesis-dependent strand annealing') have been proposed (Sung and Klein 2006). In plants and algae, nuclear located *RecA* homologues show high similarity to the prokaryotic *RecA* genes which suggests an endosymbiotic transfer from mitochondria and chloroplasts to the nucleus of ancestral eukaryotes (Lin et al. 2006). Although the introduction of foreign DNA into the nucleus occurs predominantly via random insertional mutagenesis, successful targeted homologous recombination has been reported in several algal species such as *C. reinhardtii* (Gumpel et al. 1994; Sodeinde and Kindle 1993; Zorin et al. 2009), *Nannochloropsis* sp. (Kilian et al. 2011) and *Cyanidioschyzon merolae 10D* (Minoda et al. 2004) with as little as 230-bp DNA sequence homology in the haploid cell (Gumpel et al. 1994). It has been demonstrated that the introduction of single-stranded repair DNA leads to a more than 100-fold reduction of non-homologous DNA integration in comparison with double-stranded DNA (Zorin et al. 2005). Attempts to increase the low frequency of homologous recombination in plants by the over-expression of well-characterised enzymes involved in homologous recombination such as the recA and ruvC proteins have been reported to increase homologous recombination and double-strand break repairs; however, these reports suggest that foreign gene targeting is not improved (Reiss et al. 1996, 2000; Shalev et al. 1999).

RNA Interference (RNAi) In the absence of tools for precise genome manipulation, RNAi-mediated knock-down of gene expression enables the creation of highly specific research mutants without the need for phenotypic screening or selection. RNAi techniques also enable the study of reduced levels of gene expression where total ablation would be lethal. The phenomenon of RNA interference is produced by the action of specific cellular machinery [Dicer and Argonaute (AGO) proteins (Casas-Mollano et al. 2008; Cerutti and Casas-Mollano 2006)] on mRNAs, guided by microRNAs which are naturally occurring small double-stranded RNAs (dsRNAs) in a process called mRNA cleavage (Bartel 2004). RNAi represents the use of this natural process for experimental ends by the artificial provision of small RNA molecules designed to interfere with the expression of a target gene. MicroRNAs (miRNAs) have also been discovered in *C. reinhardtii* (Molnar et al. 2009; Zhao et al. 2007) enabling a highly specific genetic tool (Moellering and Benning 2010; Molnar et al. 2009; Schmollinger et al. 2010; Zhao et al. 2007). Difficulties in achieving direct nuclear gene knockout via homologous recombination for *C. reinhardtii* (Nelson and Lefebvre 1995) led to RNAi becoming a widely used method to accomplish post-transcriptional gene silencing for gene function discovery (knock-down approaches via reverse genetics) and metabolic engineering (Fuhrmann et al. 2001; Molnar et al. 2009; Rohr et al. 2004; Schroda et al. 1999; Zhao et al. 2009). Artificial microRNAs have also been engineered to create

functional knock-downs of several nuclear genes in *Dunaliella salina* (Sun et al. 2008) and in the diatom *Phaeodactylum tricornutum* (De Riso et al. 2009). This method could potentially be used for algal genetic engineering for biofuel production (Cerutti et al. 2011; Grossman 2000; Wilson and Lefebvre 2004). The weakness of RNAi is the need to maintain the expression of the RNAi construct, typically requiring ongoing selective pressure, for example with antibiotics.

Genome Editing The ability to precisely edit the genome enables the specific deletion or mutation of genes and regulatory regions, with the resultant ability to create targeted mutations for study or industrial applications. As the resultant mutations are permanent, the stability problems inherent in RNAi knock-down constructs are eliminated. Genome editing also offers the prospect of precise mutants lacking foreign DNA, which consequently are technically non-GMO organisms. Several systems of genome editing have been developed in recent years, including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), meganuclease and the CRISPR/Cas system. Both ZFN (Sizova et al. 2013) and TALENS (Gao et al. 2014) have been used for genome editing in *Chlamydomonas*. The drawback of these systems is the substantial investment of time and effort required. In contrast, the CRISPR/Cas system promises a simpler, more facile approach. CRISPR (clustered regularly interspaced short palindromic repeats) sequences are found in prokaryotes and are short repetitive DNA sequences, corresponding to part of the DNA of bacteriophage genomes. In conjunction with a specific nuclease (Cas), they form the basis of a prokaryotic 'immune system' to recognise and eliminate viral genomes. The CRISPR RNA acts as a guide for Cas-mediated cleavage of the viral DNA or provirus. For genome editing, first described in 2013 (Cho et al. 2013; Mali et al. 2013), the addition of a targeted 'guide RNA' with co-expression of the Cas9 nuclease enables precise and specific genome editing and has been rapidly adopted in many organisms including both animals (Hsu et al. 2014) and plants (Feng et al. 2013). Although first attempts to use CRISPR/Cas in algae have encountered difficulties, it is anticipated that these will be overcome in the near future, enabling rapid and flexible genome editing in algae.

Chloroplast Transformation The chloroplast is the site of photosynthesis and storage of the resultant starch and is also important for the production of fatty acids and photosynthesis-related pigments especially carotenoids. Transformation of the chloroplast requires transfer of the transforming DNA to the interior of the chloroplast, and consequently, particle bombardment (biolistics) using DNA-coated gold or tungsten particles is the most commonly used method for chloroplast transformation. Some particles penetrate the cell wall, plasma and chloroplast membrane and deposit the transforming DNA in the plastid, which can then integrate into the local genome through homologous recombination (Boynton et al. 1988). Flanking endogenous sequences that are homologous to the targeted insertion site can make chloroplast transformation events highly targeted to any region in the genome (Rasala et al. 2013) which in *C. reinhardtii* is a great advantage over

nuclear transformation where homologous recombination occurs only at low efficiency (Sodeinde and Kindle 1993).

Homologous Recombination in the Chloroplast In the chloroplast, homologous recombination is mediated by an efficient *RecA*-type system which, due to its homology to the *Eschericia coli* RecA system, is suggested to be related to the cyanobacterial ancestors of chloroplasts (Cerutti et al. 1992, 1995; Inouye et al. 2008; Nakazato et al. 2003). In cyanobacteria, the RecA system is essential for cell viability especially under DNA damaging conditions (Jones 2014; Matsuoka et al. 2001). DNA repair is also believed to be the main function for homologous recombination in the chloroplast (Cerutti et al. 1995; Rowan et al. 2010). Several different models exist for the precise mechanism of homologous recombination and are not presented in detail here; however, the main steps of prokaryotic homologous recombination include initial strand breakage, formation of an enzyme complex to unwind the double-stranded DNA to form a single strand, followed by a mechanism called 'strand invasion' which searches for similar sequences on a homologous DNA fragment for pairing. This is then followed by DNA synthesis in accordance with the new template strand and resolution of the structure (Amundsen et al. 2007; Smith 2012).

Genome Stability in the Chloroplast With 50–100 genome copies, the chloroplast is highly polyploid, and apart from a few exceptions, each plastome shows a tetrapartite organisation containing two inverted repeat sequences that are mirror images of one another, separated by a large and a small single-copy unit. Though absent in present-day cyanobacteria and not essential for the general chloroplast genome function, the inverted repeat sequences show properties which suggest their involvement in gene maintenance and increased genome stability (Goulding et al. 1996). Chloroplast genomes can undergo homologous recombination between the inverted repeat sequences as several studies have shown two populations of plastomes in the same organism differing only in the single-copy sequence orientation (Aldrich et al. 1985; Palmer 1983; Stein et al. 1986). Furthermore, it has been observed that the inverted repeat regions accumulate nucleotide substitution mutations 2.3 times more slowly than the single-copy regions (Perry and Wolfe 2002; Ravi et al. 2008; Shaw et al. 2007). It was also demonstrated that DNA rearrangements occur more frequently when a large inverted repeat sequence is lost (Palmer and Thompson 1982). This suggests that homologous recombination between plastomes and inverted repeats contributes to this increased genome stability.

Manipulation of the chloroplast genome via homologous recombination offers the potential for exact gene deletions and insertions. Generally, chloroplast transformation vectors are *E. coli* plasmid derivatives carrying the foreign DNA flanked by DNA sequences (>400 bp each) which are homologous to the target region of the plastid DNA (Bock 2001; Hager and Bock 2000). Due to the high ploidy of the plastid genome and the fact that initially only a single plastome copy is transformed, the resulting phenotype may be weak in primary transformants and it is important to establish an efficient and suitable selection system or strategy to identify and enrich

cells containing transformed ptDNA copies, which may easily revert to wild type (Hager and Bock 2000; Rasala et al. 2013). Once the genome is homoplastomic and thus lacking template for further undesired homologous recombination events, the selectable marker can be removed (Day and Goldschmidt-Clermont 2011), though selective pressure against the transgene can still exist.

Mitochondrial Transformation Respiration and photosynthesis are coupled processes, and mitochondrial mutations are known to affect photosynthesis (Schönfeld et al. 2004) as well as many other aspects of cellular metabolism. Although in *Chlamydomonas* homologous recombination in mitochondria DNA is only detected after crosses between different mating types in mitotic zygotes (Remacle et al. 2012), it demonstrates that the cellular machinery is available for recombination-based foreign gene integration. Although the first transformation of the mitochondrion was published in 1993 (Randolph-Anderson et al. 1993) using biolistic bombardment, reports of mitochondrial transformation are rare and initially limited to the restoration of the wild-type mitochondria genome in mutant strains. In 2006, Remacle et al. reported the first and, to date, the only modification of the mitochondrial genome in vivo in a photosynthetically active organism. The work presented the introduction of a nucleotide substitution in the *cob* gene, conferring resistance to myxothiazol, and an internal deletion in *nd4* (Remacle et al. 2006). Homologous recombination was facilitated with as little as 28-bp homology between the introduced and endogenous DNA (Remacle et al. 2006).

Selection Techniques Following Transformation The identification of chloroplast and nuclear transformants can be achieved through the rescue of mutants impaired in endogenous cellular functions (non-photosynthetic, flagellar or auxotrophic mutants) or the introduction of antibiotic and herbicide resistances by point mutation of endogenous genes or the expression of heterologous resistance genes. A range of auxotrophic mutants have been developed (including mutations in arginine biosynthesis, nitrate reductase, nicotinamide biosynthesis and thiamine biosynthesis (Debuchy et al. 1989; Ferris 1995; Kindle et al. 1989; Rochaix and Vandillewijn 1982)) as well as mutants in photosynthetic capability or flagellar motility (Diener et al. 1990; Mayfield and Kindle 1990; Mitchell and Kang 1991; Smart and Selman 1993).

Classical antibiotic resistance markers include kanamycin [chloroplast: (Bateman and Purton 2000); nuclear: (Hasnain et al. 1985; Sizova et al. 2001)], spectinomycin/streptomycin [chloroplast: (Goldschmidt-Clermont 1991); nuclear: (Cerutti et al. 1997a)], neamine/kanamycin and erythromycin [chloroplast: (Harris et al. 1989)] paromomycin and neomycin [nuclear: (Sizova et al. 2001)], cryptopleurine and emetine [nuclear: (Nelson et al. 1994)], zeocin and phleomycin [nuclear: (Stevens et al. 1996)] and hygromycin B [nuclear: (Berthold et al. 2002)]. Screening for resistance to herbicides, such as atrazine, which inhibits the function of photosystem II [chloroplast: (Erickson et al. 1984)], or Basta, leading to disruption of the chloroplast structure [chloroplast: (Cui et al. 2014)], is also possible. The use of antibiotic resistance markers is not problematic for research purposes, but is less

desirable for industrial-scale production of vaccines or therapeutics, and for large-scale outdoor or agricultural production, antibiotic-free media is usually required for both sociopolitical and socio-economic reasons.

Recombinant Protein Expression Regardless of the energy return on investment (EROI) of microalgal biocrude, the low net value poses a significant problem for economic biocrude production from microalgae. One potential strategy to offset this is to exploit the fact that high-value products such as recombinant proteins are typically only a small fraction by weight of the biomass and could potentially be extracted prior to thermochemical processing of remaining biomass to biocrude. Consequently, an additional and lucrative revenue stream can arguably be obtained to subsidise the process without sacrificing overall biofuel productivity. The demand for recombinant proteins is growing with increasing population and biotechnological applications, and ultimately, the expression systems with the greatest cost-benefit are likely to dominate these markets. In this context, microalgal systems offer significant advantages over traditional microbial fermentation or mammalian cell culture systems. So-called molecular 'pharming' is the production of pharmaceutical proteins, therapy peptides, vaccine subunits, industrial enzymes and secondary metabolites or other compounds of interest in plants, and algae also offer commodity-scale opportunities. Proteins with biopharmaceutical or biotechnological relevance can be, e.g. monoclonal antibodies, vaccines, blood factors, hormones, growth factors and cytokines.

Nucleus Although the chloroplast is the expression system of choice for high protein expression levels, the nucleus as expression location has its advantages which have to be carefully considered in the context of each product. Despite the comparatively low expression rate of 1 % of TSP claimed by commercial vendors and the risk of gene silencing, nuclear expression tools enable the fusion of the desired product to a selection marker (to avoid silencing) with subsequent self-cleavage, as well as secretion of the expressed compound into the media (Lauersen et al. 2013; Rasala et al. 2012). So far, most proteins expressed from the nucleus have been reporter genes used to quantitatively measure protein expression levels as well as the localisation and accumulation (e.g. within the *C. reinhardtii* cell) (Rasala et al. 2013). Six fluorescent proteins (blue mTagBFP, cyan mCerulean, green CrGFP, yellow Venus, orange Tomato and red mCherry) were expressed from the nuclear genome to allow protein detection in whole cells by fluorescence microplate reader analysis, live-cell fluorescence microscopy and flow cytometry.

Chloroplast The production of recombinant proteins including reporters (e.g. GUS, luciferase or GFP) (Franklin et al. 2002b; Mayfield and Schultz 2004; Minko et al. 1999; Sakamoto et al. 1993), protein therapeutics (antibodies, hormones, growth factors or vaccines) (Franklin and Mayfield 2005; Manuell et al. 2007; Mayfield and Franklin 2005; Mayfield et al. 2003; Rasala et al. 2010; Surzycki et al. 2009) and industrial enzymes has established the chloroplast as a suitable production platform for a broad range for protein candidates. The potential for high expression levels [over 40 % of TSP (Surzycki et al. 2009)] and the ability to form complex

proteins including disulphide bonds (Tran et al. 2009) allow for limited post-translational modification without interference caused by 'gene silencing' making the chloroplast the site of choice for protein expression. Furthermore, the prokaryotic characteristics of the chloroplast genome such as gene organisation into operon-like structures (Holloway and Herrin 1998) potentially allow the expression of multiple transgenes from a single operon (transgene stacking) and with this the introduction of complete biochemical pathways. The additional development of a Gateway-compatible transformation system (Oey et al. 2014) allows the rapid production of multiple transformants. Success rates and costs for successfully establishing expression of new proteins in *C. reinhardtii* are estimated to be similar to those of yeast and mammalian cells (Rasala et al. 2013), but with potentially much lower production and purification costs.

11.2.4 Transcriptional and Translational Control of Transgenes

Nucleus A major mechanism of nuclear gene regulation is transcriptional control. Strategies to enhance heterologous gene expression, which occurs at relatively low levels compared to expression in the chloroplast, are the search for more effective promoters (Rasala et al. 2013) and the enhancement of translation efficiency through codon optimisation (Fuhrmann et al. 1999, 2004; Heitzer and Zschoernig 2007; Mayfield and Kindle 1990). The glycosylation patterns of nuclear expressed and secreted proteins from *C. reinhardtii* remain to be resolved. Studies on the post-translational machinery of this alga would be helpful to exploit this as an option of algal protein production.

Chloroplast Ensuring high heterogeneous gene expression requires the identification of endogenous transcriptional and translational regulatory elements (Harris et al. 2009; Marin-Navarro et al. 2007; Purton 2007). Although chloroplast gene expression is usually regulated at the translational level (Barnes et al. 2005; Eberhard et al. 2002; Nickelsen 2003; Rasala et al. 2010, 2011; Zerges 2000), choice of promoter and 5′ UTRs sequences is of importance due to potential feedback regulations which can interfere with the heterologous protein expression and mRNA stability (Gimpel and Mayfield 2013; Hauser et al. 1996; Manuell et al. 2007). The 5′ UTRs are believed to regulate ribosome association, transcript stability and the rate of translation (Barnes et al. 2005; Marin-Navarro et al. 2007; Salvador et al. 1993; Zou et al. 2003) while 3′ UTRs influence mRNA stability (Herrin and Nickelsen 2004; Lee et al. 1996; Monde et al. 2000; Stern et al. 1991) or may interact with 5′ UTRs (Katz and Danon 2002).

Mitochondria In photosynthetic organisms, mitochondria are the organelle with the greatest diversity in size and structure, ranging from 15 kb linear DNA molecules in *Chlamydomonas* to 1.0 Mb in angiosperms. To date, *Chlamydomonas* is

the only photosynthetically active organisms for which mitochondrial DNA has been altered, which significantly limits insights into translational and transcriptional control. Attempts have been made with plant mitochondria to utilise in vitro DNA and RNA import, and electroporation of isolated mitochondria has been used to gain further information about transcription and post-transcriptional processing (Remacle et al. 2012).

11.2.5 Strategies to Improve Gene Expression Levels

Fusion Proteins Specific regions within the coding regions of chloroplast genes enhance efficient expression of foreign genes in plants and algae (Anthonisen et al. 2002; Gray et al. 2009; Kuroda and Maliga 2001b). Genetic fusion of endogenous regions to an exogenous protein of interest may represent another effective strategy for high-level transgene expression (Gray et al. 2011; Kasai et al. 2003). A disadvantage of the fusion protein approach may be reduced industrial or clinical values or increased cost of purifying the protein of interest from its fusion partner.

Codon Optimisation Codon optimisation has been shown to be an important factor of heterologous gene expression in algae (Heitzer et al. 2007). It has been demonstrated that protein expression levels can be improved in the chloroplast by up to ~80-fold (Franklin et al. 2002a) by adjusting the codon bias of the transgene to the AT-rich chloroplast codon bias. Despite this, the effects of codon bias on expression levels are largely heuristic and still not well understood at a theoretical level.

Replacement of Highly Expressed Photosynthesis Genes The highest protein expression levels in *Chlamydomonas* have so far been demonstrated in transformants carrying the *psbA* promoter and 5′ UTR for transcription and translation initiation in a *psbA* knockout background, which leads to a non-photosynthetic strain (Manuell et al. 2007; Surzycki et al. 2009). *PsbA* encodes for the D1 protein in the photosystem II reaction centre and is the most rapidly synthesised protein at high light intensity in higher plants and algal cells (Trebitsh et al. 2000). Although photosynthesis was restored by introducing the *psbA* gene at a different location, the presence of the psbA protein decreased the yield of foreign protein production (Manuell et al. 2007). This may reflect competition between the two genes, but could also be due to primary energetic or biosynthetic limitations.

Open Reading Frame Orientation In the chloroplast, 3′ UTR of the RNA often shows the potential for stem-loop formation, which serves for transcript stabilisation rather than transcription termination (Rott et al. 1998). Therefore, a degree of 'read through' from the upstream gene is possible (Oey et al. 2009), which in parallel can increase the amount of translatable RNA and thus potentially increase the amount of protein (Stern and Gruissem 1987).

Heterologous and Hybrid Regulatory Systems Rasala et al. (2011) have recently shown that an increase in protein production can be achieved by the fusion of the *16S* ribosomal promoter, which does not contain translation initiation signals such as Shine–Dalgarno sequences, to the endogenous *atpA* 5′ UTR containing the translation initiation signal. This, and the demonstration that heterologous regulatory elements can significantly induce the expression rate (Kuroda and Maliga 2001a; Oey et al. 2009; Ruhlman et al. 2010), suggests that designed regulatory elements could serve to improve expression.

Inducible Systems Environmental changes or developmental factors naturally influence the up- and down-regulation of genes. Understanding those regulatory mechanisms provides a valuable genetic tool, for example to switch protein expression automatically or under the control of specific circumstances. Examples of inducible algal promoters include light responsive genes (Falciatore et al. 1999), nitrogen starvation (Poulsen et al. 2006; Poulsen and Kroger 2005), a sulphur-regulated arylsulfatase gene and an ISG glycoprotein. Tightly controlled expression of toxic proteins (e.g. the growth factor DILP-2) is also often desirable (Surzycki et al. 2007). The expression of psbD (D2 component of PSII) is dependent on the Nac2 gene fused to the copper-sensitive cytochrome c6 promoter (cyc6) and induced in copper deficiency and repressed in the presence of copper.

Riboswitches Riboswitches that can be used to regulate protein expression at the translational level have been shown to be functional in *C. reinhardtii* and *Volvox carteri* (Croft et al. 2007) suggesting that it may be a useful technique for other algae species as well.

11.2.6 Genetic Engineering in Other Algae

Apart from *C. reinhardtii,* few algal species have been subjected to extensive genomic manipulation. As it seems unlikely that *C. reinhardtii* will be used for commercial biofuel applications, this needs to be remedied. Because of the phylogenetic and structural diversity of algae, methods established for *C. reinhardtii* cannot necessarily be easily transferred to other species and may require major adaptations. Therefore, recent efforts have been made to develop molecular toolkits to increase the range of other more suitable algal species for commercial production scenarios.

A number of algae species have been transformed successfully, and an overview is given in Table 11.2. For example, *Euglena gracilis* was transformed with an antibiotic resistance marker (Doetsch et al. 2001) and *Porphyridium* spp. using a herbicide resistance cassette (Lapidot et al. 2002) RNAi has also been used to engineer nuclear genes in the chlorophyte *Dunaliella salina* (Sun et al. 2008) and in the diatom *Phaeodactylum tricornutum* (De Riso et al. 2009). Applicable genetic modifications of green algae for industry are the transformation of *Haematococcus pluvialis* (Steinbrenner and Sandmann 2006; Teng et al. 2002), an important

Table 11.2 Amenable strains and transformation systems

Compartment	Phylum	Class	Species	Method	References
Nuclear	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	A, B, E, G, S	Debuchy et al. (1989)
					Dunahay (1993) and Kindle et al. (1989)
					Kindle (1990), Kumar et al. (2004) and Mayfield and Kindle (1990)
					Rochaix and Vandillewijn (1982), Shimogawara et al. (1998) and Fernandez et al. (1989)
					Molnar et al. (2009) and Stevens et al. (1996)
					Berthold et al. (2002) and Sizova et al. (2001)
					Cerutti et al. (1997a)
					Goldschmidt-Clermont (1991)
					Schroda et al. (2000)
			Dunaliella salina	B, E, G	Feng et al. (2009) and Geng et al. (2004)
					Sun et al. (2005) and Wang et al. (2007),
					Tan et al. (2005) and Li et al. (2007)
			Eudorina elegans	B	Lerche and Hallmann (2013)
			Gonium pectorale	B	Lerche and Hallmann (2009)
			Haematococcus pluvialis	A, B	Teng et al. (2002)
					Kathiresan et al. (2009) and Steinbrenner and Sandmann (2006)
			Volvox carteri		Hallmann and Rappel (1999) and Hallmann and Sumper (1994)
					Hallmann and Sumper (1996) and Jakobiak et al. (2004)
					(Schiedlmeier et al. 1994)
		Trebouxiophyceae	*Chlorella ellipsoidea*	PT-E	Jarvis and Brown (1991) and Kim et al. (2002)
					Bai et al. (2013) and Liu et al. (2013)
			Chlorella saccharophila	PT-E	Maruyama et al. (1994)
			Chlorella sorokiniana	B	Dawson et al. (1997) and Hawkins and Nakamura (1999)
			Chlorella vulgaris	B	Hawkins and Nakamura (1999)
	Dinoflagellate	*Dinophyceae*	*Amphidinium spp.*	S	ten Lohuis and Miller (1998)
			Symbiodinium microadriaticum	S	ten Lohuis and Miller (1998)
	Heterokontophyta	*Bacillariophyceae (diatoms)*	*Chaetoceros salsugineum*	*B*	Miyagawa-Yamaguchi et al. (2011)

(continued)

Table 11.2 (continued)

Compartment	Phylum	Class	Species	Method	References
			Chaetoceros debilis	B	Miyagawa-Yamaguchi et al. (2011)
			Chaetoceros setoensis	B	Miyagawa-Yamaguchi et al. (2011)
			Chaetoceros tenuissimus	B	Miyagawa-Yamaguchi et al. (2011)
			Cyclotella cryptica	B	Dunahay et al. (1995)
			Cylindrotheca fusiformis	B	Fischer et al. (1999) and Poulsen and Kroger (2005)
			Navicula saprophila	B	Dunahay et al. (1995)
			Phaeodactylum tricornutum	B	Apt et al. (1996), De Riso et al. (2009), Falciatore et al. (1999) and Zaslavskaia et al. (2000)
					Miyagawa et al. (2009), Sakaguchi et al. (2011) and Zaslavskaia et al. (2001)
			Thalassiosira weissflogii	B	Falciatore et al. (1999)
			Thalassiosira pseudonana	B	Poulsen et al. (2006)
		Eustigmatophyceae	*Nannochloropsis* sp.	A, E	Cha et al. (2011) and Kilian et al. (2011)
			Nannochloropsis gaditana	E	Li et al. (2014) and Radakovits et al. (2012)
			Nannochloropsis granulata	E	Li et al. (2014)
			Nannochloropsis oculata	PT-E	Chen et al. (2008), Li et al. (2014) and Li and Tsai (2009)
			Nannochloropsis oceanica	E	Vieler et al. (2012) and Li et al. (2014)
			Nannochloropsis salina	E	Li et al. (2014)
	Rhodophyta	*Cyanidiophyceae*	*Cyanidioschyzon merolae*	E, PEG	Fujiwara et al. (2013), Minoda et al. (2004) and Ohnuma et al. (2008), (2009)
Chloroplast	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	B, G	Boynton et al. (1988) and O'Neill et al. (2012)
			Haematococcus pluvialis	B	Gutierrez et al. (2012)
			Dunaliella sp	B	Purton et al. (2013)
			Scenedesmus sp	B	Purton et al. (2013)
		Prasinophyceae	*Platymonas subcordiformis*	B	Cui et al. (2014)
	Euglenophyta	*Euglenoidea*	*Euglena gracilis*	B	Doetsch et al. (2001)
	Porphyridiophyta	*Porphyridiophyceae*	*Porphyridium sp*	B	Lapidot et al. (2002)
Mitochondria	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	B	Randolph-Anderson et al. (1993), Remacle et al. (2006) and Yamasaki et al. (2005)

Methods A—*Agrobacterium*, B—biolistic bombardment, E—electroporation, G—glass bead agitation, S—silicon carbide whiskers, PT—protoplast transformation, and PEG—with polyethylene glycol

producer of astaxanthin, and *Dunaliella salina* (Feng et al. 2009; Geng et al. 2004; Sun et al. 2005; Tan et al. 2005) used for β-carotene production. Diatoms are also important commercial sources for aquaculture feedstock, specialty oils such as omega-3 fatty acids, and are used in nanotechnology due to their unique silica frustules. There has been one report of a nuclear transformation of dinoflagellates (ten Lohuis and Miller 1998). Red algae have been used for both chloroplast transformation (Lapidot et al. 2002) and nuclear transformation (Cheney et al. 2001; Minoda et al. 2004). A human growth hormone (hGH) has been successfully expressed in the nucleus of *Chlorella vulgaris* (Hawkins and Nakamura 1999) and a fish growth hormone (GH) in *Nannochloropsis oculata* (Chen et al. 2008). Transformation techniques using a cellulolytic enzyme to weaken the cell walls and make the cells more competent for the uptake of foreign DNA have been successfully applied to the green algae *Chlorella ellipsoidea* (Liu et al. 2013) and may be applicable for the transformation of other algal species with tough cell walls in future. A synthetic biology approach to engineer complex photosynthetic traits from diverse algae into a more controllable production strains has been shown using an ex vivo genome assembly to transfer genes for core photosystem subunits from *Scenedesmus* into multiple loci in the *Chlamydomonas* plastid genome (O'Neill et al. 2012).

Given the recent expansion of interest in microalgae, a broader repertoire of genome sequences and analytical and molecular engineering tools are being reported and will provide the foundation for a broad range of biofuel applications, some of which are covered in the following section.

11.3 Application of Genetic Engineering for Practical Applications

11.3.1 Strategies for GM Microalgae Relevant to Biocrude Production

The potential benefits of GM in microalgae mass cultivation systems for the production of biocrude can be broadly divided into seven strategies:

1. To increase the net photosynthetic productivity of mass cultures
2. To increase nutrient assimilation capacity
3. To modify bulk energy and carbon flows (e.g. rerouting energy flows into lipids)
4. To enhance the alga's capacity to remain dominant in contaminated cultures (e.g. resistance to predators, pathogens)
5. To enhance the harvestability and processability of the algae biomass (biology of flocculation)
6. To improve economic viability through the manufacture of high-value products and services (HVP&S—e.g. recombinant vaccines or industrially useful properties such as the ability to digest cellulose)

7. To develop enabling technologies for biotechnology (e.g. export systems for proteins, lipids, or other products; internal signalling or reporter systems and switchable effectors, for example to stop and start growth, trigger programmed cell death or dissemble the cell wall upon demand).

11.3.2 Engineering Increases to the Net Photosynthetic Productivity of Mass Cultures

The two key requirements for any commercial renewable fuel system are a demonstrable positive energy balance and financial profitability. The optimisation of photon conversion efficiency (PCE) towards the desired end fuel product is central to this and requires optimisation of the following biological processes:

1. Solar energy capture
2. Storage of the captured energy as chemical energy
3. Minimising metabolic losses
4. Targeting the photosynthates into the chosen product stream

Solar Energy Capture While the theoretical PCE of microalgae may be as high as ~8–12 % of total incident solar energy (Melis 2009; Zhu et al. 2010), conventional commercial systems fall far short of this (up to ~2 % annual average for HRPs and ~5 % for PBRs). This, however, is already a significant advance on conventional agriculture and also shows the potential for further improvement. Well-operated high rate pond (HRP) systems cultivating suitable production strains in favourable climatic locations can achieve up to ~70 T ha^{-1} yr^{-1} which is equivalent to approximately 20 g m^{-2} d^{-1} though many current systems are achieving much less than this (Downes et al. 2013). The production of up to 70 T biomass dry weight yr^{-1} makes such microalgae systems ~5 times more productive than sugar cane [global average 71 T fresh weight ha^{-1} yr^{-1} (FAO—Food and Agriculture Organization of the United Nations 2014)], which as dry mass in comparison also generally has a lower calorific value than dried microalgal biomass, especially relative to oleaginous strains. At these levels (and assuming a nominal solar energy level of ~20 MJ m^{-2} d^{-1}), HRPs are producing microalgae biomass at ~2 % PCE (average value with variance between 1 and 4 %) in comparison with the global annual average of ~0.4 % for sugar cane yields when fallowing and rattooning are considered (Stephens et al. 2013a). Although microalgae can be produced at higher productivities, the capital cost and operating cost of these algae farms result in a higher relative cost of production. For example, more advanced PBR systems with improved designs and a larger surface area to volume ratio can achieve PCE rates of ~5 % in similar outdoor conditions (equivalent to ~175 T ha^{-1} yr^{-1}), but currently, the increase in capital cost is much higher than the returned benefit of the higher productivity. Higher PCE rates have been achieved in the laboratory, but this

is under artificial conditions. Consequently, biological methods of improving the PCE rate of microalgae production without incurring additional capital expenditure could play an important part in increasing the feasibility of a wide range of microalgal production systems. As the photosynthetic machinery comprises a number of potential targets for GM approaches, it is useful first to examine the molecular mechanisms of photosynthesis.

Photosynthesis and Electron transfer Photosynthesis drives the first step of all biofuel production and as such is a major target for genetic optimisation. In particular, the light reactions of photosynthesis capture solar energy and convert it into chemical energy in the form of ATP and NADPH. The ATP and NADPH generated is subsequently used to drive CO_2 fixation (dark reactions) and so ultimately the formation of biomass (*for biomethane and biomass-to-liquid (BTL)*), oils (*for biodiesel and aviation fuel*), sugars and starch (*for bio-ethanol*), and protons and electrons (*for bio-H_2*). Optimising the efficiency of light capture and its conversion to chemical energy is thus of critical importance for the development of all high efficiency/low-cost biofuel processes. Here, the complex processes of photosynthesis are briefly summarised to highlight key areas of potential genetic optimisation.

In higher plants and green algae, light is captured by the LHC proteins, which are commonly referred to as LHCI and LHCII based on their predominant interactions with photosystems I (PSI) or II (PSII). The LHC proteins belong to a large gene family, which in the green alga *C. reinhardtii* consists of over 20 members (Dittami et al. 2010). LHC proteins have a dual role:

1. To capture light and funnel the derived excitation energy to PSI and PSII.
2. To dissipate excess energy via the processes of non-photochemical quenching (NPQ) to reduce photodamage to PSII. Under high-light operational conditions such as in Australia, NPQ can result in energy losses of $\sim$90 % of the captured solar energy (Mitra and Melis 2008; Polle et al. 2003). To achieve the highest solar-to-chemical energy conversion efficiency, it is therefore imperative to minimise these losses.

The excitation energy transferred to PSII by LHCII drives the photosynthetic water splitting reaction, which converts H_2O into H^+, electrons and O_2. Under light-limited conditions, the photosynthetic electron transport chain is thought to be in the 'State 1 Transition' in which almost all of the captured photons are used to drive the transfer of electrons along the linear electron transport chain from the PSII–-LHCII supercomplex, via plastoquinone, cytochrome b_6f, the PSI–LHCI supercomplex and ferredoxin and on to NADPH.

Simultaneously, H^+ ions are released into the thylakoid lumen by PSII and the PQ/PQH_2 cycle. This generates a proton gradient across the thylakoid membrane, which drives ATP production via ATP synthase. The H^+ and electrons are recombined by ferredoxin–$NADP^+$ oxidoreductase to produce NADPH. NADPH and ATP are used in the Calvin–Benson cycle and other biochemical pathways to

produce the sugars, starch, oils and other biomolecules that are feedstocks for biofuel products (and which collectively form biomass). Alternatively, in some photosynthetic micro-organisms such as *C. reinhardtii*, the H^+ and electrons extracted from water (or starch) can be fed to the hydrogenase HydA, via the electron transport chain to drive the direct production of H_2 from H_2O. H_2 is potentially the most efficient form of biofuel production as, in contrast to the production of oils and carbohydrates, it requires no additional ATP or NADPH. Furthermore, far from only having a future use, H_2 is already used for synthetic fuel production and has significant industrial applications.

State Transitions The highest efficiency of ATP and NADPH production is achieved under low NPQ/linear electron transport conditions. This is because almost all of the captured energy is used to drive photochemistry and only two photons (*one to excite PSII and a second to excite PSI*) are required to transfer one electron from H_2O to NADPH. Consequently, microalgae have evolved the state transition process to maintain linear electron transport under changing light conditions. The state transition process balances the absorbed light energy between the two photosystems by relocating light-harvesting complex II proteins between the two PS reaction centres thus optimising the corresponding antenna. In State 1, CP29 and LHCII trimers are attached to PSII. Under high-light conditions, the PQ pool can become over-reduced (PQH_2) if the turnover rate of PSII exceeds that of PSI. To prevent photodamage, *C. reinhardtii* undergoes a transition from State 1 to State 2 in which CP29 and LHCII trimers are thought to detach from PSII, reducing its antenna size, and couple to PSI–LHCI to yield a PSI–LHCI–LHCII supercomplex which is to capture more light (Drop et al. 2011; Kargul et al. 2005). If the process of state transition is not sufficient and the PQ pool continues to be over-reduced, or the cell has a greater requirement for ATP, NADPH-dependent cyclic electron transport is induced.

Cyclic Electron Transport Cyclic electron transport transfers H^+ from the stroma and e^- from the stromal side of PSI to the luminal surface of the thylakoid membrane. H^+ is then released into the lumen, and the electrons cycled back to PSI via plastocyanin (PC). In this way, the over-reduced PQH_2 pool is re-oxidised to PQ. Converting PQH_2 to PQ helps to protect PSII from photoinhibition as the latter is available to accept electrons released by PSII. The H^+ transported to the lumen by cyclic electron transport is used for ATP synthesis via the ATP synthase. Although cyclic electron transport is not as efficient as linear electron transport, some of the energy is utilised for the production of ATP. Recently, Iwai et al. (2010) reported the isolation of a 1 MDa 'CEF–PSI supercomplex' and suggested it as the site of cyclic electron transport. This supercomplex is thought to consist of the PSI–LHCI and $Cytb_6f$ complexes, ferredoxin–NADPH oxido-reductase (FNR) and the integral membrane protein PGRL1. Independent studies revealed that PGRL1 is required for efficient cyclic electron transfer in *C. reinhardtii* (Petroutsos et al. 2009; Tolleter et al. 2011).

Theoretical Areas for the Genetic Optimisation of Photosynthesis The involvement of light-harvesting antenna proteins and the reaction centres in the dynamic function of state transitions, cyclic electron transport and specific biofuel production modes such as hydrogen remains an active area of structural biology (electron tomography, crystallography and high-resolution single particle analysis), protein biochemistry and genetic engineering research. Established and previously introduced methods such as RNA interference, random insertional mutagenesis as well as recent advantages in the development of gene-editing tools such as CRISPR/Cas (Sander and Joung 2014) or TALEN (Gao et al. 2014; Sizova et al. 2013) systems open up the ability to knock out/knock down specific genes or conduct precise gene editing.

Knockout/Knock-down of Specific Genes Deletion and knockout of specific genes such as LHC genes allow the establishment of stable mutants for structural and phenotypic characterisation. This in turn will help to develop pseudo-atomic resolution blueprints of the photosynthetic machinery to enable structure-guided design. Although quite unstable for commercial use, RNA interference to down-regulate particular proteins has been quite successful at increasing our understanding of optimal configurations for the photosynthetic apparatus and is therefore a useful tool to identify potential targets for further investigation and potential engineering applications.

Minimising Energy Losses At low-light levels, the rate of photosynthesis is limited by light, while under high-light levels, it is primarily limited by the rate of CO_2 fixation by rubisco. Increasing the atmospheric CO_2 concentration can increase the rate of CO_2 fixation. However, even under these conditions, wild-type microalgae are already supersaturated by irradiance levels in the $\sim$100–400 μE m^{-2} s^{-1} range, which is considerably lower than maximum incident irradiance levels (up to $\sim$2500 μE m^{-2} s^{-1}) as they generally possess large (dark green) chlorophyll-binding LHC antenna systems designed to capture a large proportion of the light incident upon them. Consequently, microalgae have evolved a range of photo-protective mechanisms to prevent oxidative damage under supersaturated illumination conditions. In the wild type with large antenna, algae cells at the illuminated surface of the bioreactor that are exposed to high-light levels capture the bulk of the light energy. The energy required to drive photochemistry is used, and the remainder of this captured energy is wasted via processes such as NPQ in which the excess is dissipated as heat (Mussgnug et al. 2007; Polle et al. 2003; Tolleter et al. 2011). As a result, light penetration into the culture is compromised and the cells located deeper in the culture are exposed to decreasing levels which impairs these cells in their photosynthetic efficiency. This in turn drastically reduces the efficiency of the overall culture. Although this problem can be overcome by reducing culture density or decreasing the light path, this reduces production levels or requires a larger surface area, increasing the cost of the bioreactors. Increasing the mixing rate is also of limited benefit as energy dissipation occurs on a shorter timescale ($<1 \times 10^{-6}$ s) than transferring cells from high- to low-light zones in the bioreactor ($\sim 1 \times 10^{-3}$ s) and requires a considerable energy input, significantly reducing the energy yield of

the process. Another mechanism of the cells to avoid photodamage is the ability to partially down-regulate their antenna systems yielding a light green phenotype due to their reduced chlorophyll content at the same cell density. Although this is an effective way of reducing light capture, a remarkable amount of energy is still wasted by NPQ. Reducing the amount of light captured per photosystem to the optimum light capture needed for each cell can markedly improve photobioreactor efficiencies (Beckmann et al. 2009; Mitra and Melis 2008; Polle et al. 2003).

Engineered small antenna cell lines with reduced LHC levels offer the potential of improving the light penetration into the bioreactor and the ability to better match solar energy input with the energy requirements of each photosynthesising cell. Thus, 'small antenna' cells at the bioreactor surface absorb only the light that they need, largely eliminating the need to dissipate excess energy through NPQ and to switch into the cyclic electron transport mode. This in turn allows more light (i.e. the light dissipated in the WT) to penetrate into the bioreactor so that cells deeper in the culture have a near optimal exposure to light (Mussgnug et al. 2007; Oey et al. 2013). Although the maximum efficiency of individual cells remains unchanged under saturating light levels, the overall efficiency of the small antenna culture, and with this the bioreactor photosynthetic efficiency, increases.

Initially, studies were conducted to evaluate the effect of down-regulating LHC levels more generally than specifically. Insertional mutagenesis was utilised to generate *C. reinhardtii tla1* mutant (Polle et al. 2003) with <50% of total chlorophyll per cell and a Chl a/Chl b ratio of almost triple, relative to parental strain indicating a reduction in the chlorophyll a/b binding proteins of the LHCII antenna. *Tla1* was shown to have a similar light saturation curve to the *C. reinhardtii* cbs3 mutant (Polle et al. 2000, 2003). Crossing of the cell-wall-deficient *tla1* with a cell wall intact strain CC1068 (CW^+) led to strain *tla1*–CW^+ which had higher oxygen evolution and cell densities relative to wild type at 1500 μE m^{-2} s^{-1}. Berberoglu et al. (2008) reported comprehensively upon the radiation characteristics of these two strains as well as a new mutant *C. reinhardtii tlaX* which has significantly lower total chlorophyll and substantially lower chlorophyll b. Similar such examinations of strain-specific radiation characteristics (Berberoglu et al. 2008; Heng et al. 2014; Kandilian et al. 2013; Lee et al. 2013) provide a good framework for comparisons and further advancement.

Translational control was also studied when NAB1 was postulated to be an LHC mRNA-specific repressor protein that, when disrupted through insertional mutagenesis, led to the *C. reinhardtii* Stm3 mutant with high LHC and chlorophyll levels (Mussgnug et al. 2005). Thus, the potential up-regulation of NAB1 and similar regulatory proteins like it could assist in the art of engineering light capture. Alternatively, it was found that replacement of 1 or 2 of the NAB1 cysteine residues can perturb its deactivation mechanism, and this in vitro led to enhanced repression of LHC translation, and consequently smaller LHCII antennae (Wobbe et al. 2008). This was validated when a permanently active NAB1 variant was used to generate the mutant *C. reinhardtii* Stm6Glc4T7 (Beckmann et al. 2009) although LHC antennae reduction effect was not as pronounced as that achieved by other methods.

RNA interference was used to facilitate targeted down-regulation of particular proteins of interest. Using a common LHC protein target DNA sequence, Mussgnug et al. (2007) was able, in the *C. reinhardtii* Stm3LR3 strain, to down-regulate LHCII proteins by ~95 % and LHCI proteins by ~80 %. As the LHC proteins perform a complex set of roles, a more refined strategy was developed in which specific sequences were chosen to target only LHCBM1, 2 and 3 in *C. reinhardtii* Stm6Glc4L01 (Oey et al. 2013). Both of these strategies have been reported to be successful at improving the growth rates of high cell density cultures under high-light conditions. Perrine et al. (2012) also employed an RNAi strategy to down-regulate CAO in *C. reinhardtii* and compared CAO-RNAi (CR) cell lines to the previously developed *C. reinhardtii* cbs3. Collectively, these RNAi projects have shown that although peripheral antennae can indeed be minimised, there are advantages in having precisely engineered antennae systems (e.g. which enables state transitions to function normally) for high efficiency biotechnology applications. More recently, Synthetic Genomics and ExxonMobil have reported on their collaborative work identifying light acclimation regulator (LAR) genes Lar1 and Lar2 (Bailey 2013) and developed *Nannochloropsis* mutants that are locked in a high-light-acclimated state. In terms of future developments, in this area, the above RNAi data can be used to identify particular targets for more permanent editing, deletion and upregulation using the emerging CRISPR/Cas and TALEN approaches.

Expanding the Available Solar Spectrum The currently accepted range for photosynthetically active radiation (PAR) absorbed by chlorophylls a and b only comprises ~43 % of the total solar spectrum, however, other chlorophylls include c1, c2, d and f and utilising additional chlorophyll types could enable the capacity for microalgae to exploit a wider range of wavelengths (Blankenship and Chen 2013; Chen and Blankenship 2011). In this context, it is of note that chlorophyll d (Manning and Strain 1943; Miyashita et al. 1996) and chlorophyll f (Chen et al. 2010) are red shifted and reported to be in oxygenic organisms. This indicates that these chlorophylls may therefore be able to capture light in the infrared range which is usually excluded from conventional calculations on microalgal productivity for most oxygenic algae which more commonly incorporate chlorophylls a and b into the photosynthetic machinery.

The engineering of photosystems incorporating alternative chlorophyll molecules requires the introduction of biosynthetic pathways for these specific pigments, the accompanying mechanisms of their targeted incorporation into the photosystems and the engineering of PSI, PSII and the light-harvesting proteins themselves to enable the precise coordination of these new pigments. In this context, it is of note that the disruption of the chlorophyll a oxygenase (CAO) gene which is reported to be involved in the synthesis of chlorophyll b yielded the chlorophyll b-depleted strain *C. reinhardtii* cbs3 (Tanaka et al. 1998). Subsequently, Polle et al. (2000) showed that in the absence of chl b, some substitution by chl a occurred in *C. reinhardtii* cbs3. All apoproteins of the LHC were reported to be present, although trimeric LHCII did not appear to assemble and the total antennae size was

truncated. Furthermore, cbs3 had a higher chlorophyll per cell relative to wild type even though chl b is absent. Photon use efficiency was significantly decreased in cbs3, but P_{max} depended greatly on the carbon source. Also, there has been some evidence that reduction in carotenoid levels can also have an effect in reducing PSII antennae although results are less pronounced (Polle et al. 2001). While this suggests that it is possible to engineer cell lines with tailored chlorophyll compositions, much detailed work must be completed to achieve real increases in photon use efficiency. In addition to potentially enhancing natural photosynthesis, such genetic engineering studies coupled with advanced structural biology analysis could also yield valuable insights for the design of artificial photosynthetic systems.

Precise Gene Editing Precise engineering of genes and the creation of stable cell lines, e.g. via the replacement of individual codons for specific amino acids will enable the fine-tuning of the photosynthetic process for biotechnological applications, for example using structure-guided design and gene-editing tools to modify individual chlorophyll-binding sites to facilitate the binding of specific chlorophylls or alter cyclic and linear electron transport controls and state transitions.

Photosystem Reaction Centres Aside from work on the optimisation of the LHC antennae and chlorophyll density, there has recently been progress in the investigation of variants of the D1 protein subunit of photosystem II (Vinyard et al. 2014) in which it was reported that different naturally occurring isoforms are tuned to either high- or low-light conditions and that through introducing point mutations, photochemical design concepts could be elucidated to enable fine-tuning of photosystem reaction centres towards specific environmental conditions. This work is still at early stages but has the potential to complement the work on optimisation of antennae systems for synergistic effect.

Other Recent Developments Also, there are other more generalist strategies for improving photosynthesis that are not specifically centred around light harvesting. Zhu et al. (2010) and Evans (2013) have recently reviewed some of the most promising advancements including the improvement of rubisco [or substitution of rubisco from other biological sources, e.g. (Lin et al. 2014)], and enhanced carbon concentrating and metabolic enzymes (e.g. sedoheptulose biphosphatase) that are associated with photosynthesis. It is important however to remember the distinction between modifications that enhance the productivity of a single cell and modifications that actually compromise the cell in some way but enhance the productivity of the overall culture (this is further discussed in Sect. 11.4 in the context of government regulation).

Challenges for Effective Deployment of These Technologies in Commercial Systems While growth rates in the laboratory appear to be better for high-density cultures and high-light conditions, as yet there have been no successful demonstrations of pigment/antennae mutants proving this capacity in scale up systems. Huesemann et al. (2009) did perform the first scale up cultivation of pigment mutants of *Cyclotella* that had been developed for them by Jürgen Polle; however, the mutant culture showed no significant growth benefit over the control culture.

The complexity of the antenna systems and their dynamic interactions makes the precise improvement of light capture and utilisation without the introduction of detrimental effects challenging and underlines the consideration that random mutagenesis approaches may be most useful where subsequent screening can verify that no functional units other than the target genes have been affected. In the absence of these checks, growth could actually be compromised rather than improved. Precision strategies for genetic engineering of light harvesting address this concern (as discussed above), but either system is acceptable. Furthermore, despite strains that have been developed specifically for high-density mass cultivation and to have the highest photon to chemical energy conversion efficiency show great promise, the state of being 'locked in' to a certain growth condition can also present a challenge. For example, other strains that retain the photo-adaptive capacity and can modulate their antennae size, pigment density and appropriate reaction centres have the capacity to invade cultivations of industrial production strains and grow more competitively in the photo-limited zone. Although they may not grow fast, in long-term semi-continuous culture, they could over time alter the population distribution and affect overall culture productivity (Perrine et al. 2012).

11.3.3 Engineering Improved Metabolism Systems

The downstream metabolic pathways which utilise the ATP and NADPH generated by photosynthesis offer a second area of opportunity for genetic improvement. Essentially, biocrude is reduced carbon dioxide and photosynthesis is a process of reduction. Metabolic engineering based, for example, on flux analysis (Dal'Molin et al. 2011) offers the opportunity to increase the capture of the energy derived from solar irradiation into desired 'chemical energy-rich' product (e.g. triacylglycerides, secondary metabolites or recombinant proteins) in production models utilising extraction processes or for sale of raw biomass.

To achieve the maximum PCE for a particular product, it is important to achieve four key things:

1. The optimised channelling of the captured solar energy into the biochemical pathway producing the target product
2. The optimisation of the efficiency of the biochemical pathway producing the product
3. To minimise energetic losses through cell division and metabolism
4. To export the product to avoid inhibitory feedback loops

It could be argued that while the engineering of pathways for production models utilising extraction processes or for the sale of biomass is likely to benefit most from genetic optimisation, the benefits of metabolic engineering are attenuated when whole biomass processing strategies such as HTL to biocrude are intended. However, even in this scenario, the elemental composition of microalgal biomass is still relevant to the quality of biocrude output and biomass. For example, oil-rich

biomass can be considered to be more reduced than its more oxygenated carbohydrate-rich counterpart and as such tends to have a higher energy content or calorific value in terms of MJ kg^{-1}. Thus, for example, while TAG accumulation has traditionally been examined as a feedstock for biodiesel production, for which it is particularly suited, its accumulation can also be beneficial for the production of biocrude. Similarly, protein accumulation results in high nitrogen and sulphur levels which incurs additional nutrient requirements and poses a problem for biocrude quality. Generally, algae are considered to be ~50 % carbon; however, this is not an inflexible rule. The Redfield ratio purports that the stoichiometric ratio of main elements is expected to be 106C:16N:1P; however, this is a molar ratio, and when masses are calculated, the corresponding mass ratios would be approximately 41C:7N:1P. Thus, algae can vary significantly in their carbon content, and where algae can accumulate large amounts of lipid stores, then a reasonable carbon range could be anticipated from ~40–60 % carbon. In this respect, where carbon assimilation can be increased without compromising total productivity, metabolic engineering can play an important role in strain development for whole biomass processing.

Balancing Carbon Storage in Microalgae (Case Studies) Studies have shown that the content and biochemical composition of microalgae can vary significantly as their metabolic pathways shift in response to environmental stimuli and changes in nutritional conditions—protein (6–52 %), carbohydrate (5–23 %) and lipid (7–23 %) (Brown et al. 1997; Chen et al. 2011; Guschina and Harwood 2006; Johnson and Alric 2013; Poerschmann et al. 2004; Roessler 1990). While some algae use lipids as energy storage, e.g. diatoms, for many species cultivated under nutrient replete normal growth conditions, carbon reserves are preferentially stored as starch (as in higher plants) which are readily catabolised to glucose for ATP (Johnson and Alric 2013). Though in nutrient deplete unfavourable growth conditions where temperature, light, salinity, pH, etc., may be suboptimal, the carbon can be stored in the form of triacylglycerols as a reserve for future use (Roessler 1990; Sheehan et al. 1998). By taking a nutritional and/or an environmental approach, the regulatory processes involved in these carbon storage schemes can be manipulated, although the effects may be species specific. While some of these approaches may not be practical in a commercial context, it is interesting to understand genetic activities related to such phenomena.

Nitrogen depletion was first demonstrated by Spoehr et al. in *Chlorella pyrenoidosa* and has since been applied to a number of other strains as the standard for lipid induction (Ben-Amotz et al. 1985; Spoehr and Milner 1949). Studies have shown significant increases in lipid content (from 1 to 85 % of cellular mass) in microalgal cultures at the expense of cellular growth (Rodolfi et al. 2009; Roessler 1990; Siaut et al. 2011). As the synthesis of proteins and nucleic acids rely on nitrogen, these pathways inevitably cease to function and the flow of carbon diverts to storage compounds (Berges et al. 1996; Roessler 1990). Similarly, silicon

depletion in diatoms such as *Cyclotella cryptica* has been observed to induce a multifold increase in ACCase activity leading to increased lipid accumulation, while a reported 50 % reduction in carbohydrate storage was noted. Pulse chase experiments suggested in the first 12 h after silicon depletion, ~55–68 % of the lipids produced via de novo synthesis (Roessler 1988a, b and 1990).

The effects of salinity on metabolism have been mainly studied in halotolerant species such as *Dunaliella* which is found to draw upon starch reserves to physiologically respond to osmotic stress (Craigie and McLachlan 1964). As salinity within the medium is increased, *Dunaliella* cells contract rapidly, and the cells then metabolise glucose and fructose into a glycerol pool within the cytoplasm in order for the cell to regain its volume (Baird and DeLorenzo 2010). On the other hand, when salinity in the medium is decreased, the available glycerol pool is metabolised back into starch reserves (Ben-Amotz and Avron 1973; Borowitzka and Brown 1974). Other growth factors such as temperature stress, pH variation and light intensity have also been reported to influence the lipid composition in a range of species (Guckert and Cooksey 1990; Roessler 1990).

Knowledge in how different species respond to different nutrients and environmental conditions will greatly aid researchers in developing a greater understanding of mass culture and biological response. The disadvantage of these simple strategies for increasing lipid content is that they are fundamental responses to stress, represent a loss of net productivity and can be subject to operational limitations. However, elucidating the genomic changes elicited by these responses can enable engineering strategies which offers the prospect of more rapid, direct and controllable ways to siphon off the biological gains of photosynthesis into a desirable form.

Engineering of Lipid Pathways While much is known about lipid metabolism in higher plants from research models such as *Arabidopsis* (Beisson et al. 2003), lipid metabolism in microalgae is substantially different relative to higher plants and also between microalgal genera. The neutral and polar lipids, and the enzymes and metabolic pathways involved in their biosynthesis and catabolism have been recently described with the current focus being upon gene identification to enable proper metabolic engineering (Dal'Molin et al. 2011; Guschina and Harwood 2013; Khozin-Goldberg and Cohen 2011; Liu and Benning 2013; Rismani-Yazdi et al. 2011). *C. reinhardtii* remains the most extensively studied model for microalgal lipid metabolism (Liu and Benning 2013; Merchant et al. 2012), but it does not appear to use phosphatidylcholine as a substrate in TAG synthesis or to accumulate TAG unless under stress conditions (unlike *Nannochloropsis* which can synthesise TAG under normal cultivation conditions) or in starch accumulation (*sta*) mutants (Li et al. 2010; Work et al. 2010; Zabawinski et al. 2001). Recently, the metabolic pathways of *Dunaliella tertiolecta* (Rismani-Yazdi et al. 2011) and *Monoraphidium neglectum* (Bogen et al. 2013) have been reported, and there is some knowledge of other microalgae (Guschina and Harwood 2013), but knowledge of specific enzymatic processes and the genes involved requires further advancement before

effective metabolic engineering strategies become commonplace and metabolic flux models will also assist with this (Dal'Molin et al. 2011).

In early work on acetyl-CoA carboxylase (ACCase) in which Dunahay et al. (1996) transformed the diatom *Cyclotella cryptica* with additional copies of the ACCase gene within the TCA cycle to increase the flux of carbon towards lipid biosynthesis, the resultant increase in enzyme activity did not increase lipid accumulation. Similarly, the recent engineering of increased expression DGAT strains in *C. reinhardtii* (La Russa et al. 2012) saw enhanced DGAT mRNA levels but failed to increase intracellular TAG accumulation. Thus, claims that this field of engineering lipid metabolism in microalgae is still in its infancy are valid (Merchant et al. 2012), and much more work needs to be completed before we are likely to see the potential gains in industrial production candidates that have been anticipated. Possible reasons for this are that microalgae have some form of feedback regulation and that they have multiple and divergent DGAT2 isoforms (Chen and Smith 2012), and while all algal species have at least one DGAT2 from the animal clade, it currently appears that only green algae have DGAT2s similar to higher plants. Thus, single gene engineering strategies may be of limited application when dealing with a gene network which we do not currently comprehend sufficiently. Chen and Smith (2012) call for further investigation of DGAT2 enzymatic characteristics as functionality and substrate preferences are currently not fully understood. PDAT is the other of the two enzymes involved in the final step of TAG production and it has recently been examined (Boyle et al. 2012; Yoon et al. 2012). Yoon et al. (2012) demonstrated that PDAT is indeed involved in TAG biosynthesis in *C. reinhardtii* through RNAi-induced PDAT knock-down mutants. Thus, both DGAT and PDAT represent valid targets for metabolic engineering, but more must be understood about the other metabolic processes acting in the TAG 'neighbourhood'. By simultaneously manipulating all of the critical genes that influence the metabolic flux, success is far more likely. RNAi and new CRISPR/Cas and TALEN technologies offer the potential to dissect these pathways and indeed optimise individual catalytic steps through genetic editing and the amino acid level.

Another promising genetic approach has focused upon the engineering of lipid catabolism rather than biosynthesis. Using this approach, Trentacoste et al. (2013) incorporated antisense and RNAi into the diatom *Thalassiosira pseudonana* targeting a newly identified gene Thaps3_264297, which was reported to be a multifunctional lipase–phospholipase–acyltransferase, which showed consistent decrease in microarray transcript abundance throughout the lipid accumulation phase of silicon withdrawal. Thaps3_264297 is homologous to human CGI-58 whose mutation in humans can lead to excessive accumulation of neutral lipid droplets in various tissues. Trentacoste et al. (2013) found that these knock-down mutants had increased accumulation of TAG droplets and total lipid production without negatively affecting cell division and biomass growth. Examples of other targetable enzymes may include malate dehydrogenase (*mme* gene), pyruvate formate-lyase (*pfl* gene) or the fatty acid synthase complex (FAS) to drive carbon

towards fatty acid synthesis (Perez-Garcia et al. 2011; Yu et al. 2011), and these targets will be further refined with the improvement of metabolic flux models.

Metabolic engineering is not just about increasing the flux towards fatty acid synthesis and TAG accumulation, but also qualitatively concerning the types of lipids that are produced. For example, metabolic engineering has been successful in altering the fatty acid profile of *Phaeodactylum tricornutum* (Radakovits et al. 2011) to yield shorter acyl chains. The capacity to manipulate both chain length and the degree of saturation has significant potential for adjusting fuel properties.

Engineering of Carbohydrate Pathways Similar to the metabolic models for fatty acid biosynthesis and catabolism, models for carbohydrate metabolism are also under-development, for example, in *Phaeodactylum* (Kroth et al. 2008). For bio-crude production, increasing the carbohydrate concentration in cells can also increase the total carbon content although oxygen content increases and this strategy seems secondary relative to strategies maximising lipid content. In the *C. reinhardtii Stm6* mutant, the deletion of the *Moc1* gene via random gene insertion resulted in modified respiration metabolism with the downstream effect of accumulating large starch reserves within the chloroplast (Schönfeld et al. 2004). There are also ambitions for microalgae, in particular cyanobacteria, to produce carbohydrates at the industrial scale (Ducat et al. 2012; Wijffels et al. 2013), and the company Algenol that utilises GM algae to produce and secrete ethanol is a good example of this.

Challenges for Effective Deployment of these Technologies in Commercial Systems Where microalgae are accumulating energy storage compounds, they become better candidates for production; however, within the ecology of mass cultivations, they also become better candidates for predation, increasing the energy return for micro-organisms grazing upon them.

11.3.4 Engineering Improvements to Process Streams and Economics of Algal Biotechnology

While ultimately the hard physical metrics for microalgal biocrude are essentially the energy returned on investment (EROI) and the economic viability, the maturing and scaling of the technology still require further development. During this early development phase, commercial viability requires a profitable path to technology deployment. Several approaches are possible for dealing with this problem.

High Value Products and Services (HVP&S) Algal GM is in its infancy compared to other systems. A challenge of generating GM strains for HVP&S production is to provide useful products and services that cannot be easily generated in more mature technologies. There is no rational point to replicating in algae a service that can be easily and economically performed by yeast or *E. coli* aside from reasons such as marketing appeal. The relative advantages of algae as GMO vehicles must therefore

be carefully considered on a case by case basis. Recombinant products such as peptides larger than those able to be chemically synthesised, but small enough to be extracted with relatively harsh techniques, may be particularly suitable. Many HVP&S GM strains will be designed to operate under heterotrophic conditions which simplify reactor design.

The difference between these approaches is that modification of bulk mass and energy flows is focussed on energy production and is strictly limited by the thermodynamics of light harvesting and carbon fixation, whereas HVP&S approaches are less thermodynamically constrained (and indeed, may not even utilise photoautotrophic systems) but are focussed mainly on economic gains.

Enabling and Supportive Technologies Given the constraints outlined above, it is clear that GM strains for HVP&S and those for biofuels applications will have little in common and it is unlikely that a single strain (or industrial facility) will serve both purposes, which argues against the 'biorefinery' concept if it is confined to a single strain or process. Nonetheless, the common biology underpinning all algal systems means that most of the enabling technologies invented in this space will apply similarly to a multitude of different algal biotechnology systems, yielding substantial cross-fertilisation. It is here that the biorefinery concept may be most profitable.

Many supportive technologies will therefore need to be developed before the industry matures, and GM can make major contributions to these. Protein and lipid export systems, for example, may reduce internal product inhibition while reducing harvest costs; modified photosynthetic systems may improve the efficiency of utilisation of incident light; and fluorescent signals may be generated to monitor internal biochemical processes. None of these technologies would intrinsically compromise the ability to convert light to fuel, but might greatly simplify or reduce costs for other biotechnological aspects. Clearly, there is a vast creative space for innovative GM approaches in this area. To the extent that such technologies reduce energy wastage during production, they can improve the EROI even without an alteration of the fundamental light-harvesting efficiency.

Advantages of Algae as Heterologous Expression Systems Algae as heterologous expression systems are comparable to plant systems primarily for their ability to produce proteins with post-translational modifications. They may not replace the established and commercialised bacterial and mammalian expression systems but offer the potential for biological products which are difficult to produce in an active form in prokaryotic systems and are expensive to make in eukaryotic systems (e.g. antibodies). They also offer advantages over conventional systems to be chosen for new products which cannot be produced in other systems [e.g. anti-cancer toxin (Tran et al. 2013)] and therefore provide a valuable opportunity for the industry.

One advantage that can make transgenic microalgae systems competitive in the field of pharmaceutical proteins is that many algae lack endotoxins or human pathogens (Mayfield and Franklin 2005; Walker et al. 2005) and are therefore 'Generally Recognized As Safe' (GRAS). This could allow for a reduction of

necessary purification steps during downstream processes as well as simplify quality control and therewith allay production costs. Another advantage of algae compared to higher plants is vegetative reproduction, leading to uniform clones with comparable production rates. This relates to product quality, e.g. demonstrated as certain beneficial post-translational modifications, product stability or biosafety. Microalgae systems display high growth rates and need only a short time from transformation to product formation so that scale up could be implemented within a few weeks within commercial processes. The cultivation can be inexpensive due to the relatively low costs of typical mineral media needed, therefore supplying a large-scale robust growing system which can yield cheaply extractable high-volume production. This provides possible cost savings during production processes, which could play a role in special fields, where large quantities of products are required at low costs such as recombinant antibodies or veterinary products.

Microalgae have already been established as biotechnological production systems and approved by the US Food and Drug Administration for a number of secondary metabolites useful as food additives or cosmetics (Administration 2003, 2004, 2010a, b, 2011, 2012; Plaza et al. 2009) and for the production of carotene using *Dunaliella salina* (Hosseini Tafreshi and Shariati 2009) and lutein as an antioxidant and food colourant. Antiviral activities have been shown. Vaccination concepts for a large number of diseases prevalent in developing nations based on recombinant antigen expression in microalgae could result in inexpensive production and distribution as well as long-term storage at room temperature (Dreesen et al. 2010; Specht et al. 2010). Edible vaccines are a possible field of application for algal expression systems, combining biosafety issues with inexpensive production and storage and therefore opening up making products accessible for less developed countries (Gregory et al. 2013). In the context of regulatory aspects in the pharmaceutical sector, novel expression systems have to offer enormous advantages over conventional systems to be chosen for new products. The possibility to use a closed photobioreactor system contributes to reducing the risk of contamination and prevents transgenes dispersing into the environment.

11.4 Regulatory Considerations in the Risk Assessment of GM Microalgae

The responsible production of genetically modified (GM) microalgae and its appropriate regulation in many ways parallels the previous emergence of GM crops utilised in terrestrial crop-based systems. GM crops have been in field testing for approximately three decades now and with their global scale now approaching almost 200 million hectares, their benefits have been demonstrated, although they have been beset by much controversy, and there are also some cautionary lessons

learned. There are some important distinctions between the two forms of production (i.e. aquatic versus terrestrial), and microalgae systems are generally capable of much greater containment than conventional cropping systems. In order to preface this discussion, it is important to first examine the current issues with wild (non-GM) algae, both in the environment and in commercial production systems, and the current state of regulatory oversight.

Wild Algae in Aquatic Ecosystems 'Toxic algae blooms' are a regular headline in the mainstream media resulting in a public perception that algae are a menace. In water treatment industries, this fear of algal toxins is also relatively well established. In reality, the number of algae that produce any toxins is a tiny fraction of the existing biodiversity. Almost all of the known toxins attributed to algae are actually found in certain types of cyanobacteria and dinoflagellates, with a much smaller representation from some bacillariophytes (diatoms), haptophytes, pelagophytes and euglenoids. In some cases, there are groups who are cultivating specific species, e.g. dinoflagellates, to utilise toxic compounds for applications such as biomedical cytotoxins (generally under laboratory conditions), but this is the exception rather than the rule, and the overwhelming majority of the industry is focused upon avoiding toxic species. For example, the cultivation of the cyanobacteria *Arthrospira* (*Spirulina*) for human food consumption must be free from the cyanobacteria *Microcystis*.

Environmental algal blooms, while an ongoing concern, are usually the result of anthropogenic nutrient outflows or natural processes of nutrient cycling. They are not generally the result of well-managed microalgae farming practices. Such blooms can occur during periods of elevated nutrient levels due to either natural processes (e.g. weather effected nutrient run-off from land or oceanic currents and upwellings) or from anthropogenic nutrients (e.g. municipal, agricultural or industrial waste waters), with the latter being more closely correlated with the increase in the frequency and the intensity of environmental algal blooms.

Algal blooms can be broadly divided into classes as (1) blooms that are transient and innocuous (2) both transient and persistent blooms that are generally considered to be harmful, and (3) blooms that are clearly detrimental and disruptive to ecosystems. As the algae themselves are by and large ubiquitously present, the primary underlying issue is the management of nutrients and eutrophication processes. While innocuous algae blooms are generally rapidly consumed by organisms higher up the food web (e.g. plankton and filter feeders) and are therefore transient, harmful algal blooms (HABs) (Anderson 2009; Anderson et al. 2002; Van Dolah et al. 2001) and ecosystem disruptive algal blooms (EDABs) (Sunda et al. 2006) can be comprised of algal species that are generally unpalatable to aquatic herbivores or that contain toxins. This is important because it disrupts the food web and the concordant transition of nutrition and chemical energy to higher trophic levels which can result in a loss of ecosystem biodiversity (with ecosystem biodiversity being closely correlated with ecosystem resilience). Historically, these problems are largely caused by agricultural nutrient outflows, and there has been significant

analysis of how outflows of nutrients and chemicals from agricultural production can vary greatly in their 'pollution footprint', e.g. (Hill et al. 2006). The potential for reducing the pollution footprint is one of the strong benefits of microalgal production systems (Smith et al. 2010) in that they generally have no chemical outflows, and due to greater containment relative to fields of crops in soil, they can have much lower nutrient outflow, and in some cases a negative footprint where they utilise anthropogenic nutrients from other systems, e.g. wastewater integration and bioremediation systems. Nevertheless, forward thinking risk management strategies are needed to ensure that microalgae production systems at very large scale do not induce similar concerns to those experienced in traditional agriculture.

Proper Management of Microalgal Production Systems Proper management of microalgal systems is an important aspect of any commercial operation. This will be increasingly important as systems are scaled for large-scale production and the varieties of engineered strains used increase. The establishment of production models aiming to exploit the benefits of GM microalgae contributes additional complexity to prudent regulatory frameworks. There is a duality to the responsible management of GM microalgae production systems in that (1) from a product perspective, farmed microalgae cultivations must be maintained at adequate purity and free from contaminants that can compromise product quality (e.g. in the *Arthrospira* example given above), and (2) from an environmental perspective, the release of nutrients or microalgal biomass must be properly managed in order to mitigate any risk to local ecosystems. Given that for the production of biocrude, biomass will be subjected to thermochemical processing, it is the latter point which is central to this discussion.

Both the type of release (nutrients or biomass) and the scale of release are important parameters in a proper risk assessment. Gressel et al. (2013) have added to the discussion on mitigating spills and propose that spills from large-scale cultivations will be inevitable—however, there is an important consideration here regarding the terminology moving forward in this discussion. We expect that implementation of proper standards in prudent farm management should be able to mitigate the chances of large-scale spills into the environment; however, it is widely agreed that microalgae have a relatively high capacity for dispersion (e.g. microscopic size, and potential to form aerosols). Thus, if some aerosolised cells escape to the environment, it is certainly a release, but is this considered a spill? In terms of nutrients, the scale and/or persistence of release is generally the most important variable in terms of subsequent eutrophication potential and the corresponding risk assessment, but in terms of biomass, a single cell escaping as an aerosol particle has the potential to establish itself outside of the farm boundary even if there is no 'spill'. Thus, in this respect, species release is indeed inevitable, and it is in this context that any discussion of GM strains must be conducted. Hence, if small-scale release cannot ultimately be avoided, then the discussion is inexorably dependent upon the biological character of what is released.

GM Microalgae and Their Regulation Considering the inevitability of release, risk assessments of GM microalgae must be conducted on a case-by-case basis, with specific attention to the nature of the modification and whether it actually conveys a competitive advantage of some kind to the strain in question when it is relocated within a natural ecosystem or whether the modification can result in disruption to ecosystems in some other way. Henley et al. (2013) have recently published an excellent examination of GM algae risk assessment which should serve well as a foundation study for this evolving discussion. They rightly stipulate that for a GM-specific environmental risk assessment, primary considerations of potential ecological impact include the following:

1. The potential of GM microalgae to be more highly competitive in natural ecosystems.
2. The potential of GM microalgae to result in altered communities of aquatic herbivores in terms of composition, dominance or biodiversity.
3. The potential of GM microalgae to be involved in horizontal gene transfer (HGT) to other micro-organisms.

Given that it is anticipated that, for the most part, new algae producers will not be cultivating microalgal species that are invasive or toxic—the primary considerations will be the genetic modifications themselves rather than the host strains (indeed popular host strains such as *C. reinhardtii* are quite easily outcompeted by many wild-type species); however, it has previously been seen that some potential production candidates have already been involved in bloom events that have resulted in their classification as EDABs (Sunda et al. 2006). Thus, we encourage a careful and iterative investigation of all aspects of microalgae production, but emphasise that in this discussion it is the specific genetic modifications relevant to high-density microalgae cultivations that is in need of far greater discussion in the literature. Consequently, we discuss here the implications of the engineering applications highlighted in section two, with respect to associated risk of species establishment, dominance and ecosystem disruption. Much can be gleaned from the parallels with GM crop species, especially pollen dispersal; however, there are distinct differences between terrestrial crops and communities of aquatic micro-organisms.

For microalgal strains engineered to have varied light-harvesting and photosynthetic efficiency, the general desire is to increase net biomass productivity. As discussed above, this can be achieved through different methods. The down-regulation of LHC proteins or pigmentation can provide an overall net benefit to high-density cultures in high-light conditions (i.e. the artificial farming environment), e.g. (Oey et al. 2013); however, this generally makes individual cells less competitive in natural ecosystems where competing wild-type cells retain the capacity to modulate their antennae size and pigmentation levels. In theory though, genetic modifications that unilaterally increase total productivity (e.g. a higher efficiency rubisco enzyme or strains that can utilise a wider range of the spectrum) could potentially convey a competitive advantage irrespective of the growth environment.

Where strains are photosynthetically superior irrespective of environment, they could potentially affect ecosystem dominance and diversity, and while microalgae composition might not be significantly changed, the increased availability of these microalgae could result in additional effects like changes in plankton composition. In contrast, LHC/pigment-reduced cells could lead to some immediate compositional changes when consumed, but this would be insubstantial at the community level, and as these strains are outcompeted by wild-type organisms, there would be no net change to dominance or biodiversity. Again, the real concern for HGT would be that microalgae with superior generic photosynthetic efficiency would be capable of transferring this trait to other phototrophic organisms enabling them to also have greater competitiveness in the natural ecosystem. The transfer of disabled antennae/pigment modulation would not convey an advantage to other species.

GM strains that have a greater capacity for nutrient scavenging may have an increased competitiveness if released, but there is already a diverse range of strategies for nutrient uptake and usage among naturally occurring algae (Henley et al. 2013). Thus, while the predicted risk for these modifications is considered to be low, there has not yet been sufficient data from field trials to properly draw a conclusion.

Metabolic engineering is intended to alter the composition of microalgal biomass. While for biocrude producing systems, this will ideally result in strains that have higher overall carbon content, and it is not producing strains outside of the range of what occurs in nature. Nevertheless, if the available proportion of the population containing high carbon (i.e. abundance of GM microalgae relative to wild-type microalgae) is shifted, there is potential for an effect even if the consequences are low. If the nutritional value of the microalgae is altered, then this could also lead to changes in the nutritional value of plankton and filter feeders and subsequently lead up the food web to higher trophic levels. By the ecological risk assessment proposed by Henley et al. (2013), this risk is considered to be very low; however, this should be monitored in the longer term to obtain confirmation. In general, the accumulation of energy storage products in the form of reduced carbon molecules does not convey a competitive advantage to GM microalgae and it is likely that they will also be outcompeted by wild types within natural ecosystems.

GM traits that enhance the capacity of a microalga to remain dominant in the presence of predators, pathogens and competitors are varied in their approach and range from resistance to chemicals (e.g. herbicides and pesticides) to the use of allelopathy and toxins to maintain dominance. The use of chemicals is unlikely to become widespread for low-value commodity products such as biocrude due to the economic pressure it places upon business models; however, the engineering of endogenous chemicals into GM microalgae that prevent contamination is a potential risk that must be properly examined. Henley et al. (2013) propose that the risk of this approach is low to moderate depending upon whether the allelopathic chemical is naturally occurring or novel; however, we suggest that the range of potential risk assessment outcomes can be as variable as the potential allelopathic chemicals that can be engineered and that even for relatively low-level allelopathy, at the very large scales of production proposed for addressing fuel demand, even

mild allelopathy could have ecosystem disruptive effects. Thus, we advise a strict examination of these strategies; though to the best of our knowledge, these strategies have not yet been employed. We do agree though that where traits are selected for from large populations and then elucidated and reproduced through engineering (rather than engineering of novel chemicals), the risk will be attenuated.

Other GM strategies to increase the harvestability and processability of microalgae are unlikely to affect their dominance in natural systems, and the risk for these traits is considered very low. Similarly, where protein expression is used to create a primary revenue stream from a high-value product before HTL of residual biomass, these strains are unlikely to compete in natural systems due to diversion of much of their energy flow towards a product that is not useful to the microalga.

The theoretical risk assessment discussed here and that presented by Henley et al. (2013) can be quite informative, relying on an analysis of whether similar traits are already part of the ecosystem. However, a physical risk assessment strategy will be more convincing where laboratory-scale simulated ecosystems are developed from natural water bodies and the long-term survivability of GMO algae in mixed culture can be evaluated, e.g. by PCR.

11.5 Conclusion

The commercially profitable production of algal biocrude, at scale, will represent the culmination of a long and parallel development of algal agronomy, biology, GM, bioreactor engineering, harvesting and chemical conversion processes and the development of suitable sensors and control systems, along with their associated modelling and control software. No one innovation will suffice to overcome the formidable challenges faced by this nascent industry, and no actor will have ownership of all the important intellectual property. Since the most significant competitive challenges are between algal technologies and other fuel systems and secondary markets, the field of algal biotechnology stands to benefit greatly from relative openness of sharing data, technology and experience. This suggests that the modified algal strains used for biocrude production in the future will be heavily modified fuel factories equipped with streamlined metabolism, externally controllable cellular programs, and both sensors and reporting systems for monitoring the state of the system. Biocrude production appears, at this stage, to offer one of the most promising production pathways for algal biofuel production, and genetic manipulation offers a powerful tool for fine-tuning microalgal biofuel production all the way along the development pathway.

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Chapter 12
Harvesting of Microalgae by Means of Flocculation

Koenraad Muylaert, Dries Vandamme, Imogen Foubert and Patrick V. Brady

Abstract Large-scale production of microalgae for biofuels is still facing several major challenges to become competitive with other forms of renewable and non-renewable energy. A major challenge is harvesting, which requires the separation of a low amount of biomass consisting of small individual cells from a large volume of culture medium. Flocculation is seen as a promising low-cost harvesting method for microalgae biomass. In this chapter, the challenges and potential advantages of using flocculation as a harvesting method for microalgae are reviewed.

12.1 Introduction

Microalgae have attracted attention in recent years as a promising new source of biofuels (e.g., Chisti 2007). In addition to biofuels, microalgae have a high potential for other innovative applications. They have a high content of proteins with a favorable amino acid composition and are potentially interesting for use as animal feed (Draaisma et al. 2013). Microalgae can absorb nitrogen and phosphorus from wastewater and used in wastewater treatment (e.g., Park et al. 2011a, b). Microalgae

K. Muylaert (✉) · D. Vandamme
Laboratory of Aquatic Biology, KU Leuven Kulak—University of Leuven,
Etienne Sabbelaan 53, 8500 Kortrijk, Belgium
e-mail: Koenraad.muylaert@kuleuven-kulak.be

I. Foubert
Research Unit Food and Lipids, Department of Molecular and Microbial Systems Kulak,
KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium

I. Foubert
Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven,
Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

P.V. Brady
Sandia National Laboratories, Geoscience Research and Applications Group,
P.O. Box 5800, Albuquerque, NM 87185, USA

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can also produce a range of high-value chemicals that cannot be derived from conventional crops (e.g., specific fatty acids, carotenoids, or natural pigments) (Pulz and Gross 2004). Despite this enormous potential, commercial applications of microalgae are today still limited to production of value-added biochemicals or nutritional supplements. To use microalgae for low-value applications such as biofuels, animal feed, or wastewater treatment, the cost and energy inputs in the production process need to be decreased by at least an order of magnitude (Wijffels and Barbosa 2010; Christenson and Sims 2011). One of the major challenges is to develop a low-cost and energy-efficient harvesting method (Mata et al. 2010; Rawat et al. 2012).

Microalgae are generally small (2–20 μm) and have a specific density that is close to that of their culture medium. This precludes the use of simple screening or sedimentation methods for harvesting microalgae. Similar challenges exist with harvesting of other microorganisms, such as yeasts or bacteria produced heterotrophically in fermentors. An important difference is that biomass concentrations of phototrophically cultivated microalgae are more than an order of magnitude lower than those of bacteria or yeasts. This is because high biomass concentrations in microalgal cultures result in self-shading and light limitation of photosynthesis. Microalgal biomass concentrations range from 5 g L^{-1} dry weight in closed photobioreactors to only 0.5 g L^{-1} in open raceway ponds. The low biomass concentration implies that large volumes of culture medium need to be processed to harvest the biomass. For example, a 10-ha facility that produces microalgae in raceway ponds and that produces 30 tons of dry microalgal biomass ha^{-1} $year^{-1}$ should process about 2000 m^3 of microalgal broth per day. This is in the same order of magnitude as a water treatment plant processing wastewater of 10,000 people. To realize large-scale production of microalgae, a harvesting technology is required that is capable of processing large volumes of culture medium at a minimal cost (Molina Grima et al. 2003; Uduman et al. 2010; Christenson and Sims 2011; Benemann et al. 2012; Vandamme et al. 2013).

12.2 Flocculation as Part of a Two-Stage Harvesting Process

Flocculation may play an important role in developing such a low-cost and high-throughput harvesting process (Brentner et al. 2011). During harvesting or dewatering of microalgae, a microalgal paste is produced with a dry matter content of 20 %. This implies a 400–40 times concentration from 0.5 or 5 up to 200 g dry matter L^{-1}. Theoretically, this can be achieved in a single step using mechanical methods such as high-speed centrifuges or ultra- or micro-filtration membranes. Due to the large volumes of culture broth that need to be processed, however, the cost and energy inputs are extremely high. The energy inputs for harvesting by means of centrifugation are 14 MJ kg^{-1} of dry biomass, which is about 55 % of the energy content of the biomass (Norsker et al. 2011).

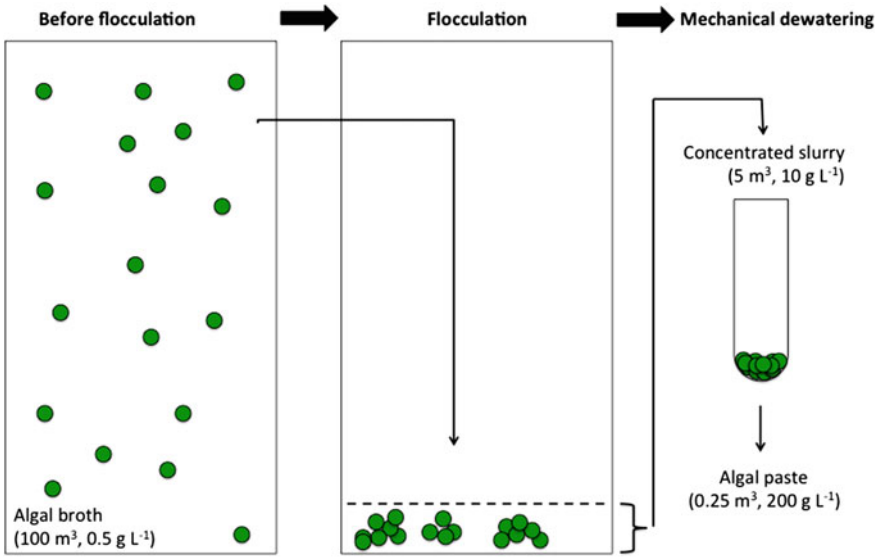

Fig. 12.1 Two-stage process for harvesting microalgae that includes a flocculation step. In the first step, 100 m^3 of a dilute microalgal suspension (0.5 g L^{-1}) is pre-concentrated by flocculation followed by sedimentation or flotation. A 20 times concentration yields a microalgal slurry with a biomass concentration of 10 g $^{-1}$ and a volume of 5 m^3. This 5 m^3 of microalgal slurry is then further dewatered using a mechanical dewatering method such as centrifugation or filtration to yield an microalgal paste with a dry matter content of 200 g L^{-1}

Several authors have suggested that low-cost harvesting of microalgae can be achieved by means of a two-stage harvesting process in which the biomass is pre-concentrated by means of flocculation prior to final dewatering (Pahl et al. 2013; Kim et al. 2013; Weschler et al. 2014). In flocculation, individual microalgal cells form larger aggregates or flocs. These flocs have much higher settling rates than individual cells and can easily be separated from the medium by means of gravity sedimentation to yield a microalgal slurry (Fig. 12.1). This slurry can be completely dewatered using a mechanical method such as centrifugation or filtration. Due to the large size of flocs compared to individual microalgal cells, the energy demand for mechanical dewatering will be much lower than for culture broth with freely suspended cells. Flocs have higher sedimentation rates than individual cells and can be separated from the medium with a low centrifugal force (Xu et al. 2012). When filtration is used, flocculation prior to membrane filtration results in higher membrane fluxes and thus a lower energy demand (Lee et al. 2012a, b).

12.3 Stability of Microalgal Suspensions

Because small particles suspended in water have a higher interfacial energy than large particles, small particles have a tendency to form larger aggregates or flocs. Spontaneous flocculation of particles, however, is often prevented by electrostatic repulsion. This electrostatic repulsion occurs when particles carry a surface charge. Microalgae usually have a negative surface charge, the result of carboxylic, phosphoryl, and amine/hydroxyl groups on the microalgal cell surface. Most important are the carboxylic groups, which are deprotonated at pH above 3–4, and thus have a negative charge. Phosphoryl groups become anionic above pH 7–8. Amine and hydroxyl groups lose a proton below pH $\sim$ 10 and become, respectively, uncharged and negatively charged. The overall effect is net negative microalgal surface charge in most natural waters (Hadjoudja et al. 2010). Few studies, however, have investigated the contribution of active groups on the microalgal cell surface to the surface charge, and more fundamental research is needed to better understand the linkage between microalgal surface charge and flocculation (Brady et al. 2014).

The electrical double layer consists of the negatively charged surface of the cell and the positively charged cloud of counterions close to the cell surface. The zeta potential, or ζ potential, is the potential difference between the bulk solution and the slip plane in the electrical double layer (Fig. 12.2). The counterions that are between the surface and the slip plane remain associated with the cell when the cell is moving through the solution. The sign of the ζ potential and the surface charge are the same. The ζ potential is relatively easily quantified by measuring the mobility of the charged particles in an electric field (Ozkan and Berberoglu 2013) and is a useful measure of the stability of microalgal suspensions. A ζ potential of −25 mV or less generally indicates a stable suspension, while a ζ potential between −10 mV and 0 mV points to a low stability and will generally coincide with flocculation.

The negative surface charge of microalgal cells is largely controlled by deprotonation of carboxylic functional groups. When pH is decreased in a microalgal suspension to levels below 3–4 where carboxylic groups are uncharged, the ζ potential approaches 0 mV (Hadjoudja et al. 2010). Indeed, flocculation can be induced by reducing the pH to below 4 (e.g., Liu et al. 2013). Chemical modification of functional groups on the cell surface through oxidation by ozone, chlorine, potassium permanganate, or potassium ferrate addition may also facilitate flocculation (Sukenik and Shelef 1984; Henderson et al. 2008b).

The ionic strength of the medium has an important influence on the ζ potential. At high ionic strength (e.g., seawater), the electrical double-layer surface is compressed and the ζ potential becomes less negative, allowing particles to approach it other and to flocculate. For example, a suspension of clay particles will flocculate when the ionic strength of the medium is increased. High concentrations of divalent cations (e.g., Ca^{2+} or Mg^{2+}) can also compress the double layer and, if present in sufficient concentrations, reverse the otherwise negative charge of surfaces. Despite the strong influence of ionic strength on the electrical double layer, there are no indications that suspensions of microalgae that live in seawater are less stable than

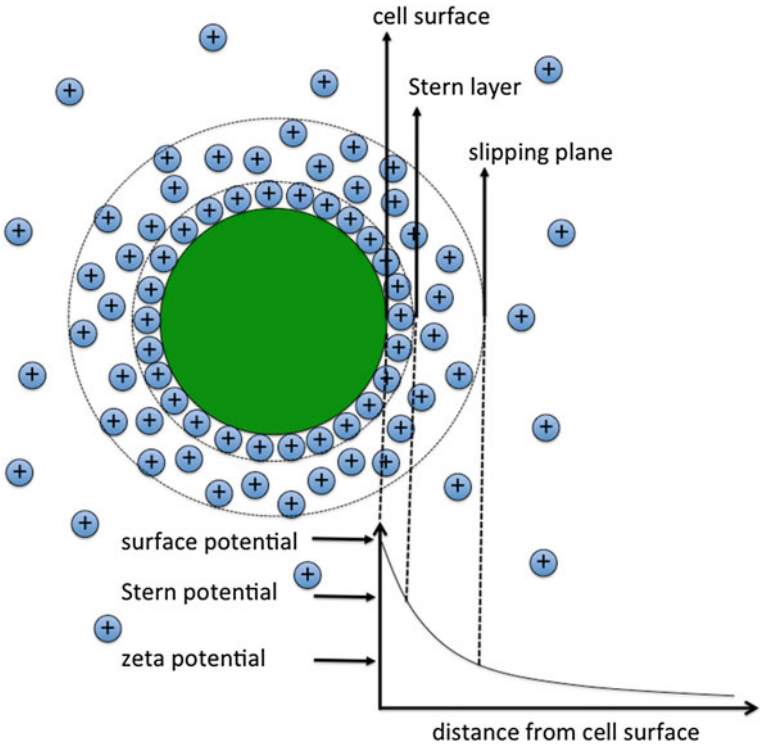

Fig. 12.2 Structure of the electrical double layer of charged ions in solution surrounding a negatively charged microalgal cell, and the potential difference between the particle and the bulk fluid as a function of the distance from the particle surface (Vandamme et al. 2013)

those of freshwater microalgae. This suggests that factors other than electrostatic repulsion also contribute to the stability of microalgal suspensions.

One factor that might play a role is steric stabilization by polymers that are associated with the microalgal cell surface (Fig. 12.3). Large polymers such as polysaccharides that are attached to the microalgal cell surface can extend into the

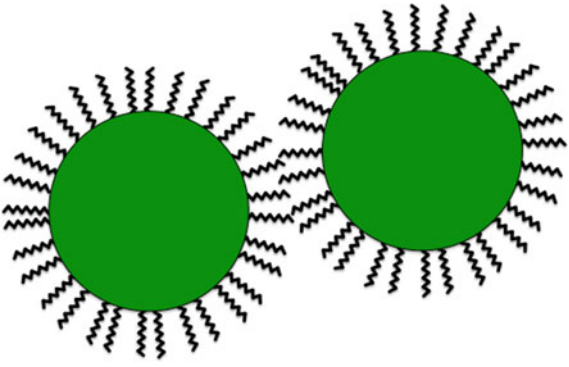

Fig. 12.3 Steric stabilization of particles in suspension by polymeric substances associated with the particle surface

surrounding medium. These polymers may prevent cells from approaching each other and can therefore stabilize microalgal suspensions. More research is needed to better understand what factors contribute to the observed stability of microalgal suspensions, particularly in seawater.

12.4 Mechanisms of Flocculation

Flocculation can be induced by several mechanisms (Fig. 12.4). A first mechanism is charge neutralization, or neutralization of the negative surface charge by adsorption of oppositely charged ions such as Mg^{+2} or Ca^{+2} to the cell surface. When the surface charge of the cell is neutralized, the electrostatic repulsion between the cells disappears. As a result, cells can approach each other and will connect through Van der Waals interactions resulting in the formation of flocs. In charge neutralization, the flocculant dose is a linear function of the surface area that needs to be neutralized. When dosage exceeds the optimal dose for charge neutralization, the surface charge

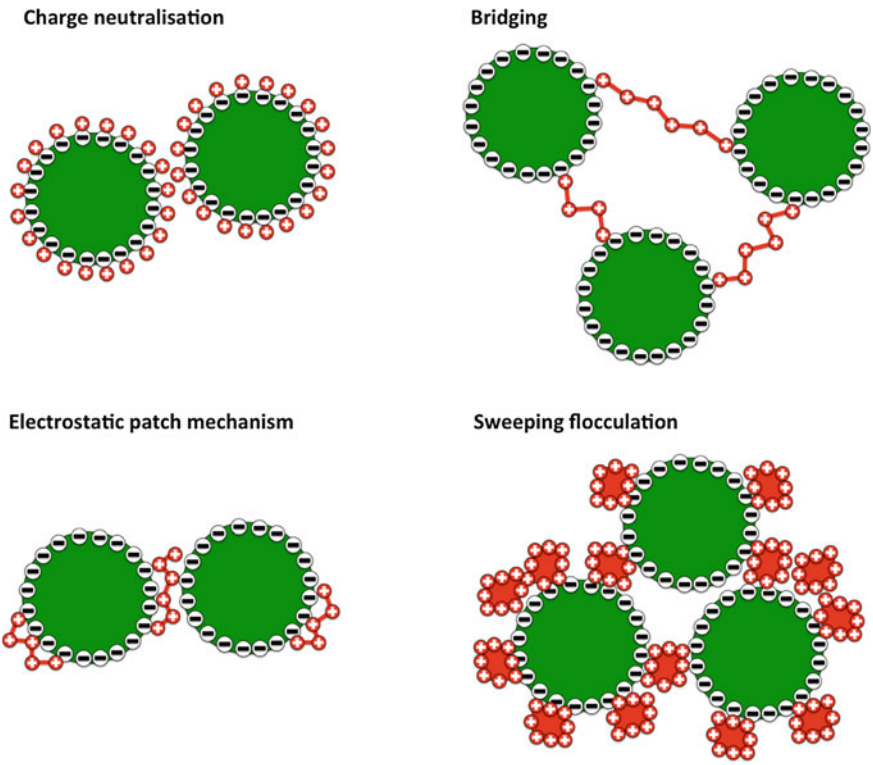

Fig. 12.4 Schematic illustration of four flocculation mechanisms: (1) charge neutralization, (2) bridging, (3) electrostatic patch mechanism, and (4) sweeping flocculation. The *white circles* with '−' sign indicate negative charges on the microalgal cell surface. The *red circles* with '+' sign indicate positive charges

may shift from negative to positive, and the cell suspension may become stabilized again; a phenomenon that is known as dispersion restabilization. Flocs formed by charge neutralization are often small and unstable (Gregory 2006).

A second flocculation mechanism is bridging by polymers. A polymer carrying positive charges, for example, chitosan, may bind to the negatively charged cell surface with the polymer chain extending from the cell surface. If the polymer connects to another cell, bridges can be formed between cells and flocs are formed. Longer polymers and weakly charged polymers are often more efficient than shorter and highly charged polymers. When a high polymer dose is used, the cell suspension may become stabilized again. This dispersion restabilization can be due to an inversion of the surface charge from negative to positive, but it can also be due to steric hindrance caused by the polymer chains that are associated with the surface (steric stabilization). Flocs formed by bridging are often large and stable (Fellows and Doherty 2006; Bolto and Gregory 2007).

A third mechanism of flocculation is the electrostatic patch mechanism. Here, positively charged polymers connect to the negatively charged cell surface and form patches on the cell surface, where the surface charge is inversed from negative to positive. Positively charged patches on one cell can connect to a negatively charged patch on another cell and flocs can be formed. Short and highly charged polymers often cause flocculation by the electrostatic patch mechanism (Bolto and Gregory 2007).

The final mechanism reviewed is sweeping flocculation or flocculation by enmeshment. In this mechanism, the flocculant forms a precipitate that enmeshes the microalgal cells. The cells then will settle or flocculate together with the precipitate. The precipitate often works as a ballast that facilitates the settling of the cells (Yahi et al. 1994). In sweeping flocculation, the flocculant dose is often independent of the concentration of particles that is flocculated. Flocs formed by a sweeping mechanism are often more stable than flocs formed by charge neutralization.

It is often not straightforward to conclude which mechanism is responsible for flocculation. A polymer flocculant may induce flocculation through charge neutralization, bridging or electrostatic patch neutralization. A metal salt coagulant may induce flocculation through charge neutralization or a sweeping mechanism (Duan and Gregory 2003). In many studies on flocculation of microalgae, one or a combination of mechanisms is often proposed, but few studies so far have demonstrated unequivocally which mechanism is responsible for inducing flocculation.

12.5 Flocculation Methods

12.5.1 Metal Salts

Metal salts such as alum (aluminum sulfate) or ferric chloride are among the most widely used flocculants in wastewater treatment. When dissolved in water, aluminum or ferric iron forms metal hydroxides that are positively charged and can

induce flocculation by charge neutralization. At higher doses, the hydroxides form precipitates that cause flocculation through a sweeping mechanism (Wyatt et al. 2012). Metal salts work well for harvesting microalgae, but the doses required for effective flocculation are very high (usually >100 mg L^{-1}). Even higher doses are required in seawater compared to freshwater (Sukenik et al. 1988). Another disadvantage is that the use of metal salts results in contamination of the harvested biomass with metals.

12.5.2 Electro-Coagulation–Flocculation

A method that is similar to the use of metal salts is electro-coagulation–flocculation or ECF. In ECF, metals are electrolytically released from a sacrificial anode. At the cathode, hydrogen gas and hydroxyl anions are produced. The metal cations react with the hydroxyl anions and form metal hydroxides that can cause flocculation in a similar way to metal salts. Due to the high conductivity of seawater, energy demand is much lower when ECF is used in seawater than in freshwater, which makes this method attractive for harvesting marine microalgae (Vandamme et al. 2011). The overall efficiency of the process may be improved by changing the polarity of the electrodes during operation (Kim et al. 2012). Although ECF may be promising for harvesting marine microalgae, contamination of the biomass with metals remains an issue. Recently, an electrolytic method was proposed for flocculating microalgae that uses inert carbon electrodes (Misra et al. 2014).

12.5.3 Polymer Flocculation

Polymer flocculants cause flocculation by bridging, or by the electrostatic patch mechanism. For bridging flocculation to occur, the polymers should be sufficiently long to be able to form bridges between individual cells. The length of the polymer chains can be significantly reduced by coiling of the polymer chains. Coiling is more prevalent in high-conductivity medium. Therefore, polymer flocculants are often less effective for harvesting marine microalgae (Bilanovic et al. 1988). Polymer flocculants are attractive because they generally require low flocculant doses and produce large and stable flocs (Granados et al. 2012). In wastewater treatment, synthetic polymers based on polyacrylamide are widely used. Because polyacrylamide contains potentially toxic acrylamide residues, contamination of the harvested biomass is an issue. Therefore, flocculants based on biopolymers are preferred over synthetic polymers. These biopolymers should be positively charged in order to allow interaction with the negatively charged microalgal cell surface. Positively charged biopolymers, however, are relatively rare in nature. Examples of natural positively charged natural flocculants are poly-γ-glutamic acid, a polymer produced by *Bacillus subtilis* (Zheng et al. 2012), and flour from the seeds of the

Moringa oleifera tree (Teixeira et al. 2012). Some studies suggest that uncharged natural polymers such as starch may also be capable of inducing flocculation in microalgae (Rakesh et al. 2013). Uncharged natural biopolymers may be modified with cationic functional groups to improve flocculation efficiency. A well-known example is chitosan, which is prepared from chitin by de-acetylation of the acetylamine groups, leaving amine groups that are protonated, and thus positively charged at low pH. Chitosan is an effective flocculant for microalgae but is only effective at low pH (Morales et al. 1985; Lubián 1989; Rashid et al. 2013). Starch that is modified by addition of quaternary ammonium groups is also an effective flocculant and can be used as an alternative for chitosan (Vandamme et al. 2010; Gerde et al. 2014). Moreover, the positive charges of the quaternary ammonium groups of cationic starch are less sensitive to pH, allowing the starch to operate over a broader pH range than chitosan. Other biopolymers that have been cationically modified for microalgae harvesting include guar gum (Banerjee et al. 2013) and cassia gum (Banerjee et al. 2014).

12.5.4 Autoflocculation

Microalgal cultures can flocculate spontaneously when pH rises as a result of photosynthetic depletion of carbon dioxide from the medium. This spontaneous flocculation has been referred to as autoflocculation but is in fact caused by precipitation of calcium and magnesium salts at high pH (Schlesinger et al. 2012; Vandamme et al. 2012a; González-Fernández and Ballesteros 2013).

When pH in microalgal cultures increases to pH 8.5–9, the first mineral to precipitate is often calcium phosphate. Calcium phosphate precipitation can be induced by photosynthetic depletion of carbon dioxide alone and does not require addition of a base to increase pH (Sukenik and Shelef 1984). High concentrations of calcium and phosphate are required for flocculation to occur. Therefore, this flocculation method can only be used in relatively hard waters with a high phosphate concentration. The amount of phosphate required for autoflocculation is higher than the phosphate required for microalgal biomass production. Because phosphate is a mineral resource with a limited supply, this flocculation method is unsustainable unless perhaps when microalgae are used for treating wastewaters with excessive phosphate concentrations. Beuckels et al. (2013), however, showed that it is possible to recover the phosphate from the harvested biomass by dissolving the calcium phosphate through mild acidification.

When pH is further increased above 10.5, magnesium will precipitate from the medium as magnesium hydroxide. Like aluminum or ferric hydroxide, magnesium hydroxide is a metal hydroxide and may cause flocculation through a similar mechanism as metal salts (Smith and Davis 2012). Magnesium hydroxide precipitates are positively charged up to pH 11 and can cause flocculation through charge neutralization (Wu et al. 2012; García-Pérez et al. 2014). Microscopical observations of flocculated microalgae, however, suggest that sweeping flocculation may be

equally important as charge neutralization (Besson and Guiraud 2013). Under some conditions, magnesium hydroxide precipitation may be induced by photosynthetic depletion of carbon dioxide (Spilling et al. 2011), but more often addition of a base is needed to increase pH to a sufficiently high level to initiate precipitation. A low concentration of magnesium is sufficient to induce flocculation (about 10 mg Mg L^{-1}; Vandamme et al. 2012a, b). Seawater contains very high magnesium concentrations (1300 mg Mg L^{-1}). In seawater, overdosing of base may result in massive precipitation of magnesium hydroxide and a very large sludge volume, which poses problems for further dewatering (e.g., Şirin et al. 2011). Therefore, it is recommended to control addition of the base rather than target a specific pH level to maximize flocculation efficiency and minimize the sludge volume (Besson and Guiraud 2013; García-Pérez et al. 2014). The amount of base that needs to be added will be a function of the quantity of magnesium hydroxide required to induce flocculation, but also of the buffering capacity of the culture medium (Garzon-Sanabria et al. 2012). Repeated recycling of the culture medium after flocculation may result in depletion of magnesium from the medium. However, Vandamme et al. 2014a demonstrated that 95 % of the precipitated magnesium hydroxide could be recovered by mild acidification, allowing it to be recycled back into the culture medium. The repeated addition of base to induce flocculation and of acid to neutralize the medium can result in accumulation of salts (Castrillo et al. 2013). To avoid accumulation of salt, ammonia may be used as a base to induce flocculation (Chen et al. 2012). Solubility calculations that take account of aqueous speciation and the high initial solubility of freshly formed precipitates are useful means for predicting and refining autoflocculation end-points (Brady et al. 2014). Chapter 13 describes primary dewatering of microalgae using autoflocculation at an industrial scale.

At high pH, calcium may also precipitate as calcium carbonate. So far, few studies have demonstrated that calcium carbonate precipitates are effective autoflocculation agents (Ayoub et al. 1986). Calcium carbonate precipitation is a slower process and may take more time than flocculation by magnesium hydroxide or calcium phosphate. Moreover, precipitation of calcium carbonate may be limited by carbonate concentrations in the medium. Calcite surfaces will be positively charged and able to autoflocculate microalgae only under restricted conditions, namely when Ca^{+2} and Mg^{+2} levels are relatively high and/or sulfate levels are low (Brady et al. 2014).

12.5.5 Bioflocculation

Some microalgae flocculate spontaneously without addition of flocculants or precipitation of minerals, and the phenomenon is generally referred to as bioflocculation. Bioflocculation can occur spontaneously in microalgae-based wastewater treatment systems, including high-rate microalgal ponds where it is often used in biomass harvesting (Park et al. 2011b; Benemann et al. 2012).

Bioflocculation is often due to a dominance of microalgae species with high settling rates, often colonial and/or large species of microalgae. For instance, high flocculation efficiencies are observed when the large chlorophyte *Pediastrum* is dominant (Park et al. 2011a). By recycling part of the harvested biomass, the dominance of this species in the community can be effectively maintained over long periods of time (Park et al. 2013). High flocculation efficiencies are also observed when filamentous cyanobacteria are prominent in the microalgal community (Su et al. 2011). Spontaneously flocculating microalgae can also be used to harvest other, non-flocculating species of microalgae by mixing bioflocculating and non-flocculating microalgae (Salim et al. 2011, 2012). Some bioflocculating species of microalgae such as the diatom *Skeletonema* appear to produce infochemicals that are capable of inducing flocculation in other microalgal species (Taylor et al. 2012). Cultures of otherwise non-flocculating species microalgae can be 'trained' to induce bioflocculation. For instance, Su et al. (2012) repeatedly removed all microalgae that remained in suspension and kept only the rapidly settling microalgae and after one month obtained a culture that flocculated spontaneously. A major advantage of spontaneous flocculation of microalgae is that no chemicals are added during the process and the harvested biomass is free from contaminants. In order to be able to use bioflocculation as a reliable harvesting method though, more research is needed to better understand the underlying mechanisms that cause the phenomenon.

Bioflocculation may also be caused by other microorganisms in the microalgal culture, such as bacteria or fungi. For example Lee et al. (2012c) showed that bacteria present in cultures of the chlorophyte *Chlorella* prompted autoflocculation. Bacteria or fungi can be cultured separately and added to a microalgal culture-induced flocculation (Zhou et al. 2012, 2013). Alternatively, bacteria or fungi can be co-cultured with the microalgae, in which case the culture medium should contain a carbon source to support heterotrophic growth of bacteria or fungi (Lee et al. 2008; Gultom and Hu 2013). Bacterial bioflocculation may be a particularly promising flocculation method in wastewater treatment systems, as wastewater often contains a carbon source to sustain bacterial growth. However, an optimal balance between heterotrophic bacterial and autotrophic microalgal production is required for optimal flocculation. This can be controlled by optimizing the ratio of organic carbon over inorganic carbon in the medium (Van Den Hende et al. 2011, 2014).

12.5.6 Novel Approaches for Flocculation

Several recent studies have investigated the potential of magnetite nanoparticles for flocculating microalgae. The nanoparticles can induce flocculation of microalgae and separation of the microalgae from the medium in a magnetic field. This technology thus combines flocculation and separation of flocs in a single process step. In some studies, the magnetite nanoparticles are used as such, without functionalization (Xu et al. 2011; Procházková et al. 2012). In other studies, the

nanoparticles are functionalized by coating the surface with cationic functional groups (Lim et al. 2012; Seo et al. 2014). By using functional groups that have a pH-dependent charge, it is possible to remove the nanoparticles after flocculation by adjusting pH and to reuse them in a second round of harvesting (Cerff et al. 2012; Seo et al. 2014)

In estuaries, flocculation of natural populations of microalgae is sometimes observed in the presence of high concentrations of sediment particles. This flocculation has been ascribed to interaction between microalgae and clay minerals (Avnimelech et al. 1982). Clays have been used successfully to induce flocculation of natural blooms of microalgae in coastal waters (Sengco and Anderson 2004; Padilla et al. 2010). So far, however, only a few studies have explored the potential of clays for harvesting microalgae. One study explored the use of synthetic organoclays with specific surface properties for flocculating *Chlorella* (Lee et al. 2013b). Farooq et al. (2013) successfully demonstrated aminoclay harvesting of *Chlorella vulgaris* and *Nannochloris oculata*. Aminoclays were used recently as a template for nanoscale zerovalent iron synthesis and were shown to be efficient for harvesting *Chlorella sp* (Lee et al. 2014).

12.6 Interplay Between Microalgal Biology and Flocculation

Microalgae are a highly heterogeneous group of microorganisms belonging to many different evolutionary lineages. Different species of unrelated microalgae can have very different surface properties (Eldridge et al. 2012). When the functional groups on the cell surface differ between species of microalgae, this will cause differences in the flocculation behavior (Henderson et al. 2010). Cell surface properties may even differ between strains of the same species (Cheng et al. 2011). For example, mutant *Chlamydomonas* strains that lack a cell wall were found to flocculate more easily than wild type strains (Scholz et al. 2011). In addition, smaller species have a higher surface to volume ratio and require a higher flocculant dose per unit of biomass (Vandamme et al. 2010).

Flocculation of microalgae differs from flocculation of inorganic colloids and particles in that microalgae are living organisms that can modify their surface properties and interact with their environment through uptake or excretion of substances (Pieterse and Cloot 1997). The flocculation behavior of microalgae can depend on the culture conditions. Zhang et al. (2012) showed that the cell surface of stationary-phase *Chlorella zofingiensis* cells had lower concentrations of carboxylic groups than during exponential phase and required a lower dosage of alum to induce flocculation. Because the microalgal ζ potential changes from exponential to stationary growth, the flocculation efficiency is likely to vary with the growth phase (Danquah et al. 2009b).

Microalgae excrete a substantial fraction of the photosynthesis products as extracellular organic matter. Hulatt & Thomas (2010) showed that up to 17.3 % of the organic matter produced during photosynthesis is excreted in the culture medium. Organic matter concentrations in microalgal culture medium are often 10–100 mg C L^{-1}, depending on species and culture conditions. This organic matter consists mainly of polysaccharides and proteins (Henderson et al. 2008a). The secreted material has a strong inhibitory effect on flocculation, as is evident from much higher flocculant dosages in media with microalgal organic matter compared to media without the organic matter (Bernhardt et al. 1989; Vandamme et al. 2012b). Most of the chemical demand required for flocculating microalgae may be due to the microalgal organic matter present in the media rather than the microalgal biomass itself, as well as impurities present in the medium, such as humic matter (de Godos et al. 2011; Beuckels et al. 2013). Chen et al. (2008) suggested that microalgal organic matter may cause complexation of free metal ions and as such prevent flocculation by metal hydroxides. It is also possible that negative charges on the extracellular organic matter compete with the microalgal cell surface for the positive charges of the flocculants (Garzon-Sanabria et al. 2013).

12.7 Further Dewatering and Processing

After flocculation, the flocculated microalgae can be separated from the culture medium by sedimentation. The efficiency of the entire flocculation–sedimentation process depends on the flocculation efficiency as well as the settling rate of the flocs and the volume of sludge that is formed. Most academic research on microalgae flocculation so far has focused mainly on the flocculation efficiency and thus evaluated mainly whether a novel flocculation technology is capable of inducing significant flocculation of the microalgae in suspension. When applying flocculation in large-scale systems, however, the technology should also be capable of generating flocs that settle rapidly and produce a small sludge volume. Properties such as floc size, settling rate, and sludge volume can differ substantially between different microalgae species and different flocculation methods (Fig. 12.5). Some flocculants like chitosan generate large flocs with a high sedimentation rate while other methods like alum produce small flocs that settle more slowly (Vandamme et al. 2014b). Flocculation by cationic starch generates a small sludge volume and allow for a 50-fold concentration of the biomass, while flocculation by chitosan generates a large sludge volume and allows only for a 20-fold concentration. The flocculation conditions or dosage can also influence flocculation process. For example, when applying autoflocculation by magnesium hydroxide in seawater medium, overdosing of base may result in massive precipitation of magnesium hydroxide and will result in a very large sludge volume (Şirin et al. 2011; Besson and Guiraud 2013; García-Pérez et al. 2014).

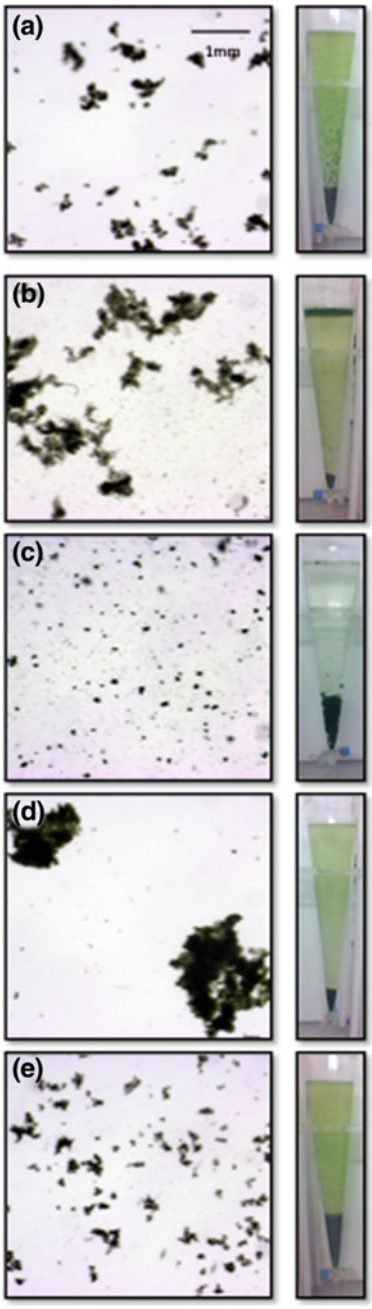

Fig. 12.5 Illustration of differences in the size and shape of flocs, and volume of sludge produced by different flocculation methods: **a** aluminum sulfates, **b** electro-coagulation-flocculation using aluminum anodes, **c** cationic starch, **d** chitosan, and **e** pH-induced autoflocculation. The same culture (*Chlorella vulgaris*, 0.5 dry weight L^{-1}, Wright's cryptophyte medium) was used for each flocculation method (Vandamme et al. 2014a, b)

Subsequent to flocculation, sedimentation is generally used to separate the flocs from the culture medium. Sedimentation in a batch tank or a flow-through settling basin is relatively inefficient because these tanks are relatively deep and require a long settling times. As a result, a large area is required for the settling tanks. In inclined settlers or lamella separators, the settling depth is reduced and the surface area for settling is increased, resulting in a much smaller settling tank footprint (Smith and Davis 2013; Wang et al. 2014). Flocs can also be separated from the culture medium by means of flotation (suspended air flotation or dissolved air flotation). Flotation generally results in a smaller sludge volume than sedimentation (Kwon et al. 2014). Membrane filtration can also be used to separate concentrate flocs into a slurry that can be further dewatered (Gerardo et al. 2014).

After pre-concentration of the biomass by means of flocculation followed by sedimentation or flotation, the algal slurry that is obtained should be further dewatered using a mechanical method. This will require transport of the slurry from a settling or flotation system to a centrifugation or filtration system. Because transport and processing of microalgal slurries generally require pumping, changes in viscosity, and rheological behavior as a result of up-concentration of the biomass should be taken into account. As long as biomass concentrations remain below 2 %, microalgal slurries display a Newtonian behavior. When biomass concentrations exceed 4 to 8 %, however, shear thinning may occur in slurries of some species of microalgae (Wileman et al. 2012). Few studies have investigated the rheological properties of microalgal slurries that are produced by flocculation. Sirin et al. (2013) noted that pH-induced flocculation had no significant effect on the viscosity of the microalgal slurry that was obtained.

12.8 Contamination of the Biomass by Flocculants

When flocculation is used in mining, wastewater treatment or fermentation the resultant sludge is disposed of as a waste product. Yet, when flocculation is used to harvest microalgae, the flocculated biomass is the product. The flocculant ends up in the harvested biomass, which may have consequences for the further valorization of the biomass. Contamination of the harvested biomass is an important factor to consider when considering the use of a flocculation method for harvesting microalgae. For instance, if the flocculant is toxic or contains toxic residues, the harvested biomass or fractions thereof cannot be used for applications such as food or animal feed. This is important when microalgal biomass is used in a biorefinery context where part of the biomass is used to generate energy (e.g., lipids), and other biomass fractions are used as animal feed (e.g., protein fraction) or high-value ingredients for the food or health industry (e.g., carotenoids) (Wijffels et al. 2010). Rwehumbiza et al. (2012) showed that aluminum added during alum-based flocculation did not contaminate lipids and fatty acid methyl esters, but it might remain in the protein fraction. Contamination of the biomass with flocculants may also influence the recovery efficiency of certain bioproducts. Rios et al. (2013) observed

a lower lipid extraction yield in microalgae harvested by flocculation (by pH increase) than in microalgae harvested by membrane filtration. Borges et al. (2011) noted that the choice of the flocculant used for harvesting microalgae influenced the ratio of saturated over unsaturated fatty acids in the biomass.

12.9 Costs Associated with Flocculation-Based Harvesting

When using flocculation in wastewater treatment or fermentation, the cost of the flocculant is related to the volume of treated water or medium. But when flocculation is used for harvesting microalgae, the cost is calculated relative to the amount of biomass that is harvested, not relative to the volume of microalgal broth that is treated. A given dose of flocculant capable of removing 0.5 kg of particulates from an aqueous medium containing 0.5 g L^{-1} of particulates will yield 1000 L of treated water in the case of wastewater treatment, but only 0.5 kg of biomass in the case of microalgae harvesting. As a result, the cost of the flocculant is a critical factor when selecting a flocculation method for microalgae harvesting.

When evaluating the cost of flocculation-based harvesting, the cost of the chemical flocculant in relation to the dosage needed is obviously important. Some flocculants have been used since many years in various industries and are low-cost commodities that are available in bulk. An example is alum, which is commonly used in wastewater treatment. Although alum is cheap, the high dosage that is required to flocculate microalgae makes its use relatively expensive (about 300 \$ ton^{-1} harvested biomass) (Molina Grima et al. 2003; Uduman et al. 2010). Chitosan requires lower dosages, but it is a more expensive chemical and therefore has a similar overall cost (about 500 \$ ton^{-1} harvested biomass). Autoflocculation by magnesium hydroxide is often proposed as low-cost method for flocculation. As most waters contain sufficient magnesium, the main cost for flocculation is the cost of the base required to increase the pH. This cost is very low when calcium hydroxide or slaked lime is used as a base (about 30 \$ ton^{-1} harvested biomass) (Schlesinger et al. 2012). When evaluating the cost of ECF, not only the electricity cost for electrolysis should be taken into account, but also the dissolution of the sacrificial anode (Lee et al. 2013a). Aluminum anodes generally have a higher flocculation efficiency than iron anodes, yet iron electrodes may nevertheless be preferred due to a lower anode cost and lower energy consumption (Dassey and Theegala 2014). Some novel flocculants such as for modified iron oxide nanoparticles are not yet available on the market, which makes it difficult to estimate their cost. No chemicals are required to harvest microalgae using bioflocculation. When flocculation of microalgae is induced by addition of cultured bacteria, fungi or other microalgae, the cost to cultivate these flocculating microorganisms should be taken into account. It is important to realize that the dosage of flocculant needed and thus the cost of the flocculant depend strongly on the species of microalgae that is harvested and on the culture conditions. The amount of algal organic matter excreted in the culture medium can result in a strong increase in the flocculant dosage.

When estimating the cost of a flocculation method, not only the cost of the flocculant should be taken into account, but also the energy demand for mixing and pumping (Elmaleh and Jabbouri 1991; Danquah et al. 2009a). Intensive mixing of the flocculant solution with the microalgal culture broth is essential to achieve good flocculation efficiency (e.g., Lee et al. 2013a, b). Other indirect costs are costs related to the sedimentation or flotation system (construction costs, operational cost, land cost) (Richardson and Johnson 2014). In life cycle analysis or LCA studies, the environmental burden associated with the production of the flocculant should be taken into account as this cost can differ substantially between different flocculants (Brentner et al. 2011). Examples of flocculants with a potentially high environmental burden might include those that are Al-based.

The primary aim of using flocculation for harvesting microalgae is to pre-concentrate the microalgal biomass and to reduce the volume of broth that needs to be processed by a mechanical dewatering method such as centrifugation or filtration. The higher the degree of pre-concentration that can be achieved during the pre-concentration stage, the higher the energy savings in the mechanical dewatering stage (Milledge and Heaven 2012). A flocculation method that can produce a small volume of algal sludge will require less energy for mechanical dewatering than a method that produces a large sludge volume. Pre-concentration of the biomass can be achieved by combining flocculation with sedimentation or with flotation. Flotation has higher investment costs and requires more energy than sedimentation, but it can achieve a higher degree of biomass concentration (Besson and Guiraud 2013).

The use of flocculation for harvesting may interfere with other stages of microalgae cultivation and processing. For instance, the flocculant may prevent recycling of the culture medium, which can result in high costs for water to prepare fresh culture medium or a high cost for treatment of the spent medium before discharge into the environment. The flocculant can interfere with downstream processing of the biomass, e.g. lipid extraction. Some flocculants form toxic residues in the harvested biomass and can limit the use of the protein fraction as animal feed, and thus limit the income that can be generated from the microalgal biomass. These indirect costs of using a certain flocculation method should also be taken into account. More information on the economics of harvesting and downstream processing is given in Chap. 14.

12.10 Conclusions

It is clear that the energy requirements for harvesting microalgae could be reduced by at least an order of magnitude if the biomass could be pre-concentrated using flocculation. There are several technologies available to flocculate microalgae, including metal salt coagulants, electro-coagulation–flocculation, polymer flocculants, or the use of clays or iron oxide nanoparticles. Flocculation can also be induced by a pH increase (autoflocculation; result of precipitation of Ca or Mg salts) or it can occur

spontaneously (bioflocculation). The main disadvantage of using flocculation for harvesting microalgae is that, in most cases, the biomass is contaminated with a foreign substance. This can limit the use of the biomass or can interfere with downstream processing. Therefore, the flocculant should ideally be non-toxic or, better still, it should be possible to remove the flocculant from the biomass after harvesting. Because cost reduction is an important issue in production of microalgal biofuels, the cost of a flocculation technology is an important criterion. Microalgae excrete organic matter in the culture medium that may interfere strongly with flocculation. This interference of microalgal organic threatens the applicability of flocculation for harvesting microalgae. So far, no ideal universal flocculation method has yet emerged. It is most likely that the optimal flocculation method will be species specific and depends on the final application of the biomass.

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Chapter 13
Industrial-scale Microalgae Pond Primary Dewatering Chemistry for Energy-efficient Autoflocculation

Patrick V. Brady, Mark P. McHenry, M. Carolina Cuello and Navid R. Moheimani

Abstract Industrial-scale microalgae production will likely require large energy-intensive technologies for both culture and biomass recovery; energy-efficient and cost-effective microalgae dewatering and water management are major challenges. Primary dewatering is typically achieved through flocculation followed by separation via settling or flotation. Flocculants are relatively expensive, and their presence can limit the reuse of de-oiled flocculated microalgae. Natural flocculation of microalgae—autoflocculation—occurs in response to changes in pH and water hardness and, if controlled, might lead to less-expensive "flocculant-free" dewatering. A better understanding of autoflocculation should also prompt higher yields by preventing unwanted autoflocculation. Autoflocculation is driven by double-layer coordination between microalgae, Ca^{+2} and Mg^{+2}, and/or mineral surface precipitates of calcite, $Mg(OH)_2$, and hydroxyapatite that form primarily at pH > 8. Combining surface complexation models that describe the interface of microalgae:water, calcite:water, $Mg(OH)_2$:water, and hydroxyapatite:water allows optimal autoflocculation conditions—for example pH, Mg, Ca, and P levels—to be identified for a given culture medium.

P.V. Brady (✉)
Geoscience Research and Applications Group, Sandia National Laboratories, Albuquerque, USA
e-mail: pvbrady@sandia.gov

M.P. McHenry
School of Engineering and Information Technology, Murdoch University, Perth, Australia

M. Carolina Cuello · N.R. Moheimani
Algae R&D Centre, School of Veterinary and Life Sciences, Murdoch University, Perth, Australia

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13.1 Introduction

Microalgae achieve a much higher biomass productivity when compared to conventional terrestrial biofuel crops (Fon Sing et al. 2011), although several issues that must be resolved include developing cost-effective dewatering and processing technologies, and the associated commercial and environmental challenges (Griffiths and Harrison 2009; Moheimani et al. 2011; Vasudevan and Briggs 2008). Cost-effective and energy-efficient dewatering of microalgae, nutrient recycling, and control of effluent wastewater are becoming major challenges to microalgae producers (Borowitzka and Moheimani 2010; Charcosset 2009; Clarens et al. 2010; Wyman and Goodman 1993; Xiong et al. 2008). Open pond microalgae production can become expensive due to variable capital, operational, and downstream processing costs derived from the low microalgae cell densities (Lee 2001; McHenry 2010), and if not optimised, industrial microalgae production will consume large volumes of water through evaporative loss (Chisti 2007; Clarens et al. 2010), generate effluent and become an energy-intensive process (Borowitzka and Moheimani 2010; Charcosset 2009; Clarens et al. 2010; McHenry 2013; Wyman and Goodman 1993; Xiong et al. 2008). It is generally not well understood that microalgae production will require large energy-intensive technologies for both culture and biomass recovery (Chisti 2007), including heat exchangers, scrubbing, pressurisation, pipeline construction, and pumping of flue gases (as a potential source of CO_2) to be intensively managed (McHenry 2010, 2013), and microalgae production sites must be carefully chosen to optimise industrial resources, natural resources, and environmental conditions to facilitate post-harvest processing (Borowitzka 1992).

13.2 Microalgae Flocculation and Dewatering

Commercial microalgae biofuel production is dependent on the unique microalgal species chosen, the management of biotic and abiotic conditions, production costs (energy, nutrients, water, land, chemicals, etc.), co-located industrial facilities, and downstream applications and markets (Borowitzka 1999; Kunjapur and Eldridge 2010; McHenry 2010, 2013). Because the concentration of microalgae in open pond systems is so low [usually between 0.5 and 2 g/L (Fon Sing et al. 2011)], dewatering and harvesting/processing will heavily influence the evolution of upstream microalgae production, strain selection, water/nutrient recycling technology, and other processes (Moheimani et al. 2013). Primary dewatering is typically achieved through flocculation, followed by separation from the water via settling or floatation (Moheimani et al. 2013). Some microalgae naturally flocculate, while others respond to chemical flocculants or non-chemical methods (Brady et al. 2014; Moheimani et al. 2013; Sukenik and Shelef 1984). For example, Origin Oil and Diversified Technology have used pulsed electric fields to enhance flocculation

and lyse microalgae (Moheimani et al. 2013). During post-primary dewatering, the remaining water in microalgae pastes is usually removed by heating, which if not using low grade or waste heat can also include a high-energy burden (McHenry 2013; Moheimani et al. 2013). Secondary dewatering or mechanical drying generally incudes physical separation of the water and the microalgae using centrifuges or decanters that are often energy intensive and relatively inefficient (Moheimani and McHenry 2013; Moheimani et al. 2013; Solix Biofuels 2011b). However, this area of research is a fast-moving field with some companies including Evodos achieving mechanical centrifuge electricity consumption reductions when dewatering water and microalgae to a paste (Moheimani et al. 2013). Evodos centrifuges exhibit practically zero cell shearing, thermal damage, without chemicals and collecting minimal bacteria requiring only <1 kWh/m, and 5.5 kWh/kg DW (Evodos 2011). Nonetheless, for industrial-scale volumes of water, this energy consumption per unit of output remains prohibitively high.

There are numerous competing approaches focussed on reducing dewatering energy demands in microalgae production (McHenry 2013). For example, General Atomics, a long-time defence contracting company based in California, is evaluating Algaeventure Systems microalgae "harvesting, dewatering, and drying" (HDD) technology with the ability to reduce dewatering energy consumption considerably using unique conveyer centrifugal technology (Li 2012; Moheimani et al. 2013). Algaeventure Systems Inc. is a spin-off company from Univenture, Inc., which is a plastic packaging manufacturer, a large-potential high-value microalgae-based co-product potentially cross-subsidising biofuel production (Moheimani et al. 2013). Taking another unique method to dewater and process biofuel precursors is the company Algenol, which has developed a unique DIRECT TO ETHANOL® process using a 15-m-long and 1.5-m-wide semi-transparent polyethylene film outdoor photobioreactor containing treated sea water, microalgae, nutrients, with a volume of air above the water. Algenol uses a "hybrid" microalgae that reportedly produce ethanol intracellularly which diffuses through the cell wall into the culture medium and evaporates along with water into the internal air volume. The water/ethanol solution condenses on the inner surface of the photobioreactor and is collected, concentrated, and distilled (Moheimani and McHenry 2013). Algenol states that their DIRECT TO ETHANOL® process produces around equal amounts of freshwater and ethanol, with collaborators membrane technology research (MTR) using their Bio-Sep™ technology—a membrane distillation technology. Similarly, Solix's from Colorado has developed a "Lumian^{TM} AGS^{TM}," photobioreactor which is a series of water-filled metal tanks supporting semi-submerged transparent microalgae cultures circulated by weighted rollers, with independently controlled air and CO_2 from a co-located coal-bed methane production facility (Moheimani and McHenry 2013; Solix Biofuels 2011a). The twenty 36-m-long 200-L bags sum to a total culture capacity of around 4 kL (Solix Biofuels 2011c). Solix's Colorado Coyote demonstration facility can produce a maximum of 28 kL/ha/year of microalgal oil, with culture peak yield rates equivalent to around 19 kL of oil/ha/year, or around 5 g of oil per m^2 per day (Moheimani and McHenry 2013). However, Solix is primarily a culturing technology provider and has not focussed on downstream energy and dewatering technology.

Another unique production approach is underway in New Zealand, where a private company, Aquaflow, is specialising in harvesting wild microalgae from municipal sewage ponds and high-nutrient waters. Aquaflow solely relies on water remediation and equipment sales rather than microalgae as their business model. In 2008, Aquaflow partnered with Honeywell UOP to use UOP/Eni Ecofining™ and the Canadian company Ensyn's rapid thermal processing (RTP™) fast biomass pyrolysis (~75 % bio-oil output by volume) to provide liquids for Honeywell's Green Diesel™ and Honeywell Green Jet™ fuel production (Ensyn 2011), using hydroprocessing to produce catalysts and thermal energy for output separation (Moheimani et al. 2013; Regalbuto 2011). Aquaflow currently operate with around 60 ha of open mixed sewage and municipal and agro-industrial waste oxidation ponds (serving a population of 27,000 and water flows of 5 GL/year.) The ponds are continuously harvested using systems built inside a 40 ft sea container using dissolved air flotation and polyelectrolyte flocculation (70–90 % recovery), followed by a belt press for extraction, processing wastewaters at a rate of 35 m^3/h to produce a wild microalgae liquid concentrate at 8–10 % microalgae by volume (Moheimani et al. 2013).

To avoid the associated high energy and material costs of dewatering, integrated microalgal biorefineries have received much attention in the literature (Chisti 2007; Wyman and Goodman 1993), and recent approaches have focussed on hydrothermal liquefaction of relatively low-lipid biomass from mixed microalgae species to produce a crude oil replacement in the presence of water at medium temperatures and pressures (Biller and Ross 2011). de Boer et al. (2012) showed that in situ hydrolysis and esterification of wet biomass and hydrothermal liquefaction was the most energetically feasible process in a comparison of four of the most promising methods to convert microalgae into biodiesel, including pulsed electric field-assisted extraction, transesterification, and in situ acid catalysed esterification of dry biomass. However, the present fundamental diversity of approaches for microalgae dewatering and the associated energy efficiency options demonstrate that microalgae dewatering is far from a mature field on the verge of commercialisation. This research discusses an alternative approach that is a potential tool for achieving low-cost primary dewatering of microalgae for economic biofuel production using inexpensive "chemical-free" approach to autoflocculation that is appropriate to large-scale industrial production systems using existing bulk agricultural commodities.

13.3 Autoflocculation

Autoflocculation works by Mg^{+2} and Ca^{+2}, and/or positively charged $Mg(OH)_2$ surfaces, bridging negatively charged microalgal surfaces, causing the latter to clump together making them easier to separate from solution. Formation of calcium phosphate also prompts microalgal autocoagulation, as does calcite formation under some conditions. Microalgae autoflocculation by Mg^{+2} requires adequate dissolved

Mg and a pH between 10 and 11; autoflocculation by calcium phosphate requires both calcium and phosphate and a pH above ~8. Microalgae autoflocculate from sea water (Mg = 52 mM) when the pH is raised from roughly pH 8 to above pH 10.4, typically in the presence of freshly formed magnesium hydroxides (Ayoub et al. 1986; Knuckey et al. 2006) and calcite (Ayoub et al. 1986). Calcite is a weak autoflocculater relative to $Mg(OH)_2$ and calcium phosphate (Folkman and Wachs 1973; Smith and Davis 2012).

At the molecular level, pH and ionic strength-dependent microalgae surface charge are controlled by carboxylate, phosphate, and amine/hydroxyl groups (Hadjoudja et al. 2010) which become deprotonated above pH 4–6 (carboxylic groups) and pH 7–8 (phosphoric groups). Microalgae carboxylate and phosphate groups are able to sorb cationic species such as Ca^{+2} and Mg^{+2} and decrease their electrostatic repulsion from other microalgae, or they can attach to positively charged $Mg(OH)_2$ surfaces which are positively charged below pH 11 (Pokrovsky and Schott 2004). In the absence of this bridging, anionic microalgae repel each other electrostatically and subsequently remain suspended in solution. The electrostatic picture of microalgae surface chemistry is complicated by the fact that microalgae surfaces are chemically heterogeneous. In particular,

1. Extracellular organic material (EOM) is secreted from the microalgae surface, particularly in the later stages of growth, complicating and possibly dominating the interfacial properties of the microalgae (e.g. Henderson et al. 2006) (EOM is thought to contain many of the same functional groups of the non-EOM microalgae surface). The presence of EOM can aid or hinder flocculation (Bernhardt and Clasen 1991; Henderson et al. 2008)
2. Microalgae can modify their surface charge and change their morphology, during flocculation, increasing flocculant demand and/or slowing flocculation (e.g. Pieterse and Cloot 1997)
3. Microalgae morphology can affect flocculation effectiveness, particularly if the microalgae are non-spherical and/or relatively large.

Physical factors also play an important role in microalgae flocculation. Flocculation is affected by the rate of alga–alga interaction which tends to increase with microalgae concentration, with shear mixing and with differential particle settling velocities; these effects have been reviewed by Bratby (2008) and Jackson and Burd (1998). Large volume fractions of microalgae or precipitates can prompt microalgae to enmesh in flocs prompting sweep flocculation that depends less on specific surface characteristics (Duan and Gregory 2003).

13.4 Autoflocculation by $Mg(OH)_2$

The requisite pH jump for autoflocculation by $Mg(OH)_2$ can be achieved through photosynthesis under CO_2-starved conditions, through lime addition, or both. For example, sea water in contact with atmospheric CO_2 at $P_{CO_2} = 10^{-3.5}$ atmospheres

has a pH of ~8.2 and a total inorganic carbon content of ~2 mM. Conversion of this carbon to biomass raises the pH to above 10 and lowers the P_{CO_2} by several orders of magnitude; part of the carbon uptake is by formation of calcite. Such alkaline swings are routinely observed in sewage treatment ponds when photosynthetic activity is high [for example see (Ayoub et al. 1986; Sukenik and Shelef 1984)]. pH boosting with slaked lime (calcium hydroxide) is relatively inexpensive, and calcium in flocculated microalgae is considered a plus if the spent microalgae are to be used as cattle feed (e.g. Schlesinger et al. 2012). Schlesinger et al. (2012) showed that lime usage could be cut by having an earlier, CO_2-starvation step.

Figure 13.1 shows calculated changes in sea water chemistry as lime is added: as pH rises, first calcite forms, and then near pH 10.3 $Mg(OH)_2$ forms. Ca levels rise as lime addition proceeds; Mg levels decrease. The curves in Fig. 13.1 were calculated using the chemical speciation code PHREEQC (Parkhurst et al. 1999) and the llnl.dat thermodynamic database. The solubility product of freshly precipitated $Mg(OH)_2$ was set to $10^{-9.2}$ (after Gjaldbæk 1924). Using the latter value predicts that sea water should precipitate $Mg(OH)_2$ at pH > 10.4. Smith and Davis (2012) saw a precipitate forming above pH 10.4 from BG-11 solution containing 9.6 mM Mg. But maximum microalgae flocculation began at pH 10.2, suggesting that flocculation was driven by charge-reversing Mg^{+2} sorption to microalgae, formation of metastable surface precipitates of $Mg(OH)_2$, or both (Smith and Davis 2012). In sum, maximum autoflocculation occurs 0.2–0.3 pH units below the pH where $Mg(OH)_2$ is predicted to form. Additional formation of $Mg(OH)_2$ does not increase flocculation (Smith and Davis 2012). It therefore seems reasonable to simply set Mg and pH autocoagulation targets to 0.2 pH units below the pH where $Mg(OH)_2$ is predicted to form. The latter will depend on the amount of Mg and to a lesser extent the amount of calcium in solution.

Smith and Davis (2012) estimate Mg requirements of autoflocculation as 1.5–2.4 % of the microalgae's ash-free mass. For a 2 g/L microalgae culture, 2.4 % is ~2 mM. There are only roughly 0.4 mM of microalgae surface sites in a 2 g/L

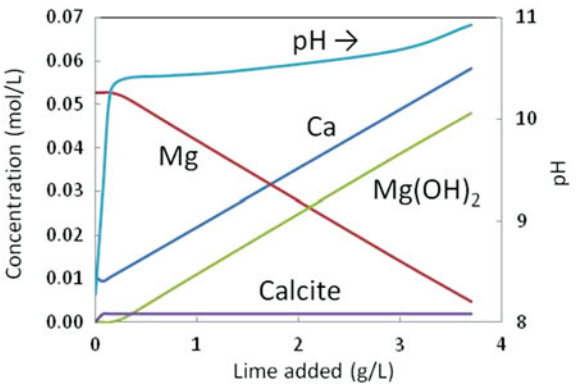

Fig. 13.1 Calculated lime addition to seawater at 25 °C. *Mg* dissolved *Mg*; *Ca* dissolved *Ca*

culture, suggesting that the bulk of the Mg forms surface precipitates of $Mg(OH)_2$ on the microalgae through the reaction:

$$Mg(OH)_2 \leftrightarrow \ Mg^{+2} + \ 2OH^- \ K \ = \ \{Mg^{+2}\}\{OH^-\}^2$$

Given a known microalgal solution Mg level, the target minimum pH, that is a flocculation critical pH—pH_c (see Sukenik and Shelef 1984)—that must be achieved, either through lime addition or CO_2-starvation, can be calculated for maximum autoflocculation to occur:

$$pH_c = 9.4 - 0.2 - 0.5\log\{Mg^{+2}\}$$

where $\{Mg^{+2}\}$ is the thermodynamic activity of dissolved Mg. High Mg fluids such as sea water will require a lower pH_c than a low Mg brackish water. This is shown in Fig. 13.2 which plots the pH_c for sea water (Mg = 0.052 mol/L; pHc = 10.06) and the pH_c for successive dilutions of sea water having lower Mg levels. High levels of EOM or bicarbonate that form aqueous complexes with Mg^{+2} will increase the autoflocculation target pH. To account for activity coefficient and solution speciation effects, the calculation is most accurately undertaken using a chemical speciation code.

A small consideration must be made regarding sea water's pH buffering capacity when it comes to high volume open ponds with sea water medium. The buffering capacity can be attributed to the reaction: $2HCO_3^- \leftrightarrow CO_2 + H_2O + CO_3^{-2}$, in which the acid or base added is consumed by the formation of HCO_3^-. Then, it will be a function of the total dissolved CO_2 [$T(CO_2)$]; that is to say $(CO_2) + (HCO_3) + (\ HCO_3^-) + (\ CO_3^{2-})$. Natural sea water representative of the oceans has a $T(CO_2)$ of around 2 mM and an average buffer intensity around 0.3 meq/L (Pytkowicz and Atlas 1975). This is a small amount when considering the cost-effectiveness of inducing autoflocculation, as it has reasonably higher values only for pH close to pKa values (bicarbonate has two pKa's, because it can

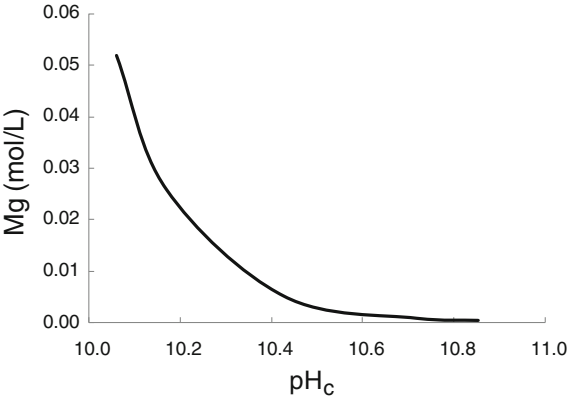

Fig. 13.2 pH of coagulation by $Mg(OH)_2$ as a function of Mg levels in seawater and progressively diluted seawater

gain a proton to become carbonic acid or lose a proton to become carbonate). According to Pytkowicz and Atlas (1975), the buffer intensity of sea water reaches a maximum at pH around 6.1 and 9.1. A starvation step prior to raising pH could solve the issue of the extra base needed to overcome the pH buffering capacity of sea water. Nevertheless, the cost of autoflocculation is very low even if a small amount of extra base is required to increase pH (Vandamme et al. 2013).

13.5 Autoflocculation by Calcium Phosphate

Sukenik and Shelef (1984) observed autoflocculation of *Scenedesmus dimorphus* from Bold's basal medium (250 mg/L $NaNO_3$, 25 mg/L $CaCl_2{:}2H_2O$, 75 mg/L $MgSO_4{:}H_2O$, 75 mg/L K_2HPO_4, 17.5 mg/L KH_2PO_4, 25 mg/L NaCl + trace elements) when photosynthesis lowered CO_2 levels, prompting the pH to increase. Sukenik and Shelef (1984) proposed that precipitation of calcium phosphate controlled autoflocculation and that the pH at which autoflocculation occurred depended upon Ca and phosphate levels. Calcium and phosphate were observed to precipitate from the solution for pH greater than 8.5, and microalgae surface charge was found to become more positive with pH when this occurred. Because the pH-dependent microalgae surface charge matched pH-dependent autoflocculation, the autoflocculation mechanism was proposed to be linking of negatively charged microalgae surfaces by positively charged hydroxylapatite precipitates. To approximate the pH dependency in Ca and P levels seen during autoflocculation by Sukenik and Shelef (1984), we write the calcium phosphate precipitation reaction as

$$Ca_5(OH)(PO_4)_3 4H^+ = H_2O + 3HPO_4^{-2} + 5Ca^{+2}$$

$$K = \left\{Ca^{+2}\right\}^5 \left\{HPO_4^{-2}\right\}^3 / \left\{H^+\right\}^4 = 10^{6.6}$$

The K was fit to the data of Sukenik and Shelef (1984) and is larger than that of a crystalline calcium phosphate. The solubility expression is then rearranged to

$$pH_c = 1.65 - 1.2\log\left\{Ca^{+2}\right\} - 0.75\log\left\{HPO_4^{-2}\right\}$$

The expression above predicts that pH_c will decrease if Ca or P levels increase. Again, accounting for activity coefficient corrections and aqueous speciation is most accurately done using a chemical speciation code. In particular, a chemical speciation code can be used to account for pH-dependent speciation of phosphate when using the above expression.

Folkman and Wachs (1973) showed that species identity was a more important determinant of lime demand for microalgae coagulation than microalgal abundance. Schlesinger et al. (2012) showed that species identity was important and also argued that the pH_c and molarity needed to induce autoflocculation are lower at

higher microalgal abundances. Clearly both species identity and abundance in the culture must be accounted for when designing and testing an autocoagulation strategy for a particular setting. Developing autocoagulation methods to inexpensively dewater microalgae is illustrated below for the case of agricultural lime addition.

13.6 Autocoagulation with Agricultural Lime

The addition of lime aims to produce two effects:

1. Raising pH to pH_c. This is species dependent and varies from 7.97 for *Chlamydomonas reinhardtii* (wild type) to 10.2 for *Nannochloris* (Schlesinger et al. 2012). The amount of lime can be reduced by ceasing CO_2 addition and allowing the available inorganic carbon to be photosynthesized to produce an increase of almost two pH units in the microalgal culture.
2. Inducing autoflocculation. This is also species dependent and, according to Schlesinger et al. (2012), varies from 6.07 μM (0.4 mg/L of $Ca(OH)_2$) for *Chlamydomonas reinhardtii* (wild type) to 16.2 μM (1.20 mg/L of $Ca(OH)_2$) for *Nannochloris.*

The amount of lime to be added to attain pH_c (and avoiding a starvation step prior to autoflocculation) is as follows:

$$\left\{\left(\frac{1}{2}\right) * \left[\left(1 \times 10^{-\mathrm{pHc}}\right) - \left(1 \times 10^{-\mathrm{pHCulture}}\right)\right] * 74 \frac{\mathrm{gr\,Ca(OH)_2}}{\mathrm{mol\,Ca(OH)_2}}\right\} \Big/ \left(\eta_{\mathrm{Ca(OH)_2}}\right)$$

As $Ca(OH)_2$ is a strong base, it completely dissociates in water, and for each mole of H^+ to be neutralised, half a mole of $Ca(OH)_2$ is needed, with 74 being the molecular weight of $Ca(OH)_2$ and $1 + \eta_{Ca(OH)2}$ the proportion of $Ca(OH)_2$ in lime. The appropriate molarity of $Ca(OH)_2$ ($M_{autofloc}$) should be added to induce autoflocculation, and experimental data show that it is at most 16.2 μM (1.20 mg/L Ca $(OH)_2$) for *Nannochloris* (Schlesinger et al. 2012). Following this, the expression becomes

$$\mathrm{Lime}\left[\frac{g}{l}\right] = \left\{\left[\left(\left(\frac{1}{2}\right) * \left(\left(1 \times 10^{-\mathrm{pHC}}\right) - \left(1 \times 10^{-\mathrm{pHCulture}}\right)\right)\right) + \left(M_{\mathrm{autofloc}}\right)\right] * 74 \frac{\mathrm{gr\,Ca(OH)_2}}{\mathrm{mol\,Ca(OH)_2}}\right\} \eta \mathrm{Ca(OH)_2}$$

Sukenik and Shelef (1984) determined that at least 0.1–0.2 mM phosphate and 1.5–2 mM Ca^{+2} must be present in the culture in order for autoflocculation to proceed. These concentrations are already present in culture mediums of sea water (Ca in sea water 411 ppm = 10 mM), particularly with the addition of N/P

fertilisers. Using this approximation, we can compare the amount of lime in various $Ca(OH)_2$ purities to be added to different microalgae cultures. Assuming the most unfavourable conditions (no starvation to raise pH, a low cell density, a high lime consuming species, a low purity (60 %) $Ca(OH)_2$ lime), we would have to add a theoretically small 2.004 mg/L (or only around 2 tonnes per GL) of lime to increase from $pH_{culture} = 7$ to $pH_c = 11$, including the 1.2 mg/L (*Nannochloris* sp.) to induce autoflocculation. This calculation ignores the losses associated with mixing for uniform lime distribution. Table 13.1 presents a list of nine microalgal cultures and required amounts of lime to initialise autoflocculation for a range of species. The authors note that the required lime per unit volume of around 2 tonnes per GL is a relatively small amount with correspondingly low associated product and transport costs, particularly when compared to the tonnes of lime applied per ha over years to mitigate acidic soils in conventional terrestrial agriculture.

Table 13.1 A list of nine microalgal cultures and lime demand

Species	Molarity of $Ca(OH)_2$ required to initiate flocculation (Schlesinger et al. 2012)		Initial pH	Final pH	60 % $Ca(OH)_2$[a] lime	70 % $Ca(OH)_2$ lime (tn/GL)	80 % $Ca(OH)_2$ lime (tn/GL)	90 % $Ca(OH)_2$ lime (tn/GL)
	(μM)	(mg/L)						
Nannochloris	16.2	1.2	7	11	2.004 mg/L 2.004 tn/L	1.718	1.503 tn/GL	1.336
Chlamydomonas reinhardtii (wild type)	6.07	0.4	7	11	0.755 tn/GL	0.647	0.566	0.503
Tetraselmis (*low cell density*)	**10.5**	**0.8**	**7**	**11**[b]	**1.3012 tn/GL**	**1.1153**	**0.9759**	**0.8674**
Tetraselmis (*high cell density*)	**10.5**	**0.8**	**7**	**9.65**[c]	**1.3009 tn/GL**	**1.1150**	**0.9756**	**0.8672**
Tetraselmis (*low cell density and CO_2-starvation* 3 h *prior flocculation*)	**10.5**	**0.8**	**10**	**11**	**1.295 tn/GL**	**1.110**	**0.971**	**0.863**
Nannochloropsis	9.2	0.7	7	9.50[c]	1.141 tn/GL	0.978	0.855	0.760
Isochrysis	5.06	0.4	7	9.30[c]	0.630 tn/GL	0.540	0.472	0.420
Synechococcus 7942 freshwater	4.04	0.3	7	9.70[c]	0.504 tn/GL	0.432	0.378	0.336
Synechococcus PCC 7002 marine	6.3	0.5	7	9.46[c]	0.783 tn/GL	0.671	0.587	0.522

[a] $Ca(OH)_2$ Molecular weight 74 g/mol

[b] pH_c cell densities $<1 \times 10^6$ (Sukenik and Shelef 1984)

[c] pH_c for each species with cell density $>6 \times 10^7$ (Schlesinger et al. 2012) Tetraselmis results are bolded to show effects of cell density and CO_2 starvation on lime demand

13.7 Conclusion

Schlesinger et al. (2012) demonstrated the economic potential of low-cost microalgal dewatering by autoflocculation. Because the underlying mechanisms are reasonably well understood, there is the potential to optimise autocoagulation strategies for a given microalgae and culture composition. Critical to success is an upfront analysis of the species-specific characteristics of the microalgae in question (Brady et al. 2014; Folkman and Wachs 1973). Note lastly that there is the potential for autocoagulation to increase the ash content of the harvested microalgae which might complicate subsequent processing. The ash content will be the lowest when autocoagulation occurs by surface charge reversal and higher when autocoagulation occurs by mineral precipitation. Mild acid rinses of harvested microalgae should lessen the ash content in both cases.

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Chapter 14
Harvesting and Downstream Processing—and Their Economics

F.C. Thomas Allnutt and Ben A. Kessler

Abstract Harvesting of dilute cultures of algae from large volumes of culture needed for production of biofuels and bioproducts is a substantial hurdle to the economic viability of algal biofuels. While centrifugation and sedimentation are already scaled to volumes that would allow direct application to algal biofuel production, their economics to the production of biofuel are not favorable. The industry has reevaluated the existing technologies and continues to innovate around the harvesting of microalgae for biofuels and bioproducts. This review discusses the historical approaches and recent advances while comparing and contrasting the different methods. An engineering estimate of comparative costs is also provided.

14.1 Introduction

A major challenge facing the microalgae industry is how to economically harvest microalgal biomass from millions of gallons of culture medium containing biomass at densities of less than 1 % total solids. Typically, open pond photoautotrophic production reaches biomass densities ranging from 0.01 to 1 gdw L^{-1}, while cell densities in enclosed photobioreactors (PBRs) range from 4 to 10 gdw L^{-1} (Chisti 2007; Stephens et al. 2010). The higher levels of biomass reported for PBRs that provided high-intensity artificial light are still low relative to cell densities that can be reached in heterotrophic cultures (which can exceed 100 gdw L^{-1}) and also introduce a different hurdle to commercial viability, the operating expense (OpEx) of the electricity for the lights, and availability of inexpensive, durable, and highly efficient lights (Chen et al. 2011). The contribution that harvesting the algal biomass makes toward the overall cost for renewable biofuel production has been estimated

F.C.T. Allnutt (✉)
BrioBiotech LLC, P.O. Box 26, Glenelg, MD 21737, USA
e-mail: fct.allnutt@gmail.com

B.A. Kessler
Phycal Inc, 51 Alpha Park, Highland Heights, OH 44143, USA

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to be between 20 and 30 % and remains a bottleneck for the industry (Brennan and Owende 2010; Dismukes et al. 2008; Gudin and Thepenier 1986).

Microalgae have been harvested by centrifugation in industrial algal applications that, to this point, have mostly focused on higher value nutritional products, such as β-carotene, astaxanthin, edible algal biomass, or higher value nutritional oils—where the high energy costs of centrifugation can be borne by a high-value product (Spolaore et al. 2006; Wijffels et al. 2013). In the case of wastewater treatment applications, where the purpose is to reduce the biological oxygen demand (BOD), algae have mostly been harvested using flocculation and either settling or flotation tanks in order to lower the overall cost. While such methods have already been operated at scales relevant to commercial biofuel production, they do not provide for preservation of the biomass for downstream processing into fuels and co-products.

It is important to note that the performance of the algal mass culturing system and the properties and value of the product portfolio being produced have direct impacts on how much the harvesting or dewatering step can cost. The reasons for this are the following: (1) In most cases, the size of the equipment is based on volumetric throughput, not dry mass throughput, and (2) in many cases, the operating costs (OpEx) are also based on volumetric throughput. Consequently, culturing systems that produce higher densities of algae and higher densities of product per unit volume of culture medium will require smaller equipment and have lower OpEx.

Additionally, the microalgal feedstock being produced cannot be degraded during the harvesting such that it cannot be used for the production of algal biofuels and any co-products necessary for commercial viability. The industry has, to this point, focused on the production of biomass that is directly converted to energy by a number of different processes (e.g., combustion, hydrothermal liquefaction, and catalytic gasification), secretion into the medium (e.g., ethanol), or biomass that is high in lipid as a feedstock for production of biofuels. But it should be noted that many of the value propositions being put forward for commercial production rely on value-added co-products (e.g., animal feeds, single-celled protein) to meet profit targets. Because of the different requirements for the growth systems, productivities, and extraction criteria, it can be difficult to directly compare harvesting technologies and their associated economics. This chapter will present qualitative considerations for the various harvesting options as well as present ranges for the associated economics.

In 1965, a study completed by Golueke and Oswald compared all of the available harvesting methods and concluded that only centrifugation and chemical flocculation were economically feasible (Golueke and Oswald 1965). However, both of these technologies have characteristics that make them less than ideal for algal biofuel production, and their commercial relevance has been reexamined. Centrifugation is energy intensive and requires expensive equipment to carry out at scale. The addition of flocculants adds OpEx in the form of chemicals and additional bulk of harvested material, and the flocculant itself can negatively impact the final product and valuable co-products (e.g., residual metals). In order to reduce the

cost impact of harvesting on production of biofuels, a number of technologies or modifications of old technologies have been evaluated and new technologies have been and are currently being evaluated and developed to reduce harvesting as a hurdle to the commercial viability of algal biofuels. This review will briefly provide an historical backdrop on algal harvesting technologies, describe the existing technologies, compare and contrast the developed technologies, and provide a description of new technologies that have begun the process of crossing into scaled use by the industry.

14.2 Harvesting Methods

Many of the available harvesting methods are not suitable for complete dewatering of the algal biomass and must be used in combination with other methods to achieve the cell concentrations necessary for the production of a feedstock for algal biofuel production. This has been referred to as either primary and secondary concentration or bulk harvesting and thickening. While the terminology is different, the processes are the same. Primary or bulk harvesting takes low concentration cultures typical in photoautotrophic mass algal culturing and brings them to roughly 2–7 % solids. For most downstream processing, a secondary or thickening step is required to increase the solid concentration. The final solid content needed varies for this secondary concentration step and ranges from 50 % solids for downstream processes that can tolerate significant moisture content (e.g., hydrothermal liquefaction and aqueous extraction) to >90 % solids for dry feedstocks needed for traditional solvent extraction.

While primary harvesting remains a huge challenge for photoautotrophically produced biomass (due to large volumes at low density), processes that bypass the higher energy secondary concentration step could provide an advantage for commercial production of biofuels. It is beyond the scope of this review to discuss methods for drying algae (for delivery of >90 % solids) and will focus on methods to deliver concentrated algal feedstocks at <90 % solids as well technologies to avoid harvesting altogether. In Table 14.1, the different primary harvesting methods are listed with a comparison of their properties and the current scale of deployment in the algal industry (not limited to biofuels).

If the technique has an advantage in a particular property, it is marked by a plus (+) sign under the applicable Properties column. For example, in settling/sedimentation, very little energy is required, so it has a (+) in that column, but since product stability is not maintained, it has a (−) in that column. If the process could have advantages for some biofuels applications and not work for another, a (±) is provided in that column. If there is not enough information to make a decision, a question mark is inserted in the column in Table 14.1. The current scale section of Table 14.1 provides an estimate of the applicability of the method to rapid commercial deployment.

Table 14.1 Comparison of features and current scale of harvesting technologies

Harvesting method	Properties						Current scale			
	Energy	Toxicity	Scalable	Viable cells	Product stability	Raw materials	Laboratory scale	Pilot scale	<10,000 L	>10,000 L
Settling/ sedimentation	+	+	+	−	−	+	✓	✓	✓	✓
Screening/ macrofiltration	+	+	+	+	+	+	✓	✓	✓	✓
Flocculation	+	±	+	−	−	−	✓	✓	✓	✓
Dissolved air flotation	±	±	+	−	±	±	✓	✓	✓	✓
Electrocoagulation	±	±	+	−	±	+	✓	✓		
Centrifugation	−	+	+	+	+	+	✓	✓	✓	✓
Microfiltration	−	+	+	+	+	+	✓	✓	✓	✓
Magnetic separation	?	+	?	+	+	?	✓	✓		
Ultrasonic separation	±	+	?	+	+	+	✓			
Hydrodynamic fluidics	?	+	?	+	+	+	✓			

+ = favorable, ± = varies, − = unfavorable; ? = unclear from the literature

14.2.1 Settling/Sedimentation/Gravity Sedimentation

One of the most straightforward methods for harvesting algal cultures is to let the cells settle naturally. This has been done for many different algal strains and has broad applicability in wastewater treatment facilities where the high bacterial load and nutrient levels tend to favor clumping and settling. Cell density and the radius of the algal cells influence its utility as well as flow rate of the system, which has been enhanced by lamellar separators and sedimentation tanks. Settling is frequently deployed with added flocculant/coagulants (Chen et al. 2011). Reliance on settling is time-consuming and is less useful in situations where one would like the harvested algal biomass or its cellular components to remain intact and/or to maintain cellular function or product integrity for further downstream processing. While it is also the slowest separation option, it is also the one with the lowest energy requirement. Therefore, settling/sedimentation is not employed for algal biofuel application without at least some type of flocculation or settling accelerant.

One of the focuses of microalgal lipid biofuel research is high lipid-containing algae. As has been reported in the past, low-density cells tend to not settle well (Edzwald 1993). The best oleaginous algae used for biofuel feedstock have between 37 and 70 % of their biomass by weight as lipid under induction. High lipid content translates into cells of low density (Eroglu and Melis 2009) that can remain suspended and make settling not useful. This type of buoyant biomass can also complicate other methods that rely on gravity for separation.

14.2.2 Filtration

14.2.2.1 Screening/Macrofiltration

A more rapid and low-cost method uses screens or filters with wide gauge pores to retain the algae and allow the medium to flow through the screen without excessive clogging. Dead-end type filters and filter presses are examples of macrofiltration technology. This harvesting method is applicable for a limited number of algae that are of large size and filamentous, properties that allow them to not form impermeable cakes, clog the screens, or flow through the pores. Large pore screens can be used to reliably capture a large percentage of the available biomass (Shelef et al. 1984). The process is widely used for larger, filamentous algae like *Spirulina* with rotating screens and back flushing, but is unlikely to be economical for non-food applications (Packer 2009). Unfortunately, most currently utilized microalgal production strains are non-filamentous and cannot be harvested in this manner; their small cell size necessitates the use of microfiltration or alternative separation methods. The addition of filtering aides, such as diatomaceous earth, sand or cellulose, can improve the effectiveness of macrofiltration methods, but not without adding significant cost and downstream processing complications.

14.2.2.2 Microfiltration

Microfiltration employs the use of filtration media with <1 μm pores and a pressure differential created by flow to dewater biomass slurry. Various conformations of filtration media that are made from various materials are available at industrial scale, including hollow fibers, plates, and spiral wound. The membranes can be made of polymers (e.g., PVDF, PES, PS), ceramics, or metals. There is an established history of using microfiltration technology for the purpose of cell harvesting, as in the fermentation industry and wastewater treatment. Advantages of microfiltration are that the algal cells retain their structure, properties, and motility (Chen et al. 2011).

Microfiltration methods have been advancing rapidly since the early studies in algal harvesting. Stacked filters that are subject to blocking have been replaced by hollow fiber filters and tangential flow filters that are now being applied to dewatering of extremely dirty solutions, such as those in wastewater treatment plants (e.g., Koch Membrane Systems PURONPlus MBR (http://www.kochmembrane.com/Engineered-Systems/Standard/PURON-MBR.aspx). Blocking of the filter pores (membrane fouling) by algal and bacterially derived materials, due to their small cell sizes, is a chief problem of any of these microfiltration methods and is a concern for microfiltration (Bosma et al. 2003). The use of hollow fiber filters and tangential flow filters provides some scouring of the membrane surface to help reduce blockage and provide high flux rates.

Not much data are publically available on the performance of membrane filtration at an industrial scale for algal biofuel systems. Important performance values include flux rate (LMH or L m^{-2} h^{-1}), recirculation rate (L min^{-1}), and filter pressures (kPa). However, engineers at Phycal, Inc. performed studies at a subpilot scale (hundreds of liters) using polymeric, hollow fiber filtration membranes with 0.2-μm pore sizes. A typical performance over an 8-h trial for the alga *Chlorella vulgaris* (Fig. 14.1.) demonstrated steady state flux rates around 150 LMH, and a final biomass concentration of 22 g/L. Other trials with similar performance metrics were able to reach 80 g/L, showing this membrane filtration system could provide primary and secondary harvesting for an algal biofuel production system that could tolerate significant water content, such as aqueous extraction and hydrothermal liquefaction. However, because flux rates are lower when biomass concentrations

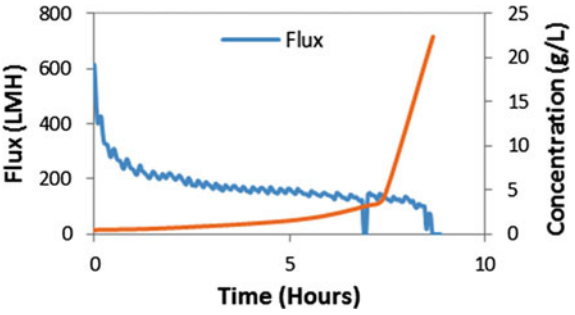

Fig. 14.1 Microfiltration flux rates on the unicellular green alga *Chlorella vulgaris*

are higher, scaled systems would have two or more filtration stages with different operating parameters, membrane pore sizes, and operating concentrations at each stage in order to optimize throughput and lower OpEx (e.g., pumping and membrane replacement).

Work on improved microfiltration in conjunction with algal biofuels continues with novel metallic membranes being developed, which are proposed to lower the membrane replacement costs (lower OpEx) and lower overall fouling to increase throughput (NAABB 2014).

14.2.3 Flocculation

Flocculation is based on the addition of materials (flocculants) or changing the medium in such a way the cells are attracted to each other and therefore more rapidly settle to the bottom of a holding tank. This is really the result of coagulation and then flocculation. Coagulants destabilize the charges and surface properties of the cells in suspension, so they do not resist agglomeration. Flocculants focus on stimulating the formation of larger aggregates from the destabilized algal cells.

Autoflocculation, chemical flocculation, bioflocculation, and electroflocculation are all examples of processes that rely on flocculation to aggregate and speed the removal of algal cells. At scale, all methods for flocculation require large amounts of space and are expensive due to costs of coagulants and/or flocculants and operators (Bosma et al. 2003) or other chemical additions (e.g., pH adjustment in autoflocculation).

14.2.3.1 Chemical Flocculation

Chemical flocculation adds flocculating and/or coagulating agents to the culture medium to speed cell aggregation. This process is often used with microalgae as a pretreatment in combination with other processes such as dissolved air flotation (DAF). While this improves the speed at which the cells are collected, it has the added complication of dosing chemicals at a specific desired concentration to achieve this rate. Generally, coagulants are used to neutralize the charges of the particles in the solution, and flocculants are the chemicals used to aggregate the particles. These chemicals (flocculants and coagulants) complicate the overall process in that they add additional cost, often add metals or other compounds that need to be disposed of in the resulting biomass, and complicate any downstream processing of the materials into primary and co-products. Negative impacts have been countered in a number of ways, including the use of degradable biopolymers as flocculants (e.g., polyacrylamide and starches) and electroflocculation where no flocculant is directly added.

Inorganic flocculants and coagulants are typically iron or aluminum based and are used to neutralize the surface charge. This method requires a significant input of

the inorganic flocculant which adds to the sludge; this adds to the OpEx both in the inputs and in processing (to remove the chemicals). Additionally, the process is sensitive to pH, usually working best at higher pH but varies with strain and culture condition. All algal strains do not respond the same to a particular chemical, so tailoring will be required to fit the organism being harvested (Chen et al. 2011). The chemical flocculants can also be a problem for downstream use of the biomass for feeds, feedstock for anaerobic digesters, and residual ions can be a problem for use of digestates for land application as a soil amendment (Christenson and Sims 2011).

An example is the use of alum (hydrated potassium aluminum sulfate) for flocculation of *Scenedesmus* and *Chlorella* cultures (Molina Grima et al. 2003). Knuckey (2006) used ferric-induced flocculation at 0.5 mg L^{-1} to concentrate algae that had been pH-adjusted to about pH 10 by 200-800-fold. The floccs needed to be neutralized after concentration. They successfully flocculated *Chaetoceros calcitrans, C. muelleri, Thalassiosira pseudonana, Attheya septentrionalis, Nitzschia colesterium, Skeletonema* sp., *Tetraselmis suecica,* and *Rhodomonas salina* all with >80 % efficiency. All were marine algae useful for aquaculture feeds (Knuckey et al. 2006). As an example of strain differences, it has been reported that *B. braunii* flocculated better at pH 11 (Lee et al. 1998).

Organic flocculants and coagulants can also be used; these are high molecular weight bridging polymers (e.g., chitosan and starch) that react with cells in the culture to make large aggregates and help speed up flocculation (Edzwald 1993). It is reported that these biodegradable polymers do not contaminate the biomass as much as inorganic coagulants and that cationic polymers are superior to anionic and neutral ones. Cationic polyelectrolytes (e.g., Dow C-31) induced algal cells to flocculate while anionic and nonionic polymers were shown to be ineffective (Tenney et al. 1969). Changing the pH was necessary to optimize the flocculation with these cationic polymers, with essentially no flocculation at high pH (above 8) and maximal flocculation between pH 2 and pH 4. The flocculation was attributed to a bridging by the polymers between algal cells to form a matrix. The chemicals necessary for pH adjustment (and neutralization) significantly add to the overall OpEx of flocculation processes. The flocc formation is inhibited by high salt concentrations, forms better at high biomass density, and is subject to shear disruption, which slows the flow rates possible.

Growth stage has also been reported to have an impact on the flocculation procedure. For example, *Botryococcus braunii* cultures flocculated easiest after two weeks of culture regardless of harvesting method (Lee et al. 1998).

14.2.3.2 Autoflocculation

Auto- and bio-flocculation are both methods that take advantage of natural means to force the cells to aggregate in floccs. In autoflocculation, environmental conditions, such as pH, temperature, or salinity, are manipulated in order to influence the cell surface charges and allow the cells to come into closer contact such that they will ultimately stick together in small groups.

Autoflocculation occurs as a result of the formation of carbonates that stick algal cells together at elevated pH. This can be induced by the addition of NaOH to the system (Chen et al. 2011). The pH at which autoflocculation occurs is species specific and will have to be determined empirically. For instance, a study comparing autoflocculation in *Phaeodactylum tricornutum* and *Scenedesmus obliquus* found that flocculation occurred at 10.5 and 11.3, respectively (Spilling et al. 2010). They were able to achieve an 8.6-fold increase in biomass density in 30 min and 10.7-fold after 12 h. The pH is the most important factor, but cell concentration and turbulence also have significant impact.

14.2.3.3 Bioflocculation

Bioflocculation refers to the use of bacteria or other algae to create or induce flocculation of the target organism. This is usually caused by secreted biopolymers or extracellular polysaccharides (EPS).

Bacteria additions to flocculate algae have been proposed as a method to reduce the cost of harvesting from dilute algal cultures. For example, a recent article describes the use of a *Bacillus* sp. RP1137 bacterium to flocculate *Nannochloropsis* (Powell and Hill 2013).

Self-flocculating algae, such as *C. vulgaris* JSC-7, have been used to promote flocculation. Significant effort has been put on the identification of potential biofuel production strains that work in mass culture but self-flocculate to improve the economics of harvesting. Co-culturing or addition of algae that produce large amounts of EPS has also been used successfully to flocculate the algal culture without addition of chemical coagulants or flocculants.

For example, the co-culture of *C. vulgaris* JSC-7 with the non-flocculating algal strains, *S. obliquus* FSP or *C. vulgaris* CNW11, increased flocculation of the non-flocculating strains to nearly the same rate as seen for JSC-7 alone. For co-culture with *S. obliquus,* the efficiency went from 28.1 to 62.7 % when diluted 1 to 2 and to 41.2 % when diluted 1 to 5 with JSC-7. For CNW11 co-cultured with JSC-7, the efficiency went from 25.6 % to 68.3 and 34.8 at 1:2 and 1:5 dilution, respectively (Alam et al. 2014). These same authors isolated and partially characterized a polysaccharide-based agent that was shown to be particularly strong in flocculating the CNW11 strain (>85 % efficiency within 1 h) with a concentration as low as 0.5 mg/L (Alam et al. 2014). Diatoms have also been used to induce flocculation in *Nannochloropsis* cultures, which were subsequently filtered and dried (unpublished; Ben-Amotz 2009).

14.2.3.4 Electroflocculation/Electrocoagulation/Electrolytic Aggregation

The use of an electric field to modify the surface charge of the algal cells and stimulate flocculation holds the potential for improved harvesting since, in theory,

the method would not require addition of chemicals and could be easily run as a continuous system. The hurdles for general applicability of these technologies are development of electrodes that do not add metals to the system and reducing the cost of the power required.

Electrocoagulation using metal electrodes employs similar chemical phenomena as tradition coagulation, but rather than introducing a dry chemical, such as aluminum chloride, metal ions are typically released from the reactive metal electrode into the water through electrolysis. The metal ions then act similarly to the dry chemicals. The use of electricity also helps influence the particle charges. Environmental parameters such as pH and salinity must be optimized for electrocoagulation to work efficiently. Therefore, the trade-offs between electrocoagulation and chemical methods are (1) higher energy cost but lower raw material cost assuming the same environmental adjustment, (2) fewer introduced counterions (e.g., chlorides), and (3) replacement costs for sacrificial plates.

One study used aluminum anodes for electrocoagulation of algae from a wastewater treatment plant. This study found that removal after 15 min reached as high as 99.5 % (based on chlorophyll content) with a power input of 550 W (Azarian et al. 2007). At a reduced energy usage (100 Wdm^{-3}), the same separation efficiency could be achieved in 30 min. They did not analyze the Al ion released but recommended this as a further issue in need of optimization before the wide use of this method.

Electroflocculation using inert electrodes does not add metal ions to the culture since the electrodes are inert. The electric force is used to drive cells to the anode where the cells lose charge and flocculate. One study showed that 80–95 % of the algal cells from a wastewater treatment plant could be removed from a 100-L vessel in 35 min (Poelman et al. 1997). These techniques are typically higher in both operating and capital costs due to higher energy requirement and more valuable metals.

14.2.4 Flotation

Flotation is a method where air bubbles and algal particles are attracted to each other and the air bubble buoyancy moves the algae to the surface where it can be collected. Flotation methods can capture particles that are less than 550 μm, making these methods particularly suitable for unicellular microalgae. There are a number of different methods applied for harvesting by flotation: DAF, dispersed flotation, electrolytic flotation, and ozone flotation.

14.2.4.1 Dissolved Air Flotation (DAF)

DAF depends on the addition of coagulants or flocculants, but the harvesting is sped up by using fine bubbles moving up from the bottom of the DAF unit to

capture small aggregates and bring them to the surface of the tank. At the tank surface, froth containing the cells is skimmed off and collected in a much more concentrated solution—100-fold increase in solids (Milledge and Heaven 2013). DAF is a well-developed method and has already been scaled to volumes that would be used for commercial algal biofuel production. However, DAF suffers from the need to add chemicals to the process that need to be dealt with in downstream processing and thus adds OpEx to the overall operation (Chen et al. 2011).

A comparison of DAF coupled with filtration to direct filtration (with or without ozonation) for removal of algae in wastewater showed that the DAF/filtration system allowed more rapid flow through and longer runs than were possible using direct filtration (Ferguson et al. 1995).

14.2.4.2 Dispersed Flotation

In dispersed flotation, 700- to 1500-μm bubbles are formed by high-speed mechanical agitator with air injection system. Additions of surfactants such as the cationic N-cetyl-N–N-N-trimethylammonium bromide (CTAB) have been used to remove *Scenedesmus quadricauda*, while non-ionic Triton X-100 and anionic SDS did not work (Chen et al. 2011).

14.2.4.3 Ozone Flotation

In a variation of dispersed flotation, ozone bubbles are injected in what is called "dispersed ozone flotation" that has been shown effective in the harvest *S. obliquus* (Cheng et al. 2011). No separation was possible with simple air flotation with this culture. The ozone air flotation separation was attributed to ozone-generated release of algal biomolecules (polysaccharides and proteins) that aided flocculation (Cheng et al. 2011). Proteins released from the cells due to the ozonation probably helped the froth formation and cell separation. Ozone dosage required to successfully separate the cells was 0.2–0.5 mg O_3/mg biomass—in a range similar to that used in wastewater treatment plants. This was tested at small scale, and it is unclear how the oxidative properties of ozone would impact downstream processing and the stability of oils and co-products.

14.2.4.4 Electrolytic Flotation

Electric field-driven water hydrolysis generates H_2 which forms bubbles that adhere to the microalgae and carries them to the surface to accelerate harvesting (Mollah et al. 2004). This process has been run in both batch and continuous mode and found that higher power positively correlated with increased algal cell separation in eutrophic lake water (Alafara et al. 2002). The advantage of this method is there is

no need for the addition of chemicals to induce separation. However, metal ions are released from the electrodes and will accumulate in the biomass. Scrapers similar to those used in the other flotation techniques can be used to remove the biomass from the surface of the water and provide concentrations in the range of 20–70 g/L. The need for electrical power and the positive correlation with increased power combine to make commercial application a challenge.

14.2.4.5 Continued Innovation Around DAF

Active research and process development are still ongoing to improve the overall cost efficiency of DAF-related systems so that they can meet the economic thresholds imposed by the biofuel marketplace. Two recent examples of this are posiDAF and ballastDAF. The term posiDAF is used to describe DAF being run with a positive surfactant added to the bubble generation system. The 2007 thesis of Henderson (2007) applied this technology to algae and is under development as a commercial product by WaterInnovate Ltd. (http://waterinnovate.co.uk/). Another technology, termed ballastDAF, uses positively charged glass beads pushed through the system instead of air bubbles to provide separation of the cells from the medium (www.rachelwhitton.co.uk/uploads/1/9/4/5/…/whitton_poster.pdf). The glass beads are collected by low-speed centrifugation (e.g., cyclones), and then, the beads can be reused for further separation. Jefferson and colleagues claimed a reduction in energy to <20 % of that required by conventional DAF.

14.2.5 Centrifugation

Centrifugation is a standard and efficient process for collection of algal biomass from a dilute solution. It is flexible and can be run continuously to handle huge volumes at scale. The major obstacles to the use of centrifugation in algal biofuel production are high OpEx and CapEx as well as being prone to mechanical problems due to freely moving parts (Bosma et al. 2003). Additionally, high lipid-containing algae are harder to centrifuge and therefore require additional energy to recover by centrifugation.

Generally, it is believed that centrifugation for the primary and secondary dewatering would only be feasible for high-value applications (Molina Grima et al. 2003). However, others feel that a continuous centrifuge would be economical at large-scale Briggs (2004). It is most likely that the use of a centrifuge for secondary concentration with improved and dependable centrifuges could be useful for biofuel production in a continuous and large-scale process.

Hydrocyclones are low-cost continuous centrifuges that have been used for clearing algae and suspended solids from ballast waters. Their efficiency increases with decrease in size of the hydrocyclone and higher flow rates, which could be an

issue for industrial scale-up. An example of this issue is a study of solid separation efficiency of 5- and 18-cm-diameter hydrocyclones that delivered 34 and 29 % removal, respectively (Martinez et al. 2007). Recent analyses suggest that the use of hydrocyclones for algal biofuels could be economical (Packer 2009).

14.2.6 Magnetic Separation

Magnetic separation of algae has been proposed in the past and is currently being investigated. The concept is to pass magnetized algal cells (or magnetic aggregates) past a magnet and remove them directly from the culture medium.

The original methods for magnetic separation used addition of magnetite and aluminum sulfate to the culture that are bound to the algal cells and were subsequently removed with an electromagnet. This was very successful at neutral and slightly acid pH waters (79–94 % removal) but less effective with water at higher pH (55–64 %) (Bitton et al. 1975). This approach has the problem of pH sensitivity as well as the dependence on flocculant and added magnetic materials.

There are other current approaches for magnetic separation of algae. One company, Advantageous Systems LLC, specializes in functionalized nanomaterials and is marketing a system for the separation of algae. Public details of their methods are not available, but it appears that they have used functionalized nanomaterials added to the algal culture that is then passed through magnetic plates to separate the algae in a rapid, continuous, and efficient manner (http://www.adsalgae.com/). Another similar approach used chitosan–Fe_3O_4 nanoparticle composites to effectively (99 % efficiency) remove *Chlorella* sp. KR-1 from the culture medium using a permanent NdFeB magnet in laboratory scale (Lee et al. 2013).

Using molecular engineering, there are efforts underway to produce algae with improved iron uptake and storage as well as being more magnetically susceptible, so that they can be separated magnetically (Sayre and Postier 2013). These authors engineered iron uptake and storage proteins into *Chlamydomonas reinhardtii* and *Auxenochlorella protothecoides* and found improved iron utilization but, to this point, have been unable to definitively show improved magnetic moment (personal communication).

Magnetic separation systems that recognize only production strains of algae and harvest them specifically while excluding bacteria and other contaminants would be advantageous in that they could provide a clean separation of suitable feedstock for the downstream processing and a cleaner final product. However, addition of magnetic materials to the system would impact downstream processes as well as add OpEx for the materials used. Currently, there are no scaled-up systems for magnetically separating algae. However, large magnetic separators are already in use in the mining industry should technological and cost advantages be overcome.

14.2.7 Ultrasonic Separation

Ultrasonic waves, in various formats, have been used to induce aggregation of algae and speed up separation of algae. The concept is to use ultrasonic energy to concentrate the algae in a specific location or node to facilitate rapid recovery. Ultrasonic separation can be run continuously, has low stress (cells remain viable), is non-fouling, has few moving parts to break down, and occupies relatively little space. However, this process was considered less cost-effective than centrifugation for industrial harvesting due mainly to the requirement for a cooling system and the low concentration factor compared to centrifugation and microfiltration (Bosma et al. 2003). It might be useful in a system where the alga secretes a metabolite, and it would be advantageous to remove live cells for reuse in the production of more material while harvesting the metabolite from the medium.

One example of the application of ultrasonic separation of algae is a process deployed with *Monodus subterraneus* UTEX 151 that was followed by enhanced sedimentation as a harvest tool (Bosma et al. 2003). The algal cells were pumped through a chamber equipped with an ultrasonic transduction chamber fitted with a resonator and reflector that provided a standing wave in the chamber. Cells were forced to the nodes and agglomerated such that when the field was removed, they quickly settled due to gravity. High cell densities and slow flow rate were most favorable for ultrasonic separation providing a maximum recovery of 92 % of the cells and an 11-fold increase in concentration.

Another application of ultrasound is the acoustic focusing method recently analyzed within the NAABB consortium's harvesting group. The use of ultrasound to both separate and lyse the cells was investigated such that both the harvesting and extraction methods could be combined. Using a low-frequency ultrasonic field, the separation of *Nannochloropsis salina, Nannochloris oculata*, and *Auxenochlorella protothecoides* was demonstrated. Their acoustic focusing harvester was scaled to 45–225 L h^{-1} and provided an 18-fold increase in biomass density (NAABB 2014).

14.2.8 Hydrodynamic Fluidic Devices

A hydrodynamic fluidic separation system based on fluidic shear as the culture stream passes through a spiral pattern in the fluidic device has been developed at the Palo Alto Research Center. This system is a spiral channel through which the culture is passed; drag from the channel walls exerts forces that separate particles from the suspending solution such that separated, concentrated, and dilute streams come out at the end of the separator. The device can be run continuously, requires no filters, is generally large enough to prevent fouling, and requires no additional materials for the separation. They have demonstrated a benefit to the use of flocculants with small algae in this system. Such a system would also have minimal

or no fouling due to the large size of the fluid channels. This device has been used with *Scenedesmus dimorphus* with chitosan as a flocculant and provided a 42-fold increase in concentration. With *Arthrospira platensis* and no flocculant, the device provided 5.8-fold concentration on one pass and 18.8-fold on two passes through the concentrator providing a 97.3 % harvesting efficiency (Hsieh et al. 2012; Volkel et al. 2011). There are currently no reports of use of this technology at large scale with algae.

14.2.9 Reduce or Eliminate Harvesting

Completely or partially eliminating the harvesting step is a direct way to reduce the cost of harvesting on the process, but is only valuable if it does not impact the downstream processing of products and co-products. There are several ways that are being explored to truncate or eliminate harvesting in algal biofuel production.

Mass culture in a biofilm. Supplying a suitable surface on which to mass culture algae without suspension in a dilute culture medium would allow the direct harvest of concentrated algae rather than trying to separate a dilute suspension of algal cells from their culture medium. In theory, this method would also allow better access to nutrients and sunlight.

A biofilm-based system is currently being commercialized by BioProcessAlgae LLC. Their Grower Harvester™ system provides a substrate on which algae are inoculated and allowed to grow under suitable conditions and then removed in a more concentrated form using a stream of water to remove the cells from the substrate (http://www.bioprocessalgae.com/technology/). Little data are publically available at this time on the economics of this process.

Directly process high moisture content biomass. Elimination or partial elimination of secondary harvesting would mean that the algal pastes do not have to be highly concentrated, leaving a large amount of water with the algal cells (>20 % moisture). A number of different technologies are applicable to process high moisture algal pastes, including hydrothermal liquefaction (Biddy et al. 2013), catalytic hydrothermal gasification (Biller et al. 2011), supercritical methanol conversion to biodiesel (Patil et al. 2011), and hydrothermal carbonization (Heilmann et al. 2011). All of these processes either require the presence of water for conversion to biofuels or can tolerate high levels of moisture in the biomass without excessive parasitic energy loss. While it is beyond this review to discuss all of these methods, one example is the use of supercritical methanol conversion to biodiesel on algal paste. Open pond-grown *Nannochloropsis* sp. biomass at <10 % solids was directly converted to fatty acid methyl esters suitable for use in biodiesel (Patil et al. 2011).

Direct harvesting of biofuel or bioproducts. An indirect approach has also found favor in the microalgal industry where the biofuels are not contained within the algal cells but secreted into the medium so that the products are harvested without

having to harvest the algal biomass itself (opening the prospect of reuse of the algal biomass for additional product generation).

One unique alga, *B. braunii*, accumulates lipid on the exterior of the cell as it becomes senescent or stressed. This alga produces some very long chain and unique lipids but grows very slowly. A suggestion that continuous harvest using a biocompatible solvent in an aqueous/solvent bioreactor has recently been proposed that would allow direct harvesting on a continuous basis to lower costs and offset the slow growth of the biomass (Moheimani et al. 2014; Zhang et al. 2011).

An example of this approach is that currently applied by Algenol for the production of ethanol from cyanobacteria (bluegreen algae). Secretion of algal lipid into the medium could allow continuous culturing of the algal biomass while removing product from the culture medium through phase separation. This has been demonstrated in bacteria (Sauer and Galinski 1998) and has been proposed for diatoms and other algae (Ramachandra et al. 2009). The company Synthetic Genomics reports that they have developed algal cells that secrete oil in a continuous manner to produce an algal biocrude (www.syntheticgenomics.com/what/renewablefuels.html) and have filed patents protecting the idea (Roessler WO2009076559). Other companies have similar ideas being applied to cyanobacteria, such as Joule Unlimited with enclosed PBRs and excreted volatile organics, although they are not publishing their methods, so it is hard to calculate the economics.

14.3 Current Usage and Industrial Utility

The process for conversion of the algal feedstock into biofuel dictates the type or types of harvesting methods that can be deployed. For instance, if the value proposition requires the use of residual biomass for animal feeds or valuable co-products, many of the simple settling, flocculation, and flotation methods may not be applicable since they require long time periods and addition of chemicals that could be detrimental to the biofuel and/or co-products.

The different techniques have different costs that must be absorbed by the final product portfolio (biofuel plus associated co-products). The overall cost hinges on the application (i.e., the process deployed by the company producing the biofuel). An in-depth study on harvesting technologies was conducted by the National Alliance for Advanced Biofuels and Bioproducts (NAABB) in 2011. Estimates for capital and operating costs associated with different harvesting techniques are provided in Table 14.2. These numbers were derived either from the NAABB report, literature, or by an engineering analysis and estimate by the authors.

Adjustments were made from the NAABB data to reflect energy and chemical costs on a per volume basis, assuming 0.5 g L^{-1} dry biomass concentration, which is a reasonable average for phototrophic cultures. In practice, costs for some methods, such as centrifugation, are impacted primarily by volume. Others, such as the chemicals required for chemical harvesting, are dependent on the mass of solids

Table 14.2 Estimated capital and operating costs for harvesting algal biomass from dilute mass cultures by select methods

Technique	Energy usage ($kWhm^{-3}$)	Energy cost ($/m^3)	Chemical cost ($/m^3)	Membrane replacement cost ($/m^3)	Total OpEx ($/m^3)	Estimated CapEx range ($/m^3/day)
Stacked disk centrifuge	3.30	0.2640	–	–	0.264	207–484
Evodos centrifuge (spiral plate)	0.950	0.0760	–	–	0.076	?
Dissolved air floatation	0.250	0.0200	0.004	–	0.024	38–89
Chitosan flocculation	0.005	0.0004	0.028	–	0.028	4–8
$AlCl_3$ flocculation	0.120	0.0096	0.023	–	0.033	160–372
Electrolytic harvesting	0.039	0.0031	0.002	–	0.023	248–579
Ni-Alloy membrane filtration	0.046	0.0037	–	0.018	0.004	80–187
Ultrasonic harvesting	0.078	0.0062	–	–	0.006	396–925
Hollow fiber membrane filtration	0.480	0.0384	–	0.105	0.144	189–442

Data were generated from NAABB final report (NAABB 2014), literature and authors' engineering calculations

in the stream. In this way, performance of the growth systems influences the types of harvesting systems that could be desired for the application.

Capital costs were scaled from the NAABB report to 100 m^3 h^{-1} throughput using the 6/10ths rule of thumb for scaling capital costs and are presented as a ±40 % range. A caveat should be noted, many of these technologies are not yet at industrial scale, meaning that the actual industrialized costs could be significantly different than these projections. Particularly, the assumptions for chitosan flocculation and electrolytic harvesting stand out as requiring additional data to determine a more accurate scaled-up cost estimate.

Some OpEx components, such as labor and maintenance, were not included in the calculation. Electricity was assumed to cost $0.08/kWh. Hollow fiber members were estimated at a cost of $250/$m^2$ with a 3-year life span, and the nickel alloy membranes were taken at $50/$m^2$ with a 2-year life span. The details of the assumptions for the chemical dosing, cost of chemicals, and supporting data can be found in the NAABB final report (NAABB 2014).

Algal biofuel and bioproduct companies are currently pre-commercial or just recently in commercial-scale production of products based on algae and relatively

closed with production data. While it is difficult to ascertain the exact process many of these companies are using for their harvesting technology, one can speculate from their Web sites, patent applications, and discussions with colleagues the probable harvesting methods being applied. Aurora Algae, Inc. (West Perth, Australia) is growing algae in open ponds photoautotrophically and harvesting using a DAF system supplied with flocculant (http://www.aurorainc.com/technology/facilities/karratha-facility/). Looking at the Sapphire Energy Green Crude Farm information, it appears they use a DAF system in their process (http://www.sapphireenergy.com/GCFvideo). Heliae (Arizona, USA) uses a spiral plate centrifuge system from Evodos for their harvesting for the production of astaxanthin (http://www.heliae.com/technology/?page=harvesting). Cellana (Hawaii, USA) uses selection of algae that settle rapidly combined with continuous centrifugation for their harvesting, and they have shifted away from biofuels to higher value products (http://cellana.com/technology/core-technology/). While Cyanotech (Hawaii, USA) is not in biofuels, they have developed a very efficient system for continuous centrifugation and washing over a moving screen in production of *Arthrospira*. Bioprocessalgae Inc. (Iowa, USA) grows their algae in enclosed systems and biofilms in order to harvest algae by washing down the biofilms and settling (http://www.bioprocessalgae.com/). Algenol (Florida, USA) has developed one of the systems that mostly bypasses the harvest of biomass and, instead, focuses on recovery of product (ethanol) through condensation on the bioreactor cover and processing of the water/ethanol product in their "direct-to-ethanol" process (http://www.algenolbiofuels.com/direct-to-ethanol/direct-to-ethanol). Joule Unlimited (Massachusetts, USA) is a bit hard to decipher but appears to produce biofuels (e.g., ethanol) and bioproducts in enclosed PBRs and to collect the products directly rather than extraction from biomass (http://www.jouleunlimited.com/).

14.4 Future Direction and Challenges

There is no universal solution to cost effectively harvesting low-density algal cultures (pond or photobioreactor) on the scale necessary for commercial-scale algal biofuel production. Traditional methods of centrifugation and drying are too costly to be applicable to biofuel production—therefore, continued innovation is necessary in this area. The diversity of the algal biofuel production processes, ranging from extraction of high lipid biomass of a unialgal culture and conversion of ~21 % solid biomass derived from a mixed culture of native grown algal strains by hydrothermal liquefaction to harvesting a secreted product either directly or indirectly, make finding a single universal harvesting method elusive. Table 14.3 summarizes the advantages and disadvantages of the various systems described in this review.

Innovative approaches are being tested both in academic and in industrial laboratories, and the expectation is that economical harvesting methods, either new or based on adaptations of existing procedures, will be developed that spur the

Table 14.3 Advantages and disadvantages of various harvesting methods

Technology	Different modifications	Advantages	Disadvantages	Open questions
Settling	Open tank settling Plate settlers	Known technology, scalable	Slow settling rate results in high capital costs; long time requirement impacts quality of harvested biomass	None
Flocculation	Autoflocculation Bioflocculation Chemical Flocculation Electroflocculation	Low capital and operating costs	Performance consistency, potential market availability issue for scale-up	Fate of chemicals downstream, quality of water for recycle
Flotation	Dissolved Air Flotation	Much more time-efficient separation than settling, known technology, scalable	Potential low harvest efficiency does not result in high concentration of cells	Fate of chemicals downstream, quality of water for recycle
Centrifugation	Spiral plate Stacked disk Spiral wound	Known technology, scalable, high solid output, no chemicals required	High capital and operating costs	Quality of water for recycle
Filtration	Screening Macrofiltration Microfiltration	Known technology, scalable, high solid output, no chemicals required, no suspended solids in permeate stream	High capital and operating costs	Membrane lifetime
Magnetic Separation	Magnetic particles Engineered cells	Potential for low operating and capital costs	Not available industrially	Scale-up, operating costs
Ultrasonic Harvesting	Acoustic focusing Force to plate, settle	Potential for low operating and capital costs, no chemicals required	Not available industrially	Scale-up, operating costs

deployment and commercial success of algal biofuels and associated co-products. All of the recent R&D focused on this area and discussed in this review indicates that innovation is ongoing, and the authors are confident that economical harvesting of dilute algal systems will be achievable in the near future.

14.5 Conclusion

Microalgal harvesting methods are quite varied, and the choice of a particular method is dictated by the ultimate use of the harvested biomass. As companies try to reduce the cost of microalgal-based biofuels by the production of high-value coproducts from the biomass, additional constraints are placed on the harvesting technology to maintain the quality of both the primary biofuel and the coproduct. A careful balance needs to be maintained to reduce the costs of harvesting dilute algal cultures to maintain a positive impact on the overall production costs of the portfolio of products. The continuing research to reduce the costs of algal harvesting will make a positive contribution to reduce the commercial threshold of biobased fuels and bioproducts derived from microalgae.

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Chapter 15
Potential of Converting Solar Energy to Electricity and Chemical Energy

David Parlevliet and Navid R. Moheimani

Abstract Chemical energy can be produced from solar energy via photosynthesis. Solar energy can also be converted into electricity via photovoltaic devices. These two mechanisms would seem to compete for the same resources. However, due to differences in the spectral requirements, there is an opportunity to coproduce both electricity and chemical energy from a single facility. We propose to introduce an active filter or solar panel above a microalgae pond to generate both electricity and chemical energy. There are several advantages to such technology including reduced heating (saving freshwater) and an independent electricity supply. Additionally, by channeling targeted illumination back into the microalgae ponds, we can double the amount of light absorbed by the microalgae. This can result in increased biomass productivity.

15.1 Introduction

There is no doubt that available fossil fuel resources are depleting. Despite new reserves of some fossil fuels, the current reserves of oil, coal, and gas will last 40, 200, and 70 years, respectively (Shafiee and Topal 2009). There is also the issue of human-induced climate change. These situations have resulted in increasing worldwide interest in the renewable energy sector. Apart from fuel, there is also an urgent need for sustainable food production for the ever growing human population.

One of these alternative and renewable energy supplies is that of photovoltaic modules (solar panels). These are solid-state devices that directly convert solar

D. Parlevliet (✉)
School of Engineering and Information Technology, Murdoch University, Murdoch, WA 6150, Australia
e-mail: d.parlevliet@murdoch.edu.au

N.R. Moheimani
Algae R&D Centre School of Veterinary and Life Sciences, Murdoch University, Murdoch, WA 6150, Australia

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energy into electricity. Photovoltaics have been used widely for many decades to convert sunlight to electricity (Wenham et al. 1994) and are a well-established technology despite faults that appear in some modules (Djordjevic et al. 2014). Photovoltaic devices take incident illumination and through the use of a charge separating junction can supply electrons to an external circuit. Although in their initial uses they were for specialized projects such as the space program and for remote area power supplies, there is an increasing demand for terrestrial and domestic systems. Solar panels are becoming a common sight on rooftops in suburban areas.

Biofuel (biodiesel and bioethanol) has been used widely as an alternative source of chemical energy (de Boer et al. 2012). It is projected that the global annual production of bioethanol and biodiesel will increase from 113×10^9 and 28×10^9 L in 2012 to 167×10^9 and 40×10^9 L in 2022, respectively (OECD/FAO 2012). There is no doubt that renewable transport fuel production using crops such as oilseeds or sugarcane has economic as well as ethical problems. This is mainly due to the competition for limited resources (freshwater and nutrients) with food crops. Therefore, there is a need for an alternative source of raw material for biofuel production.

These two energy production methods, one for chemical and the other for electrical, seem to compete for the same resource. They both require illumination from the sun to drive their different processes. However, photosynthesis and the production of biomass are largely reliant on the blue and red end of the solar spectrum, whereas photovoltaics are highly efficient in the green part of the spectrum. These differences in spectral requirements are discussed later in this chapter and can be seen in Figs. 15.2 and 15.3. This suggests that if there was a mechanism for splitting the solar spectrum between these applications, it would be possible to convert the entire solar spectrum to electricity and chemical energy.

We have previously described the scenario of placing an active filter or solar panel above an microalgae pond (Moheimani and Parlevliet 2013; Parlevliet and Moheimani 2014). We produced a conceptual framework and model that described the total amount of power provided to a microalgae culture and the subsequent electrical generation from the solar cells. In this work, we have further developed the model to examine the amount of power absorbed by the microalgae, this taking into account the varying absorption spectra of different microalgae. This work provides a more detailed description of the model and the expected increases in illumination that can be provided to the microalgae.

15.2 The Solar Spectrum

The surface of the Earth receives significant amounts of electromagnetic radiation from the Sun in the form of light. This irradiance has been well characterized (Neckel and Labs 1984) and varies in intensity with different wavelengths. For example, the peak irradiance from the Sun is in the visible part of the spectrum and

this tails off into the infrared. The intensity and spectrum of the light that are measured on the Earth's surface (the terrestrial spectrum) are significantly different from the spectrum of light that would be measured outside of the atmosphere (the extraterrestrial spectrum). This is due to absorbance within the Earth's atmosphere. With its importance to many industries and sciences, the solar spectrum has been well characterized and two standard terrestrial solar spectral irradiance distributions (ASTM 2008) have been defined. These standard distributions are used in the photovoltaics industry for testing of PV modules under standard test conditions. This allows the comparison of the efficiency and performance of different solar modules.

Two different spectral distributions are described in these standards (Gueymard et al. 2002). The first of these spectral distributions is the direct normal spectrum. This is "*the direct component contributed to the total hemispherical (or 'global') radiation on a 37°-tilted surface"* (Gueymard et al. 2002). The second applies specifically for photovoltaic modules and is a good approximation for modules that are tilted toward the equator at 37° (Gueymard et al. 2002). Average values for the atmospheric composition, aerosols, water vapor, and ozone content are taken into account in the standards defined in ASTM G-173-03 (ASTM 2008). Due to the absorbance of light in the atmosphere, the irradiance is dependent on the optical path length through the atmosphere. This is known as the air mass. The spectra in ASTM G-173-03 use an air mass of 1.5. For the midlatitudes, this is a reasonable average. This is a good approximation for modules in the United States of America and southern regions of Australia. As this spectrum is well defined, it is suitable for use in modeling the power absorbed by microalgae as well as the power produced by photovoltaic modules.

The extraterrestrial irradiance and the Global Tilted AM1.5 spectra described in the ASTM G-173-03 standard are shown in Fig. 15.1. This plot shows the irradiance in terms of W m^{-2} nm^{-1} which is an expression of the power in each part of the spectrum incident on a particular area. This can also be described in terms of μ mole photons s^{-1} m^{-2} which is often used when discussing photosynthesis. Converting from one to the other is wavelength specific and can be done using:

$$I_\mu = \frac{I_w \lambda}{hcN_A \cdot 10^{-6}}$$

where I_μ is the irradiance in μ mole photons s^{-1} m^{-2}, I_w is the irradiance in W m^{-2}. nm^{-1}, λ is the wavelength, h is Planck's constant, and N_A is Avogadro's number.

The daily global solar radiation exposure is defined as the total amount of solar energy falling on a horizontal surface per day. The daily solar radiation exposure typically ranges from 1 to 35 MJ m^{-2} and will depend on the time of year, clarity of the air, and the level of cloud cover. For example, the daily solar radiation exposure would usually be highest in clear, sunny, conditions during the summer and lowest during winter or very cloudy days. Some regions of the world have very high radiation exposures due to their location and number of cloud free days. The northern and central regions of Australia experience high levels of incident illumination.

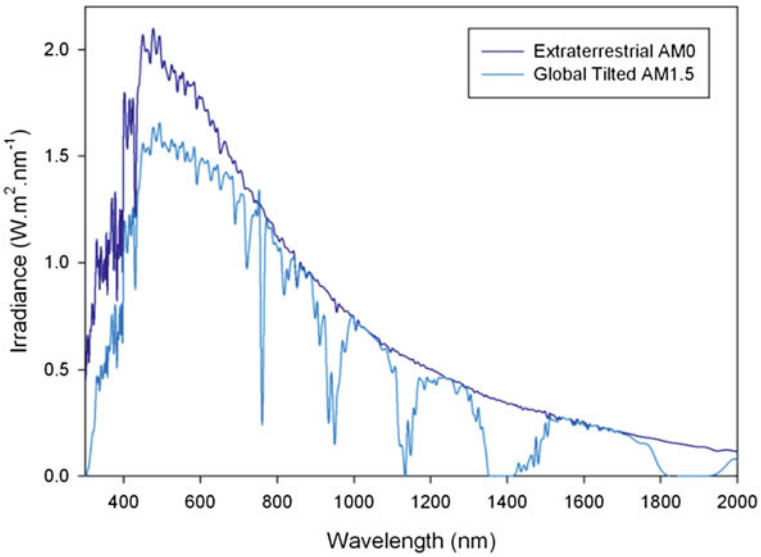

Fig. 15.1 Standard solar spectra as defined in ASTM G-173-03 (ASTM 2008)

This makes them particularly good for photovoltaic electricity production and biomass production (Clifton and Boruff 2010; Borowitzka et al. 2012). Successful conversion of solar energy into chemical energy in the way of biofuel also relies on the availability of abundant water supplies (i.e. seawater or large aquifer resource). Prime locations for algae farms and biofuel production exist where these are available and there is abundant solar radiation (Borowitzka et al. 2012).

15.3 Spectral Requirements of Microalgae

Photosynthesis makes use of solar energy to convert CO_2 into glucose. This process is vital for life on Earth. Photosynthesis can only use parts of the solar spectrum that are in the photosynthetic active radiation range (PAR) (irradiance between 400 and 700 nm). Based on the measured average solar spectrum at the Earth's surface, the proportion of total solar energy within PAR is about 48.7 % of the incident solar energy (Zhu et al. 2008). Pigments are responsible for capturing this light. Photosynthetic organisms contain several pigments. As a matter of fact, pigments are responsible for the names of different divisions and classes of algae. For instance, Cyanophyceae and Rhodophycea contain Chl *a* and phycobillins, while Haptophyceae and Bacilariophycea contain Chl *a* and *c*. It is to be noted that all photosynthetic organisms contain Chl *a* which has the strongest absorption at 430 and 662 nm.

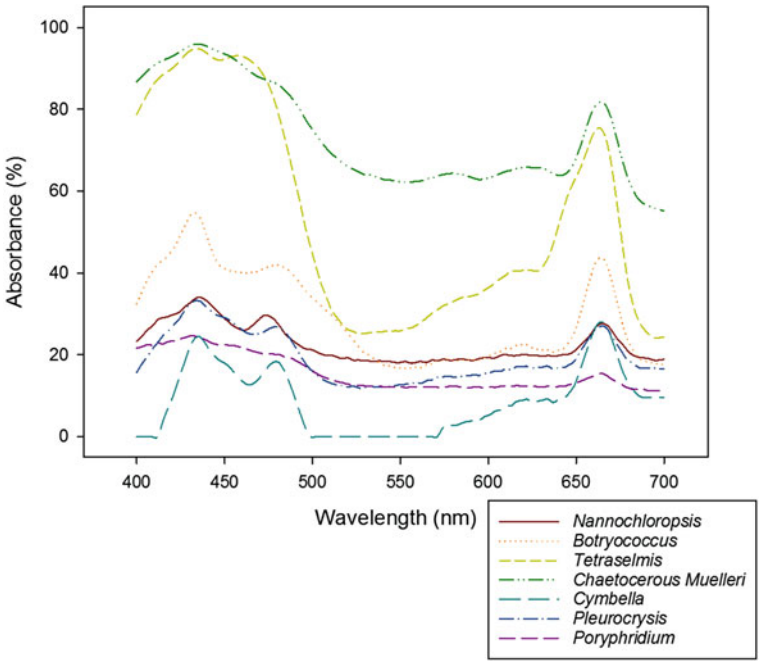

Fig. 15.2 Absorbance spectra of different microalgae species

Other accessory pigments have different absorption spectra allowing the organism to more effectively collect different spectra of light (Frigaard et al. 1996). The absorption spectra of some microalgae species are summarized in Fig. 15.2.

15.4 Spectral Requirement of Solar Cells

Solar cells are devices for producing electricity which use incident illumination to supplying electrons to an external circuit. The use of these has even been described as the "art of converting sunlight directly into electricity" (Wenham et al. 1994). There are a range of technologies and materials used to produce solar cells, each with their own benefits and drawbacks. By far, the most common and familiar example of a solar cell is that of crystalline silicon. Crystalline silicon solar cells currently dominate the world market and held over 93.5 % market share in 2005 (Singh and Jennings 2007), decreasing to 83 % in 2010 (Tyagi et al. 2013) and 86 % in 2011 (Fraunhofer 2012). Crystalline silicon solar cells have a long history and have undergone major improvements in efficiency over the years. The first crystalline silicon solar cell had a limited efficiency of 6 % (Chapin et al. 1954); however, new solar cells have been developed with efficiencies greater than 25 % in

the laboratory and 22 % in full modules (Green et al. 2012; Beardall et al. 2009). Although solar cells are generally optimized to absorb strongly across the whole solar spectrum, each individual technology will have variations in performance. These variations are due to a number of factors including the properties of the semiconductor, such as the bandgap.

15.5 The Semiconductor Bandgap

The semiconductor bandgap determines the optoelectronic properties of the semiconductor material. A semiconductor's bandgap has a significant influence on the properties (including absorption in the case of a solar cell) of devices produced from them. The bandgap defines the minimum amount of energy needed for an electron to jump from the valence band to the conduction band.

The value of the bandgap (E_g) is characteristic of each semiconductor. This value affects the properties of the solar cells produced from each semiconductor (McEvoy et al. 2003). For example, semiconductors are effectively transparent to photons of energy less than the bandgap energy as these photons have insufficient energy to excite an electron from the valence to the conduction band and hence are not absorbed.

The minimum room temperature bandgap energy values for some common semiconductors range from 0.67 eV for germanium (Lide 2005) to 1.35 eV for gallium arsenide (Lide 2005). The semiconductors used for solar cells should ideally have a bandgap energy close to the peak of the energy range of light in the AM1.5 spectrum (1–3 eV). Not all semiconductors are appropriate for the use in solar cells. The most suitable semiconductors will have a bandgap of about 1–1.6 eV(Wenham et al. 1994). Silicon, with a bandgap of 1.12 eV (Lide 2005), is a good candidate material use in solar cells. Ideally, a solar cell should have a flat response to irradiance of different wavelengths. However, this is not usually the case as each will respond differently to different parts of the spectrum. A measurement known as the spectral response can characterize the quantum efficiency of the solar cell to different wavelengths of light.

The spectral response of a solar cell is defined as the short-circuit current (output current under short-circuit conditions) per unit power of incident monochromatic light, as a function of the wavelength of the incident light (Cuevas et al. 2002). The spectral response measurement shows how the solar cell will perform under different spectral conditions and can have implications on which technology is deployed in the field. For example, Ruther et al. (2002) have shown that crystalline cells are more suitable for "red" spectra and that amorphous silicon solar cells are more suitable for "blue" spectra (Ruther et al. 2002). This can contribute to the better performance of a-Si:H cells during summer months and the better performance of c-Si cells during winter months, due to the seasonal variations in the

spectra of light received by the solar cell (Ruther et al. 2002). Comparison and analysis of the spectral response measurements for different solar cell technologies enables the most appropriate solar cell to be deployed given the spectra of light they are likely to encounter.

15.5.1 Crystalline Solar Cells

Solar cells manufactured from doped crystalline silicon solar cells are among the most widely recognized varieties of solar cells. Crystalline silicon solar cells made up about 86 % of the market in 2011 (Fraunhofer 2012). Currently, state-of-the-art single crystal silicon solar cells are reaching a conversion efficiency of up to 24.7 % (Beardall et al. 2009). A similar 24 % efficient (when measured under AM1.5 at 25 °C) passivated emitter, rear locally diffused (PERL) solar cell has been reported with a conversion efficiency of up to 46.3 % (Zhao et al. 1996). This was under monochromatic light of 1040 nm (Zhao et al. 1996). The broad spectral response of this solar cell can be seen in Fig. 15.3.

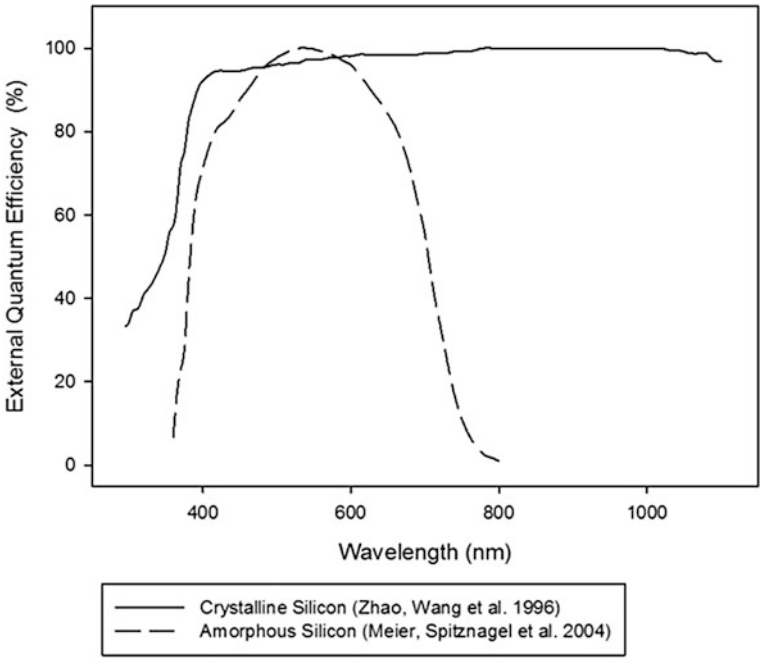

Fig. 15.3 The spectral response of two different solar cell technologies

15.5.2 Thin-Film Solar Cells

In comparison to crystalline solar cells, thin-film solar cells use significantly less material. The development of thin-film solar cells was in part driven by high material costs of crystalline silicon. Thin-film solar cells were much more efficient in terms of material usage and had potentially lower fabrication costs. Thin-film solar cells are fabricated from layers of doped semiconducting materials. These layers produce a charge separating junction often in the form of a p-i-n junction (McEvoy et al. 2003). As they use significantly less material and are inherently thin, thin-film solar cells can be semitransparent to visible light. When a transparent substrate (such as glass) is used, this can allow some irradiance to pass through the device (Shah et al. 2004). Although a single-junction thin-film solar cells can be relatively inefficient in comparison with their crystalline counterparts, several junctions can be stacked so as to produce a more efficient device (Shah et al. 2004). These multi-junction devices can be made from identical junctions or the junctions can be tuned to different wavelengths and parts of the solar spectrum so as to absorb as much of the spectrum as possible. There are a range of examples of thin-film solar cells, and one of the most well-known examples is amorphous silicon.

Hydrogenated amorphous silicon solar cells have been in development since the late 1970s (Wilson 1980). Although thought to be a good alternative to crystalline silicon, amorphous silicon solar cells had one significant drawback in the form of a light-induced degradation, photodegradation, known as the Staebler–Wronski Effect. This is the process whereby the performance and efficiency of the amorphous silicon solar cell degrade upon extended exposure to light (Staebler and Wronski 1977). The degradation occurs over a period of time as the solar cell is exposed to light. The efficiency and performance of the cell degrade asymptotically to a stabilized minimum, upon which point the stabilized cell does not degrade any further. Any additional exposure to light after this point has minimal effect on the solar cell's performance. This light-induced degradation can be revered by annealing the cell above 150 °C for a period of time (Staebler and Wronski 1977). Despite these drawbacks to amorphous silicon, a thin-film amorphous silicon solar cell has been produced on a antireflection-coated glass substrate with a reported stabilized efficiency of 9.47 % (Meier et al. 2004). The spectral response from this solar cell is shown in Fig. 15.3. As can be seen, the device had lower quantum efficiency in the blue and the infrared portions of the spectrum compared to the crystalline silicon PERL solar cell. However, it does have a comparable peak in efficiency in other parts of the solar spectrum.

15.5.3 Luminescent Solar Concentrators

While both the crystalline silicon solar cells and amorphous silicon solar cell examples shown in Fig. 15.3 are optimized to have a broad spectral response, it is

known that photovoltaic devices would work with a higher efficiency if they only had to absorb monochromatic light (Sark et al. 2008) or light from a very narrow spectral range. One way to provide a limited portion of the spectrum to a solar cell, to make the most of its peak efficiencies, is to use a luminescent solar concentrator (LSC). A LSC is a flat-plate solar concentrator made from a thin transparent polymer (such as acrylic) containing a luminescent material (Sark et al. 2008). They work by accepting light from the AM1.5 solar spectrum and directing a portion of the light toward the edges of the flat polymer sheet. Solar cells are located at the edge of this flat sheet for converting the light into electricity. Photons incident upon the polymer sheet with enough energy will excite the luminescent materials. These materials will then re-emit a photon with a longer wavelength. As the photons are emitted in random directions, a portion of these will be captured by total internal reflection in the flat sheet and transmitted to the edge where it can be collected by a solar cell (Sark et al. 2008). With the photons being re-emitted in random directions, there will be some that are transmitted out of the concentrator, and however, a portion is captured and directed toward the edge for collection. Photons without insufficient energy to excite the luminescent material will be transmitted through the concentrator with very low loss. The beauty of these flat-panel concentrators is that increasing the size of the polymer sheet will directly increase the number of photons captured via total internal reflection and able to be converted to electricity. That is, the concentration factor increases with the area of the concentrator. This increase in output from the concentrator can be achieved without increasing the size of the solar cell itself.

Although the efficiency of LSC has historically been fairly low (Sark et al. 2008), there have been devices reported with efficiencies of up to 7.1 % (Sark et al. 2008) which bodes well for the technology. This style of concentrator relies on the use of only a small portion of the solar spectrum. For example, if a luminescent material that emitted green photons was used, all incident light with longer wavelengths would be transmitted through the concentrator. The re-emitted green photons would be directed to a solar cell with a high efficiency in this part of the spectrum. This would potentially be a good match for a system that would convert solar energy into electricity and grow microalgae.

15.6 Electrical and Chemical Energy Co-production

Using the AM1.5 direct solar spectrum as a baseline, we can model the amount of energy that can be converted into electricity by a solar cell if a portion of the solar spectrum is diverted to algae production and only the remainder is provided to the solar cells. This allows us to determine the viability of a cultivation system based on this concept in terms of generating electivity or increasing the portion of specifically targeted PAR available for cultivation. The proposed lossless system places a filter or device above the algae pond to split the spectrum into the appropriate components. We do not consider the mechanism used to redirect the light or the specifics

of how the system will function. However, one candidate technology would be the luminescent solar concentrator or a variation thereof. Although the exact mechanism is not described, the model assumes all the light not provided to the algae is directed to a solar cell. The model assumes there are no losses associated with transmission of light through the filter or reflections from the surfaces of the filter or solar cell. The model also disregards electrical resistance in the transmission of the generated electricity.

The first aspect of this model is to determine the component of the spectra absorbed by the microalgae. As can be seen from Fig. 15.2, on average the main chlorophyll absorption peaks are centered at 434 and 662 nm. The portion of the spectrum transmitted to the algae was varied by changing the threshold around these peaks. For example, full-width half-maximum (50 % threshold) meant the spectra from 400 to 492 nm and 644 to 678 nm was transmitted to the algae, while for a threshold of 80 %, only the spectra from 417 to 458 nm and 656 to 670 nm were transmitted to the algae. Additionally, the light given to the algae is limited to between 400 and 700 nm as this is the region typically considered PAR (photosynthetically active radiation). All energy not transmitted to the algae is provided to the photovoltaic device for producing electricity. To calculate the power absorbed by the different microalgae species, the AM1.5 solar spectrum is multiplied by the absorbance spectrum (Fig. 15.2). The allocation of the solar spectrum as the bandwidth changes and the power absorbed by the microalgae (nannochloropsis) can be seen in Fig. 15.4.

With this allocation of the solar spectrum, we can calculate the power generated by a solar cell in hypothetical system using the reported spectral response graphs and parameters for crystalline silicon (Beardall et al. 2009) and amorphous silicon (Meier et al. 2004).

The short-circuit current density (J_{SC}) generated by a solar cell is calculated from:

$$J_{SC} = \int EQE(\lambda)\left(\frac{\Phi(\lambda)_{AM1.5}(PV(\lambda))}{q}\right).d\lambda$$

where $EQE(\lambda)$ is the external quantum efficiency as a function of wavelength, $\Phi(\lambda)_{AM1.5}$ is the photon flux density calculated from the AM1.5 (Global Tilted) solar spectrum, $PV(\lambda)$ is function defining the portion of spectrum not transmitted to the microalgae, and q is the charge of an electron.

The open-circuit voltage (V_{OC}) of the solar cell is dependent on the short-circuit current density and will vary with the irradiance that is incident upon the cell. This can be calculated from (Messenger and Ventre 2010):

$$V_{OC} = \frac{kT}{q}\ln\left(\frac{J_{SC} + J_0}{J_0}\right)$$

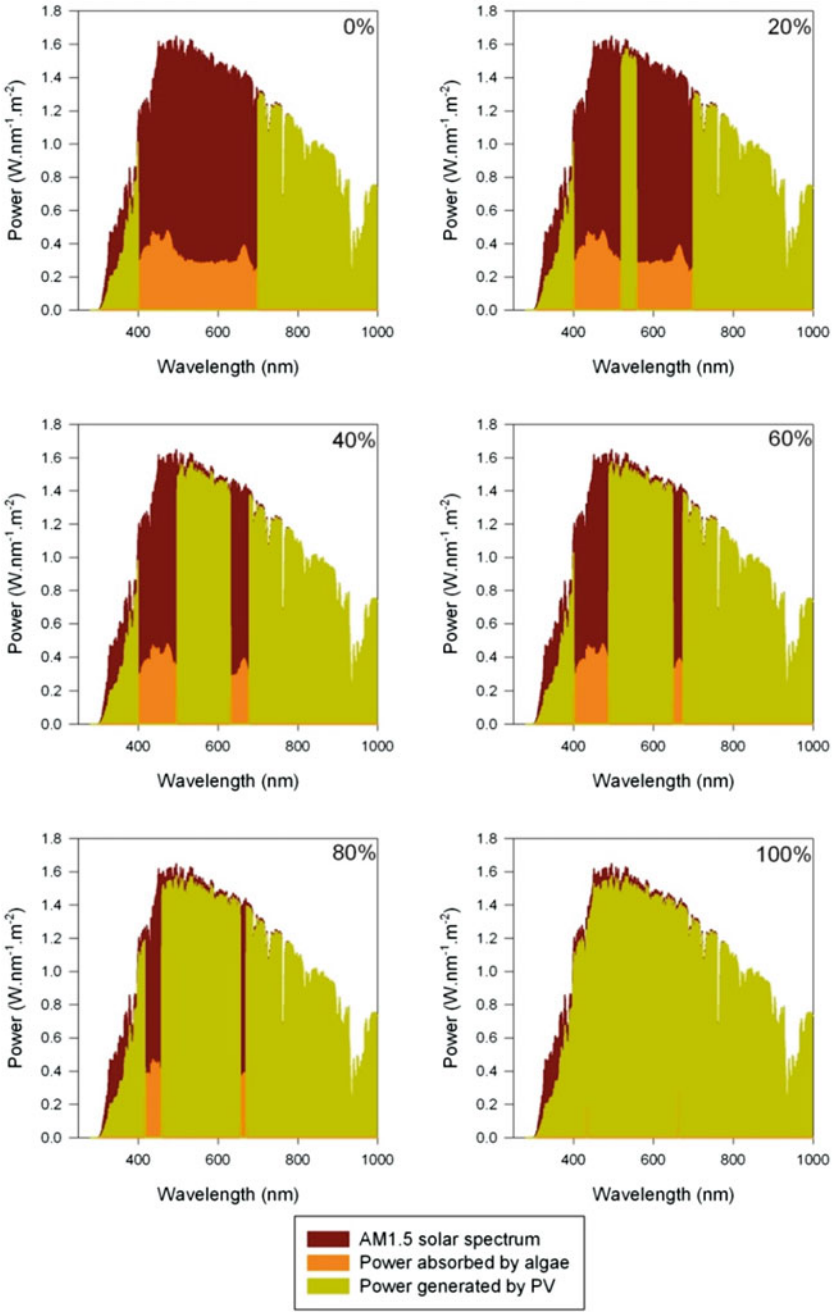

Fig. 15.4 Power absorbed by nannochloropsis and power generated by a crystalline solar cell for different bandwidths

where E_g is the bandgap of the semiconductor material, k is Boltzmann's constant, T is the cell temperature in K, and J_0 is derived from the published parameters of each device.

The power generated (P) in W.m^{-2} from the cell is then:

$$P = FF.I_{SC}.V_{OC}$$

where the fill factor (FF) is the value published in the literature for each cell type.

The power generated by the photovoltaic modules from this limited spectrum and as the bandwidth changed can be seen in Fig. 15.4. The power produced from the solar cells can be directed to the powering facilities associated with the growth of microalgae (pumps and monitoring systems) or to provide additional illumination to the microalgae. The former would reduce the costs of running the plant whereas the latter can boost growth productivity. Light emitting diode (LED) arrays can be used to most efficiently provide additional lighting to the microalgae at a specific wavelength. LEDs are highly efficient solid-state devices for converting electricity into light. They can be designed to emit light in a range of wavelengths to match the spectral. The internal quantum efficiency of high quality LEDs can exceed 99 %. This sounds extremely efficient, and however, there are difficulties in extracting the light from the LED which leads to low external quantum efficiencies (EQE) in the order of only a few percent (Schnitzer et al. 1993).

There is a significant amount of research effort into increasing the external quantum efficiencies of LEDs. As a result, LEDs are produced from a range of materials and use a variety of technologies. Some of the resulting LEDs include blue emitting InGaN-GaN LED's with a EQE of 40 % (Gardner et al. 2007), thin-film GaAs LEDs with a 30 % EQE, (Schnitzer et al. 1993), and organic LEDs with an EQE of 30 % (Kim et al. 2013). In some cases, careful texturing can improve the light extraction efficiency which yields LEDs with an EQE greater than 50 % and in some cases up to 60.9 % (Krames et al. 1999a).

The most useful LED for adding targeted illumination to a microalgae pond would be those LEDs with high external quantum efficiencies at particular wavelengths, such as those discussed by Krames et al. with efficiencies of 60.9 % (Krames et al. 1999a).

The additional power (P) in Wm^{-2} that can be produced using the power generated (P_{in}) using the system modeled above can be calculated from:

$$P = EQE_{LED}.P_{in}$$

where EQE_{LED} is the external quantum efficiency of the LED.

The additional irradiance from these LEDs is assumed to be tailored to the peak absorbance of the respective microalgae species. The total power absorbed by the algae is thus the irradiance absorbed directly by the microalgae and the peak absorbance multiplied by P.

15.7 Results of the Model Under Ideal Circumstances

The power output under constrained lighting situations from both a crystalline and amorphous silicon solar module has been calculated using the model outlined earlier. This is independent of the species of algae used as the power produced by the photovoltaics relies only on the portion of the spectrum they would receive. The threshold determines the portions of the spectrum provided to the photovoltaics as shown in Fig. 15.5. The power produced is not zero when the entire PAR is provided to the microalgae as there is an extensive part of the spectrum beyond PAR which photovoltaic devices can convert to electricity. It is clear that crystalline silicon solar cells are much more efficient in the regions outside of PAR than amorphous silicon is. This is due to the extended spectral response of crystalline silicon into the infrared part of the spectrum and the higher efficiencies of the crystalline solar cells (Fig. 15.3). The energy generated by these crystalline silicon solar cells can be used to power additional lighting to add more irradiance to the microalgae. If a LED system with external quantum efficiencies in the order of 55 % (Krames et al. 1999b) is used, a substantial amount of additional illumination can be provided to the microalgae.

The model we have created for this scenario is highly dependent on the absorption spectra of the microalgae. The power generated by the PV cells can be directed to a series of LEDs which will provide additional illumination to the microalgae. As can be seen in Fig. 15.6, the augmented power absorbed by the

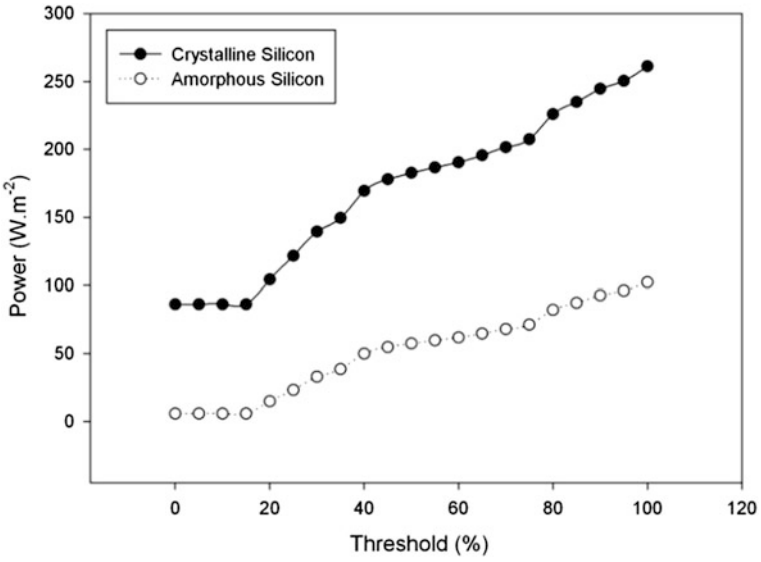

Fig. 15.5 Power produced from two different solar cell technologies as the spectrum threshold is changed

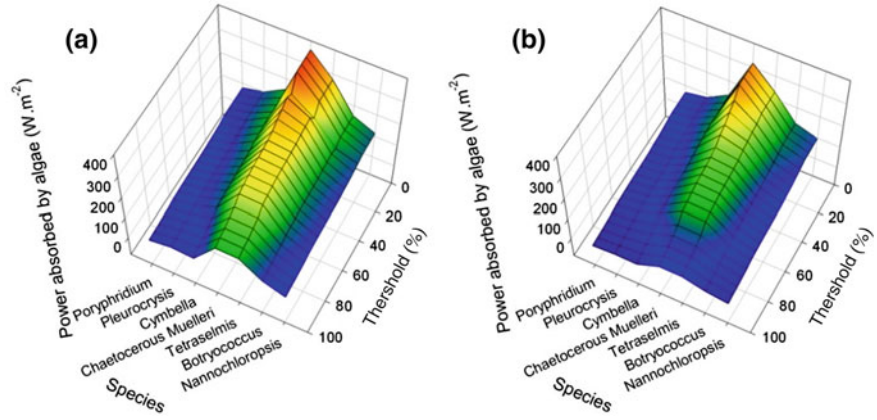

Fig. 15.6 Power absorbed by microalgae of different species when augmented by additional illumination from electricity coproduced by **a** crystalline and **b** amorphous silicon solar cells

microalgae is distinctly higher from some species when compared to others. Notably, this is chaetoceros and tetraselmis. The reason for this is apparent in Fig. 15.2. These two species of microalgae have a much greater absorbance than the other species in the measured absorbance data. This does not necessarily mean they are the most productive and only that they absorb the greatest portion of irradiance. The second trend which appears in the graphs in Fig. 15.6 is that a system augmented with electricity generated by crystalline silicon solar cells will generate more power and have a larger portion able to be provided back to the microalgae via LEDs. This highlights the importance of a highly efficient collecting device. For the remainder of this work, we will focus on results from the crystalline silicon parts of the model which are a best-case scenario.

A more useful visualization of this data is to examine the change in the amount of power absorbed by the microalgae as the threshold is changed. Figure 15.7 shows the change in power absorbed by various microalgae species when compared to the minimum situation. That is, the situation where all irradiance between 400 and 700 nm is transmitted through to the microalgae. Additional illumination is still provided by LEDs using the IR and UV parts of the spectrum. When compared to this baseline, it can be seen that even if all the irradiance in PAR is transmitted to the algae, there is a boost of 20 % in the total amount of power absorbed by the microalgae. This is from the additional irradiance provided by the LEDs which are powered by infrared radiation (>700 nm) captured by the crystalline silicon photovoltaics. As the threshold is increased, the total amount of power provided to the microalgae decreases and beyond 50 %, there would be no net benefit for this system. At first glance, this would seem to indicate there is not a great deal of advantage in filtering the light as described in this model and combining electricity and biofuel production. However, it needs to be recognized that much of the power being absorbed by the microalgae may not be assisting in photosynthesis. There is

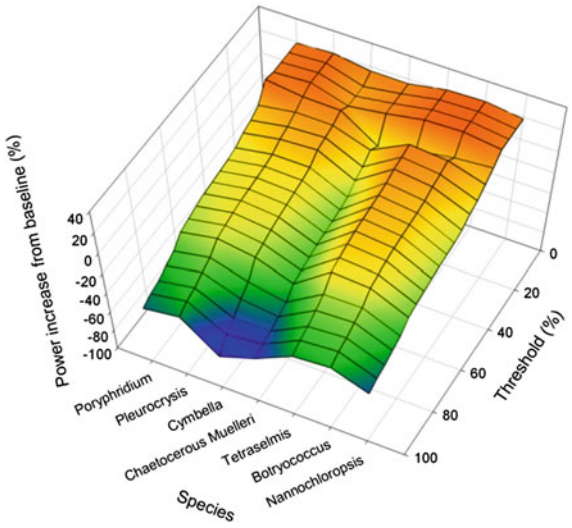

Fig. 15.7 Change in power absorbed by microalgae compared to the 0 % threshold situation

still significant absorption in other portions of the spectrum. If, for example, the green portion of the spectrum is not required for photosynthesis, it can be entirely removed from the spectrum provided to the microalgae and instead converted to blue or red light which will be absorbed efficiently by the microalgae.

A better comparison would be to the 50 % threshold. This equates to a full-width half-maximum band-pass around the main absorption peaks of the microalgae as seen in Fig. 15.2. This situation is shown in Fig. 15.8. From this figure, it can be seen that there are gains in the amount of irradiance absorbed by the microalgae by

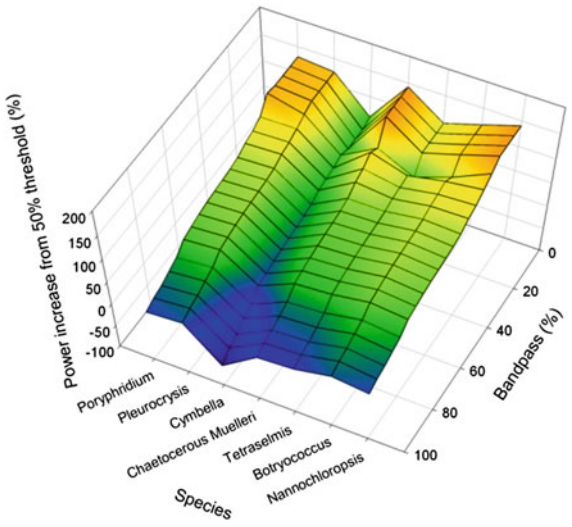

Fig. 15.8 Change in power absorbed by microalgae compared to the 50 % threshold situation

in excess of 100 % in some situations and for some species. This is a significant increase in irradiance absorbed by the microalgae and should lead to more productive growth.

A combination of these two energy production methods (solar energy and chemical energy) can efficiently use the whole solar spectrum. We recognize that an area covered by PV panels of the same scale as a microalgae farm would produce more electrical energy than the algae can store as chemical energy. However, the advantage of our proposed method is the production of chemical energy for transportation or other high value crops and can increase the productivity of microalgae systems.

This suggests that a combination of the two energy production systems would allow for a full utilization of the solar spectrum allowing both biofuel and electricity production from the one facility. This makes efficient use of available land, or it can enhance biofuel production by management of the spectrum and the addition of targeted illumination. Therefore, we propose a co-production system that uses an active filter or photovoltaic system above a microalgae pond to capture and efficiently convert the whole solar spectrum into usable energy or products. While the mechanism for splitting the spectrum is not fully determined as yet, there are several candidate options, including a specifically tailored semitransparent thin-film PV, luminescent solar concentrators, or other advanced energy harvesting flat glass panel that match the spectrum not used by the microalgae. One excellent candidate technology system that can transmit arbitrary visible light wavebands, capture the infrared part of the spectrum, concentrate it on the edge of a glass panel, and convert it to electricity has been recently developed and patented (Rosenberg et al. 2013).

15.8 Benefits of Coproduction of Electricity and Biofuel

There are several key benefits to a system that can coproduce electricity and chemical energy from the solar spectrum. Our proposed system would filter the light before it is provided to the microalgae ponds. This will reduce the total amount of energy provided to the algae in the parts of the spectrum where it is not required for photosynthesis. This in turn will reduce the heating of the ponds and subsequent evaporation of the water. It is to be noted that most places with high light irradiance, that are suitable for microalgae cultivation, also have a high evaporation rates and limited supplies of freshwater. By reducing evaporation, the use of freshwater in the production facility can be reduced. This also reduces the salinity of the microalgae ponds, allowing alga with a lower salt tolerance to be grown for a longer period of time.

Our proposed system would also generate electrical energy. This can be used to aid the production by powering motors and other electrical items at the production facility. This is advantageous in remote areas where grid connection and stable electricity supplies can be an issue. Introducing a method of cogeneration of

electrical energy has benefits in the remote areas that microalgae cultivation takes place, such as northern and central Australia. In these areas, the cogeneration of electricity would reduce the reliance on grid-supplied electricity and diesel generators. By generating some of its own electricity, rather than purchasing electricity (or diesel fuel), the costs associated with production, dewatering, and extraction of oil from microalgae can be reduced which leads to more cost-effective production of biomass.

Alternatively, the electricity can also be used augment the light received by the algae to aid their growth. As shown in the model, we have described that this additional illumination could more than double the amount of energy absorbed by the microalgae. This would result in an increase in productivity and growth.

15.9 Conclusion

While there are a number of factors that influence biomass productivity, photosynthesis places upper limits on how effectively solar energy can be transformed into chemical energy (in the form of carbohydrate, lipid, and proteins). As ultimately light via photosynthesis is the main limit to the growth and a key component of the productivity of microalgae, it is understandable that an increase in the amount light provided for photosynthesis will result in more photosynthesis and thus more productivity. Solar panels are another established mechanism for utilizing the solar spectrum. In this case, they convert sunlight into electricity. While these two methods of energy production would normally be competing for the same resource, we have shown in the model described here that they can complement each other. By allowing the portions of the solar spectrum not required by the microalgae to be diverted to highly efficient solar panels, we can generate both electrical and chemical energy from a single facility.

There are several advantages to using a filter system to divert portions of the solar spectrum to different tasks. These include a reduction in heating and evaporation, co-production of electricity, and a subsequent boost in the productivity of the microalgae. This allows for the cheaper and more efficient/sustainable production of biofuel or value-added crops in remote locations which are located away from sources of electrical power.

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Chapter 16
The Anaerobic Digestion of Microalgae Feedstock, "Life-Cycle Environmental Impacts of Biofuels and Co-products"

Andrew James Ward

Abstract Anaerobic digestion offers a potential pathway to eliminate some of the overheads for microalgae-based biofuels bio-refinery production systems. It is anticipated that the incorporation and integration of anaerobic digestion with microalgae-based biofuels production is able to attain higher efficiency and improve sustainability in the production of biofuels from microalgae. This chapter investigates several of the technical issues associated with anaerobic digestion of microalgae biomass including the low concentration of biodegradable (digestible) microalgae substrates, cell wall disruption and high lipid concentrations. Also highlighted is when the incorporation of anaerobic digestion into a biofuels bio-refinery concept, several anaerobic digestion-related issues can be addressed by the pre-treatment methods used to process microalgae for liquid and gaseous biofuels. This chapter also discusses other technical issues associated with the anaerobic digestion of microalgae including ammonia inhibition, low C/N ratio and co-digestion. Gas produced by the anaerobic digestion of residual microalgae biomass can be used for electrical or thermal energy within the microalgae biofuels bio-refinery, while the high density microalgae cultures can provide efficient biogas purification. The resulting digestate has been shown to be an ideal nutrient source for the continued growth of additional microalgae biomass, and helps to close the nutrient loop associated with large-scale microalgae biomass production. With a greater understanding of the different microalgae species and their characteristics, the anaerobic digestion of microalgae and their residues must be optimised to play an essential role in the sustainable future of clean energy derived from microalgae biomass.

A.J. Ward (✉)
Microalgae Engineering Research Group, School of Chemical Engineering,
The University of Adelaide, Adelaide, SA 5000, Australia
e-mail: Andrew.ward@adelaide.edu.au

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16.1 Introduction

Biofuel production from microalgal feedstock has several challenges to overcome as it becomes a mainstream industry capable of producing the quantity of liquid biofuel required at a competitive price (Pragya et al. 2013). One of the main challenges is how to utilise the remaining biomass after the lipid fraction has been removed for lipid-based biofuel production (Sialve et al. 2009). Generally, microalgae biomass contains 30–40 % lipid, and up to 70 % of the residual biomass is left after the extraction process (Pragya et al. 2013). Other challenges faced in large-scale microalgae production is the need for fertilisers (Fenton and Ohuallachain 2012), high energy inputs required for harvesting and dewatering biomass (Molina et al. 2003), and challenges involved with lipid extraction and conversion processes (Pragya et al. 2013). Anaerobic digestion can offer a pathway to eliminate some of the overheads of the production cycle by recovering nutrients from extracted residual biomass and the generation of electricity from methane biogas (Sialve et al. 2009). It is anticipated that the incorporation and integration of anaerobic digestion to microalgae biofuel production and bio-refinery processes will increase the cost effectiveness of the production methods helping it to become more economically feasible and sustainable (Sialve et al. 2009; Ward et al. 2014).

16.2 Microalgae and Anaerobic Digestion

16.2.1 Historical and Current Microalgae Digestion Perspective

The first authors to report anaerobic digestion of microalgae biomass was Golueke et al. (1957) where authors investigated the anaerobic digestion of *Chlorella vulgaris* and *Scenedesmus* grown as part of a wastewater treatment process. Anaerobic digestion of microalgae was then continued in a series of studies on advanced integrated large-scale wastewater pond production, with resultant microalgae biomass used for anaerobic digestion as a method of converting solar energy to chemical energy (Benemann et al. 1977; Chen and Oswald 1998; Golueke and Oswald 1963; Golueke et al. 1964; Green et al. 1995a, b; Oswald 1976; Oswald et al. 1994). In general, they identified several key challenges that could hinder the optimal digestion of microalgae biomass. Table 16.1 provides a summary of the research on several different microalgae species to date, and highlights the biogas potential from microalgae. The variation in biogas volumes produced reported in literature illustrates the variation in biogas production between both different species of microalgae and variability within the same species (Table 16.1). The production variation for a single species is highlighted for

Table 16.1 Methane biogas production from anaerobic digestion of different species of microalgae biomass

Microalgae species	C/N ratio	Methane yield	Loading rate	Reference
Arthrospira maxima	4.3–5.33	173 mL g^{-1} VS	500 mg/TS/L	Inglesby and Fisher (2012)
Arthrospira platensis	N/A	481 mL g^{-1} VS	2000 mg/TS/L	Mussgnug et al. (2010)
Blue-green algae (cyanobacteria)	N/A	366 mL g^{-1} VS	281.96 mg/VS/L	Rui et al. (2007)
Chlamydomonas reinhardtii	N/A	587 mL g^{-1} VS	2 000 mg/TS/L	Mussgnug et al. (2010)
Chlorella kessleri	N/A	335 mL g^{-1} VS	2000 mg/TS/L	Mussgnug et al. (2010)
Chlorella sp., *pseudokirchneriella* sp. and *Chlamydomas* sp.	N/A	0.28–0.60 m^3/kg/VS	402 mg VS	De Schamphelaire and Verstraete (2009)
Chlorella sp., *Scenedesmus, Euglena* and *Oscillatoria*	N/A	300–800 mL $^{-1}$ VS	N/A	Golueke and Oswald (1959)
Chlorella sp., *Scenedesmus*	N/A	170–320 mL g^{-1} VS	1.44–2.89 g/VS/L	Golueke et al. (1957)
Chlorella sorokiniana	N/A	212 mL g^{-1} VS	N/A	Polakovicova et al. (2012)
Chlorella vulgaris	N/A	403 mL g^{-1} VS	2 g/VS/L	Lu et al. (2013)
Chlorella vulgaris	N/A	286 mL g^{-1} VS	5000 mg/VS/L	Lakaniemi et al. (2011)
Chlorella vulgaris	6	240 mL g^{-1} VS	1000 mg/VS/L	Ras et al. (2010)
Chlorella vulgaris	N/A	189 mL g^{-1} VS	N/A	Polakovicova et al. (2012)
Chlorella vulgaris	N/A	0.40–0.45 L	2677–6714 mg (COD)	Sanchez-Hernandez and Trvieso-Cordoba (1993)
Dunaliella	N/A	440 mL g^{-1} VS	910 mg/VS/L	Chen (1987)
Dunaliella salina	N/A	505 mL g^{-1} TS	2000 mg/TS/L	Mussgnug et al. (2010)
Dunaliella tertiolecta	N/A	24 mL g^{-1} VS	5000 mg/VS/L	Lakaniemi et al. (2011)
Durvillea antarctica	N/A	492 mL g^{-1} VS	3000 mg/dry/TS/d	Vergara-Fernandez et al. (2008)
Euglena gracilis	N/A	485 mL g^{-1} VS	2000 mg/TS/L	Mussgnug et al. (2010)

(continued)

Table 16.1 (continued)

Microalgae species	C/N ratio	Methane yield	Loading rate	Reference
Lake Chaohu natural population consortium	N/A	295 mL g^{-1} VS	N/A	Shuchuan et al. (2012)
Macroystis pyrifera and *Durvillea Antartica* (50 % blend)	N/A	540 mL g^{-1} VS	3000 mg/dry/TS/d	Vergara-Fernandez et al. (2008)
Macroystis pyrifera	N/A	545 mL g^{-1} VS	3000 mg/dry/TS/day	Vergara-Fernandez et al. (2008)
Microcystis sp.	N/A	70.33–153.51 ml	1500–6000 mg/VS	Zeng et al. (2010)
Nannochloropsis oculata	N/A	204 mL g^{-1} VS	N/A	Buxy et al. (2013)
Nannochloropsis salina (lipid extracted biomass)	4.4	130 mL g^{-1} VS	2000 mg/l/VS	Park and Li (2012)
Phaeodactylum tricornutum	N/A	0.35 L g^{-1} COD	1.3 ± 0.4–5.8 ± 0.9	Zamalloa et al. (2012)
Scenedesmus obliquus	N/A	287 mL g^{-1} VS	2000 mg/TS/L	Mussgnug et al. (2010)
Scenedesmus obliquus	N/A	240 mL g^{-1} VS	2000 mg/VS/L	Zamalloa et al. 2012)
Scenedesmus sp.	N/A	170 mL g^{-1} COD	1000 mg/COD/L	Gonzalez-Fernandez et al. (2012c)
Scenedesmus sp. (single stage)	N/A	290 mL g^{-1} VS	18,000 mg/VS/L	Yang et al. (2011)
Scenedesmus sp. (two stage) *Note* 46 ml/g/VS hydrogen	N/A	354 mL g^{-1} VS	18,000 mg/VS/L	Yang et al. (2011)
Scenedesmus sp. and *Chlorella* sp.	N/A	16.3–15.8 cu ft	7.8–9.2 cu ft/lb (VS)	Golueke et al. (1957)
Scenedesmus sp. and *Chlorella* sp.	6.7	143 mL g^{-1} VS	4000 mg/VS/L	Yen and Brune (2007)
Spirulina Leb 18	N/A	0.79 g/L	72,000 mg/L/TS	Costa et al. (2008)
Spirulina maxima	4.16	0.35–0.80 m^3	20–100 kg/m^3 (VS)	Samson and Leduy (1986)
Spirulina maxima	N/A	320 mL g^{-1} VS	910 mg/VS/L	Chen (1987)
Spirulina maxima	N/A	330 mL g^{-1} VS	22,500 mg/VS/L	Varel et al. (1988)

(continued)

Table 16.1 (continued)

Microalgae species	C/N ratio	Methane yield	Loading rate	Reference
Spirulina platensis UTEX1926	N/A	0.40 m^3 kg	N/A	Converti et al. (2009)
Tetraselmis	7.82	0.25–0.31 L g^{-1} VS	2000 mg /VS	Asinari Di San Marzano et al. (1983) C/N ratio- Lourenco and Barbarino (1998)
Tetraselmis	N/A	252 mLg^{-1} VS	5400 mg/VS/L	Ward and Lewis (2015)
Waste water grown community	N/A	497 mL g^{-1} TS	2.16 g/L/TS	Salerno et al. (2009)
zygogonium sp.	N/A	344 mL g^{-1} TS	N/A	Ramamoorthy and Sulochana (1989)

C. vulgaris, with the anaerobic digestion in five separate experiments yielding five different biogas volumes per gram of volatile solids (VS) for each experiment, ranging from a low of 189 mL/g/VS up to 450 mL/g/VS. It also highlights how the pre-treatment, digester configuration and resultant microbial communities can impact on the final biogas production.

When investigating biogas production from microalgae biomass, several different terminologies are utilised to report the biogas production from microalgae substrates. Units range from biogas production for grams of COD destroyed, biogas produced for each gram of VS added to digester and biogas produced for each gram of total solids added to digester. The standard methods used to determine VS are also the same as standard methods used to determine the organic weight (ash free dry weight—AFDW) of microalgae (Clesceri et al. 1998). Ash free dry weight is used extensively by phycologists to report quantities of microalgae biomass. When reporting microalgae biomass, the organic weight or the VS (digestible component) of the microalgae biomass is only a percentage of the total solids and ash content varies between species. The variation in AFDW and VS can differ by up to 50 % between species and can significantly affect calculating or modelling the biogas production from different microalgae species (Ward et al. 2014). Several key issues have been identified in scientific literature that may be detrimental to the anaerobic digestion of microalgae biomass and result in reduced biogas production. The following sections highlight the major factors that can influence biogas production from microalgae.

16.3 Technical Restraints on Microalgae Anaerobic Digestion

16.3.1 Low Concentration of Biomass

One of the major factors hampering anaerobic digestion of microalgae biomass is the low concentration of algal biomass under culture conditions. Most outdoor microalgae cultures are very dilute and may only contain 1 g L^{-1} of solid biomass (Golueke et al. 1957; Stephans et al. 2010); too dilute for anaerobic digesters, potentially causing bacterial washout due to biomass and excessive water addition to achieve the required VS solid loading rates (De Schamphelaire and Verstraete 2009; Golueke et al. 1957; Parkin and Owen 1986). This problem is overcome by harvesting, concentrating and dewatering microalgae cultures to concentrate the biomass. This is a relatively expensive and time consuming production requirement for most microalgae biofuel production methods (Harun et al. 2010; Pragya et al. 2013; Stephans et al. 2013; Ward et al. 2014). However, when anaerobic digestion is integrated with a microalgae biofuel production system, the energy requirement for harvesting and dewatering processes is offset by resultant methane production from the anaerobic digestion process (Sialve et al. 2009). Many different methods are used for the harvesting, concentration and dewatering microalgae, including settling of biomass (Collet et al. 2010), use of chemical flocculants (Golueke et al. 1964; Kalyuzhnyi et al. 1998), centrifugation (Benemann et al. 1977), alum (Golueke and Oswald 1963) and electro-flocculation (Pragya et al. 2013). The above listed harvesting methods have been shown to be nontoxic to anaerobic digestion and may improve digester performance due to better retention of solids (Callander and Barford 1983; Campos et al. 2008; Golueke and Oswald 1963; Kalyuzhnyi et al. 1998; Krishnan et al. 2006). However, with the high cost associated with some of these harvest methods, many new laboratory and pilot scale methods are under development, the resulting impact to anaerobic digestion is yet to be assessed for toxicity effects causing inhibition of anaerobic digestion processes (Ward et al. 2014).

16.3.2 Cell Wall Permeability

Many species of microalgae are encapsulated by a rigid cell wall and can be problematic in anaerobic digesters (Chen and Oswald 1998; Golueke and Oswald 1959; Gonzalez-Fernandez et al. 2012a, b; Mussgnug et al. 2010; Samson and Leduy 1983; Sialve et al. 2009; Zamalloa 2012). Anaerobic digester associated bacteria need to be able to access the contents of the microalgae cells to allow the digestible components to be converted to methane biogas (Ward et al. 2014). The microalgae cell wall material and chemistry have a major influence on the biogas potential (Ras et al. 2010), and some microalgae species have been shown to survive intact after 6 months

within an anaerobic digester, changing to heterotrophic growth rather than phototrophic growth (Mussgnug et al. 2010). Several studies conclude that a pre-treatment step is required to disrupt the cell wall to increase bacterial hydrolysis before addition to the anaerobic digester (Chen and Oswald 1998; Golueke and Oswald 1959; Mussgnug et al. 2010; Sialve et al. 2009; Zamalloa 2012). Within an integrated system, cell lysis or disruption is also essential for solvent extraction of the lipid fraction of microalgae biomass, allowing solvents to react with internal cell lipids (Lee et al. 2013). Therefore, microalgae cell wall disruption processes are essential for both lipid-based biofuel applications and for optimal anaerobic digestion. The multiple methods of disruption and cell lysis include mechanical, physical, thermal, chemical and enzymatic methods and have the twin applicability of allowing solvent to react with lipids increasing lipid yield in the extraction process and also microbial hydrolysis of the cell contents during anaerobic digestion (Ward et al. 2014). However, the cost of the extra pre-treatments must be considered within the anaerobic digester and biofuel production systems as the energy consumption for the pre-treatment of microalgae biomass can be equal or higher than the energy gained from the microalgae cell (Lakaniemi et al. 2011; Lee et al. 2012, 2013; Lu et al. 2013; Sialve et al. 2009; Yen and Brune 2007).

16.3.3 Ammonia Inhibition

The proximate composition of microalgae can have a major influence on the biogas productivity. For instance, lipid and protein play an important role in anaerobic digestion as they breakdown during the hydrolysis stage (Chen et al. 2008). High protein concentrations lead to the formation of ammonia compounds within the digester (Chen et al. 2008; McCarty 1964). Ammonia is produced from the biological breakdown of nitrogenous matter (i.e. protein). High concentrations of ammonia being formed within the digester can lead to inhibition of the bacterial community (Buswell and Boruff 1932). Ammonia toxicity has been shown to affect the methanogenic bacteria in two separate ways. The first is by the ammonium ion directly inhibiting the methane synthesising enzyme. The second is by the hydrophobic ammonia nitrogen (NH_3–N) molecule diffusing passively into the bacterial cell causing an imbalance and/or a potassium deficiency within the microbial cell (Kayhanian 1999; Sialve et al. 2009; Ward et al. 2014). Furthermore, NH_3–N in the gaseous form has been shown to be more toxic at lower concentrations than the aqueous ionised form (McCarty 1964). Ammonia is extremely toxic at levels above 3000 mg/L and can be moderately inhibitory at level of 1500–3000 mg/L (McCarty 1964). On the other hand, maintaining NH_3–N concentrations between 50 and 200 mg/L have been shown to be beneficial for the bacterial population as ammonia nitrogen is an essential nutrient required by the microbial community (Parkin and Owen 1986). As the utilisation of volatile fatty acids must balance the production of volatile fatty acids by hydrolytic and acetogenic bacteria to maintain digester stability, efficient digester performance is therefore dependant on maintaining the

NH_3–N concentration below the inhibitory limits for all associated anaerobic digestion bacteria (McCarty 1964; Ward et al. 2014).

16.3.4 Low C/N Ratio

Microalgae have a relatively low carbon to nitrogen (C/N) ratio when compared to other biomass substrates and wastewaters utilised in the anaerobic digestion process (Sialve et al. 2009; Vergara-Fernandez et al. 2008). The ideal C/N ratio for anaerobic digestion is approximately in the range of 20–30 (Parkin and Owen 1986). In contrast, microalgae have a C/N ratio, between 4 and 8 (Ward et al. 2014). When a substrate has a C/N ratio below 20, it can cause an imbalance in an accumulation of NH_3–N within the digester and associated inhibition (Sialve et al. 2009). To overcome low C/N ratios associated with microalgae, co-digestion with other waste streams high in carbon (i.e. paper, glycerol, pig manure, cow manure, lipid rich fats oils and greases, municipal waste, soybean oil) emolliates the high NH_3–N build-up (Ehimen et al. 2009; Gonzalez-Fernandez et al. 2011; Saxena et al. 1984; Shouquan et al. 2009; Yen and Brune 2007). For example, when paper was added to microalgae in a digester, the C/N ratio increased from 6.7 to 36.4 with the best co-digestion biogas production with a mix of 50 % paper and 50 % microalgae at a C/N ratio of 18.0 (Yen and Brune 2007). When the biogas production from the C/N ratio treatment of 18 was compared to the lower C/N ratio of 6.7, a 50 % increase in biogas productivity was recorded (Yen and Brune 2007). Such co-digestion ratio productivity is highly dependent on the species of microalgae being digested as well as the secondary substrate being co-digested (Ward et al. 2014). When a high C/N ratio is used, there is a risk that the bacterial population may become NH_3–N limiting, causing inhibition with the methanogen bacterial community (Chen et al. 2008; Parkin and Owen 1986). When considering the co-digestion of microalgae with other substrates, the availability, seasonality and location of the secondary co-digestion substrate must also be considered to ensure continuous availability and low cost associated with procuring and transportation (Ward et al. 2014). Furthermore, balancing both the C/N ratio and all co-digested substrate, degradation rates are essential to balance the carbon and nitrogen release within the digester. To balance the C/N ratio, the co-digestion substrate should have a similar degradation rate as the primary substrate being digested (Ehimen et al. 2009; Kayhanian 1994).

16.3.5 Lipids

Lipids contained within microalgae are attractive for the production of methane biogas. Lipids have a higher theoretical methane potential when compared to proteins and carbohydrates (Zamalloa 2012). However, due to the chemistry of

lipids and the low alkalinity and buffering capacity associated with them, high lipid concentrations can be inhibitory to anaerobic digestion (Park and Li 2012; Ward et al. 2014). Inhibition by lipids is caused by the intermediate products produced during their breakdown, such as long chain fatty acids and VFAs (Park and Li 2012). When the lipid content of the microalgae is below 40 %, it has been suggested that the direct conversion of microalgae to methane by anaerobic digestion is more energetically favourable when compared to lipid removal from microalgae biomass (Sialve et al. 2009). However, lipid concentrations have been reported to be inhibitory to aerobic digestion at concentrations of 31 % or higher (Cirne et al. 2007). When considering many of the microalgae species used commercially for biofuel production, many have been purposely selected for a lipid concentration of 30 % or higher. Therefore, the removal of the lipid fraction for lipid-based biofuel production is highly beneficial and crucial for the anaerobic digestion process. (Cirne et al. 2007; Sialve et al. 2009). The lipid extraction methods utilised to extract microalgae lipid can also have an effect on the digestibility of the residual biomass (Ehimen et al. 2009). The solvents butanol, hexane and methanol have been shown to have no detrimental effect or inhibition to the anaerobic microbial community if the residual biomass is heated sufficiently to remove any entrained solvents (Ehimen et al. 2009). However, chloroform from the Bligh and Dyer extraction process has been shown to be detrimental to the anaerobic digester microbial community (Bligh and Dyer 1959; Thiel 1969).

16.3.6 Digestate and Nutrient Recycling

Nutrients in the form of commercial fertilisers are an expensive input for mass production of microalgae biomass (Collet et al. 2010), particularly nitrogen and phosphorous (Fenton and Ohuallachain 2012; Lyovo et al. 2010; Vaccari 2009). Large nutrient requirement can increase cost of microalgae biofuel production and compete with agricultural demand (Erkelens et al. 2014; Fenton and Ohuallachain 2012). Fertiliser prices are highly dependent on fossil fuels price's (Fenton and Ohuallachain 2012; Vaccari 2009). Increase in fossil fuel price coupled with the higher worldwide agricultural demand resulted in increased fertiliser costs (Fenton and Ohuallachain 2012; Stephans et al. 2010; Vaccari 2009; Ward et al. 2014). Therefore, nutrient recovery from residual and waste products from the microalgae production is essential to allow sustainable product development (Erkelens et al. 2014; Sialve et al. 2009; Stephans et al. 2010). Anaerobic digestion can offer a solution to the fertilisers input problem by recycling extracted biomass and any by-products (Erkelens et al. 2014). Anaerobic digestion of algal biomass produces a clear liquid digestate that is nutrient rich containing both nitrogen and phosphorous (Stephans et al. 2010). Anaerobic digestion digestate nutrient content of 546–2940 mg/L ammonia nitrogen and 141–390 mg/L phosphorous have been reported from anaerobically digested microalgae (Collet et al. 2010; Erkelens et al. 2014; Ward et al. 2014; Zamalloa 2012). A further benefit of the integration of anaerobic

digestion into microalgae production is the ability to utilise the microalgae cultures to purify the biogas produced from anaerobic digestion (Converti et al. 2009; Green et al. 1995a). The concentration of methane in biogas produced from microalgae is in the range of 30–50 % (Sialve et al. 2009); generally too low to utilise in its current form, and purification of the biogas is needed before utilisation (Vergara-Fernandez et al. 2008). Due to the low solubility of methane and high solubility of CO_2, the uptake of CO_2 in microalgae cultures is able to purify the biogas to a higher energy density, with the added benefit of stripping other gasses such as sulphur and ammonia (Green et al. 1995a; Ward et al. 2014). As methane has been shown to be non-detrimental to microalgae growth, the dual purification of biogas as a consequence of supplying microalgae cultures with additional nutrient in the form of CO_2 achieves multiple productivity benefits (Green et al. 1995a; Sialve et al. 2009).

16.4 The Future Prospects of Anaerobic Digestion in Microalgae Production

Current development of new anaerobic digester designs and configurations can play an important role in the future of hybrid microalgae production and anaerobic digestion facilities. New digester reactor designs that decouple the hydraulic and solid retention times can be extremely beneficial for anaerobic digestion of microalgae (Ward et al. 2014) and can be achieved by upflow anaerobic sludge blanket reactors, anaerobic membrane reactors, anaerobic filters and bed reactors, in-pond fermentation pits and also by two-stage anaerobic digestion (Gao et al. 2007; Goodwin et al. 2001; Green et al. 1995a, b; Haridas et al. 2005; Inglesby and Fisher 2012; Shin et al. 2010; Varel et al. 1988; Vergara-Fernandez et al. 2008; Zamalloa 2012; Zhou et al. 2009). New reactor designs allow better control, breakdown and conversion of organic matter within the digester (Vergara-Fernandez et al. 2008). In particular, two-stage anaerobic digestion physically separates stages of digestion into separate reactors, allowing much better control over the anaerobic digestion process (Dinsdale et al. 1996; Dugba and Zhang 1999; Shin et al. 2010; Varel et al. 1988; Vergara-Fernandez et al. 2008; Yu et al. 2002). Furthermore, molecular research underway is providing new insights into the microbial communities associated with the anaerobic digestion process and aim to understand the impact of internal environmental conditions and change within the digester community (Keyser et al. 2006; Shin et al. 2010; Supaphol et al. 2011). For example, new molecular methods in the anaerobic digestion process (such as “poly chain reaction denaturing gel gradient electrophoresis” and “real time poly chain reaction”) are under investigation to improve metabolic pathways within the digester and the abundance and species composition of bacterial populations (Patil et al. 2010a, b; Shin et al. 2010; Skilman et al. 2009; Supaphol et al. 2011; Ward et al. 2015; Zhang et al. 2012; Ziganshin et al.

2011). A greater understanding of the anaerobic digestion processes at the molecular level can optimise the anaerobic digestion process to specific microalgal biomass and co-digestate substrates (Ward et al. 2014).

16.5 Conclusions

The integration of anaerobic digestion with microalgae-based biofuels production is able to attain higher efficiency and improve sustainability of the production of microalgae-based biofuels. Several of the technical issues including the low concentration of biodegradable (digestible) microalgae substrates, cell wall disruption and high lipid concentrations can be overcome by the pre-treatment methods used to process microalgae for liquid and gaseous biofuels. Gas produced by the anaerobic digestion of residual microalgae biomass can be used for electrical or thermal energy within the microalgae biofuels bio-refinery, while the high density microalgae cultures can provide efficient biogas purification. The resulting digestate has been shown to be an ideal nutrient source for the continued growth of additional microalgae biomass and help to close the nutrient loop associated with large-scale microalgae biomass production. With a greater understanding of the different algae species and their characteristics, the anaerobic digestion of microalgae and their residues can be optimised to play an essential role in the sustainable future of clean energy derived from microalgae biomass.

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Chapter 17
Economic and Energy Analysis of Large-Scale Microalgae Production for Biofuels

Karne de Boer and Parisa A. Bahri

Abstract Microalgae biofuels have been under development for the last 40 years; however, in the last 6 years, this development has intensified due to higher oil prices and wider acceptance of anthropogenic climate change. Despite the excellent potential of algal biofuels, they are not yet commercially viable. The reason for this lack of progress is examined in this chapter by firstly reviewing the range of different technology options for biofuels from microalgae. Secondly, an analysis of the available techno-economic and energy assessments is performed highlighting the effect that each system element has on the overall viability.

17.1 Introduction

Microalgae are a feedstock of great interest for the production of energy, fuels, food, high-value nutritional supplements and specialty chemicals. Microalgae attract this attention because they have high photosynthetic efficiency, can be grown at massive scale on non-arable land, grow rapidly, can thrive in salt or brackish water and naturally produce a range of compounds with commercial value (Borowitzka and Moheimani 2010). Due to the high potential of fuel production from microalgae, research and investment into microalgae development typically occur in earnest after oil price spikes, e.g. the aquatic species program in the USA after the 1970s oil embargo (Sheehan et al. 1998) and the rush of new algae companies and research after the rapidly increasing oil prices between 2007 and 2012 (Ribeiro and da Silva 2012).

Despite these promising characteristics, no one has been able to commercially cultivate and process microalgae at scale for the purpose of producing fuels. The central reason for this slow progress is that it is not economically viable

K. de Boer (✉) · P.A. Bahri
School of Engineering and Information Technology, Murdoch University, Murdoch, WA 6150, Australia
e-mail: karne@regenerateindustries.com

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(ANL et al. 2012; Benemann et al. 2012; DOE 2014). That is, it costs more to grow and convert the algae into fuel than the fuel is worth (Stephens et al. 2010).

This chapter reviews the existing techno-economic models and life cycle analyses (with a focus on energy consumption) that have been published on the large-scale production of algal biofuels. The purpose of this review is to provide readers with a clear grasp on future trends in microalgae economics and opportunities for improvement. This review is focused on the economic and energetic feasibility of the biofuel production process as these are the barriers to large-scale commercial deployment.

17.2 Microalgae Production Systems for Biofuels

A cursory examination of literature or commercial websites related to biofuel production from microalgae makes it clear that there are almost as many different ways of growing and processing microalgae for the purpose of producing fuel as there are species of microalgae. Figure 17.1 provides a block flow diagram of the typical process for converting microalgae into biofuel.

The numerous process variations arise from different technologies applied at each of the unit operations listed below:

1. Growth
2. Harvesting
3. Dewatering
4. Extraction
5. Conversion

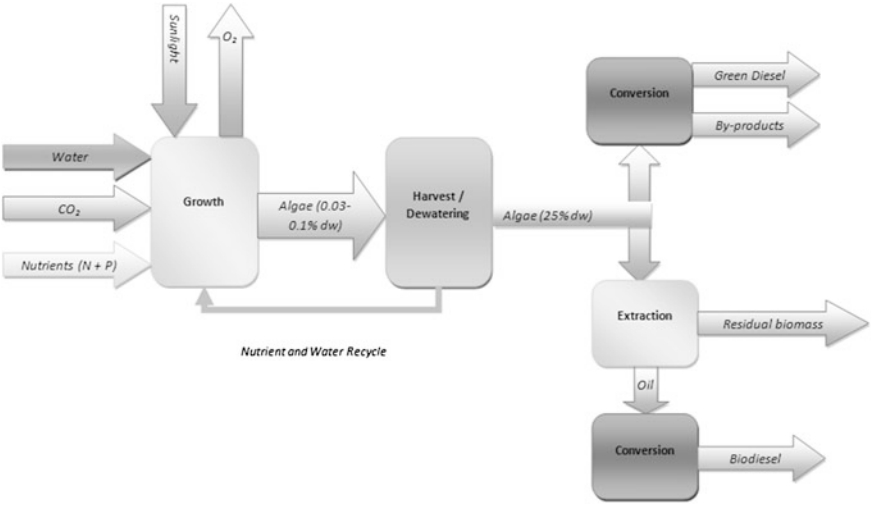

Fig. 17.1 Microalgae to biofuels block flow diagram (de Boer et al. 2012)

In some cases, harvesting and dewatering are combined as one step or extraction and conversion can occur simultaneously. An overview of the various technologies proposed for this is provided in Table 17.1.

17.2.1 Growth Systems

There are two main types of growth systems, open ponds and closed photo-bioreactors. As the name suggests, open ponds are growth systems that are essentially open stretches of water that are naturally occurring or artificially made, naturally circulated (by temperature differences and wind) or mechanically agitated (usually paddle wheel driven). The most common types for large-scale microalgae production are artificially designed naturally circulated ponds (e.g. Hutt Lagoon—Western Australia) or artificially constructed open raceway ponds (e.g. Sapphire energy—Mexico). The open raceway or high-rate pond utilised by most microalgae companies/researchers is considered to be the lowest capital cost and lowest energy consumption approach for microalgae growth and is typically based on the design and investigation work conducted by researchers in the aquatic species research program (Benemann et al. 2012). In this approach, shallow (15–35 cm deep)-oval-shaped ponds are gently agitated by a paddle wheel to achieve maximum productivity.

In closed Photo-bioreactors (PBR), microalgae are cultivated in a controlled environment to maximise productivity. There are numerous types of photo-bioreactors with major categories including:

- Tubular photo-bioreactors
- Flat plate bioreactors
- Bag reactors

A subclass of PBR is attached reactor systems or biofilms in which the microalgae are grown on a substrate that is wet by the growth medium rather than being grown in solution (Ozkan et al. 2012). In the first two of these designs, construction can be either of plastic or glass, while in the latter, construction is of flexible plastics. The major stated advantages of PBR are the ability to control temperature, CO_2 content and pH and provide protection from predators. Furthermore, water loss is minimised through low evaporation rates; however, evaporation loss is counteracted by the need to control temperature with water. The major disadvantages for closed PBR are the preventatively high capital costs (Benemann et al. 2012; Borowitzka and Moheimani 2010).

Biofilm or attached systems are fundamentally different to both open ponds and closed PBR as the microalgae grow on a substrate rather than in suspension in the liquid growth medium (Ozkan et al. 2012). The advantages of these systems are the reduction in water volume, energy consumption and the ease of harvesting; however, like PBR systems, they will have a higher capital cost than open ponds.

Table 17.1 Technology options for microalgae to biofuel unit operations

Unit operation	Technology option
Growth	*Open ponds*
	• **High-rate ponds (paddle wheel)**—Large raceway ponds are used to cultivate algae with low energy mixing provided by paddle wheels • **Natural circulation**—Large open ponds are naturally mixed via temperature difference and wind induced currents
	Photo-bioreactors
	• **Flat plate**—Microalgae are grown between or on top of inclined flat plates • **Tubular**—Algae are circulated through glass or plastic tubes • **Plastic bag**—Algae are cultivated in plastic bags that are typically suspended in greenhouses or in water bodies • **Biofilm/Attached**—Instead of growing in water suspension, microalgae are grown on a substrate in which water is passed over the microalgae
Harvest	• **Gravity settling**—The microalgae naturally settle when agitation of the growth medium stops • **Flocculation**—Microalgae are drawn into clumps to enhance gravity settling or flotation through the use of a flocculant (chemical or biological) or electrical charge (electro-flocculation) • **Dissolved air flotation (DAF)**—Individual microalgae or flocculated clumps are floated to the surface using microbubbles
Dewatering	• **Belt filter**—Water is squeezed out of algae via a belt filter press • **Centrifugation**—Centrifugal force is used to separate water from the microalgae biomass due to density differences • **Thermal drying**—Excess water is removed from microalgae biomass through heating and evaporation
Extraction	*Extraction*
	• **Cell disruption**—The cell wall is broken down via chemical, electrical or mechanical means to facilitate oil extraction • **Dry solvent extraction**—Oil is extracted by contacting dried microalgae biomass with a solvent • **Wet solvent extraction**—Oil or other metabolites from wet microalgae biomass are extracted • **Mechanical extraction**—Oil is extracted using physical means (screw press)
Conversion	*Whole cell*
	• **Hydrothermal liquefaction**—Green diesel is produced from wet algae paste • **Digestion**—Biomethane is produced from wet microalgae biomass using anaerobic digestion • **Pyrolysis**—Bio-oil is produced from dry microalgae biomass • **Gasification**—Syngas (and potentially Fischer-Troph) fuels are produced from dried algae biomass
	Metabolite
	• **Transesterification**—Extracted vegetable oil is converted to biodiesel via methanolysis • **Fermentation**—Extracted carbohydrate is converted to ethanol via fermentation • **Hydrotreating**—Extracted vegetable oil is converted to green diesel via hydrotreating

The fundamental goal in any growth system is the low-cost production of biofuels from microalgae, which is achieved by maximising productivity (species specific), reducing capital cost, minimising energy consumption and maximising nutrient (N, P, trace minerals and CO_2) utilisation efficiency. In general, open ponds provide the lowest capital cost and energy consumption, while PBR (closed systems) offer greater protection from predators (weeds and pests), greater control and higher productivity. Numerous PBR have been trialled; however, history has demonstrated and continues to demonstrate that open ponds are the only viable methodology for large-scale microalgae production, even for high-value nutraceuticals (Benemann et al. 2012). As a result, the trend in research has been to identify/select or genetically modify microalgae to provide consistent high yields in the challenging environment (e.g. increasing salinity due to evaporation, predators, changing temperatures) of open ponds rather than continually refine PBR's.

17.2.2 Harvesting and Dewatering

The critical role of harvesting and dewatering is best understood by examining the volume of water associated with different microalgae concentrations in microalgae growth systems. Table 17.2 provides a comparison of the water volume required to produce 1000 kg of microalgae biomass at different stages of the process. The concentrations shown in this table are typical or representative of concentrations achieved at the respective growth or harvesting phase.

The massive volumes of water required in the growth phase result in high capital costs and subsequently high energy costs because of the need to pump the growth medium to the processing equipment and then remove it. As a result, the majority of research efforts have been focused on low energy and low-cost methodologies for water removal. The water volumes required and the subsequent capital/energy burden are one of the major impediments to economic biofuel production (de Boer et al. 2012). The importance and thus associated research undertaken in this field are reflected in this book with three other chapters providing significant detail on dewatering and harvesting processes.

Table 17.2 Water volume for 1000 kg of biomass

Stage	Concentration (%)	Water volume (L)
Open pond growth (Delrue et al. 2012)	0.05	1,999,000
Photo-bioreactor growth (Delrue et al. 2012)	0.50	199,000
Post-harvest (de Boer et al. 2012)	1	99,000
Post-dewatering (de Boer et al. 2012)	25	3000

17.2.3 Extraction and Conversion

Once the microalgae biomass has been removed from the water (typically to 20–30 % dw biomass), a number of options are available for the conversion of the biomass into fuel or valuable co-products. These approaches can be broadly categorised into two system types. The traditional approach is to extract the metabolite of interest (typically lipids or vegetable oil) and then convert the oil into a fuel via transesterification (biodiesel) or hydrotreating. The extraction process requires the use of a solvent such as hexane that requires the biomass to be completely dried; as a result, further thermal energy and capital expenditure are required to dry the biomass to below 10 % moisture to allow solvent extraction (Xu et al. 2011). Recently, wet solvent extraction methods have been proposed, which negate the need for drying; however, these have yet to be proved on a large scale (Kanda and Li 2011).

The further cost of drying has led researchers to investigate in situ conversion methods that convert the oil contained in the biomass into biodiesel directly with the biodiesel subsequently separated from the biomass (Ehimen et al. 2010; Johnson and Wen 2009; Levine et al. 2010; Wahlen et al. 2011). Again, these methods have limitations including high solvent consumption, higher processing temperatures and low yields (de Boer et al. 2012).

The challenges associated with extraction have led to further investigation into conversion methods that use the whole algal cell (Biller and Ross 2011; Liu et al. 2013). Again, approaches can be split into wet and dry, with dry methods requiring the whole biomass to be dried and then converted to biofuels via traditional thermal methods, e.g. pyrolysis and gasification (Babich et al. 2011). In this case, the dried microalgae biomass simply becomes one of many potential feedstocks that can be used in existing biomass to liquid processes. Again, the need to dry the biomass and the fact that these processes are uneconomical with existing low-cost traditional biomass sources has resulted in this approach being all but abandoned for microalgae.

Another method, which is currently being investigated by research institutions and implemented by companies throughout the world, is hydrothermal liquefaction. In this process, originally developed by Shell, wet biomass is converted directly into a hydrocarbon liquid (bio-oil) under relatively mild temperature and pressure conditions, with the oil to be subsequently upgraded to produce biofuel (Goudriaan et al. 2005, 2008). This process uses wet biomass and produces oil at approximately 35–40 % yield (dry biomass basis). The oil can be used as a blend stock in traditional refining equipment, and it is very attractive from an economic viability viewpoint. The major weaknesses in this approach are the complete loss of any valuable co-products and the challenges associated with recycling nutrients (Liu et al. 2013).

An overview of the different extraction and conversion processes discussed above is provided in Fig. 17.2.

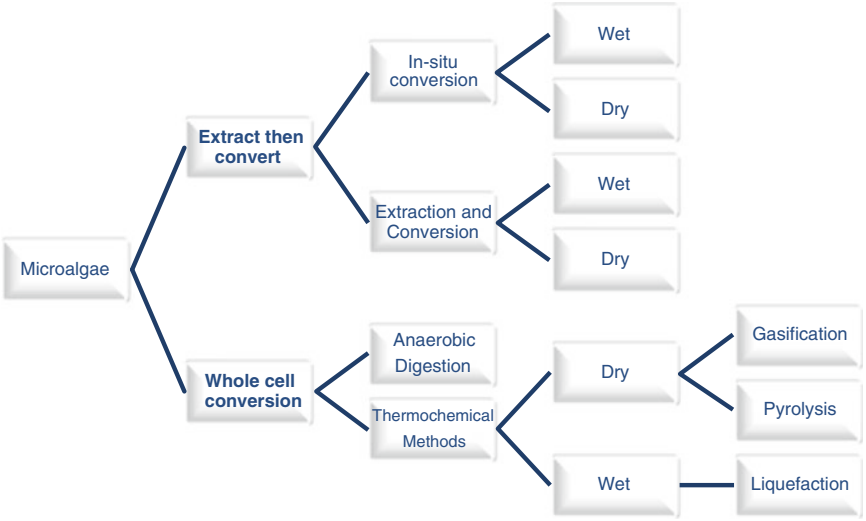

Fig. 17.2 Technology options for different systems (de Boer et al. 2012)

17.3 Economics

Due to the significant potential of microalgae as a feedstock for biofuels, numerous economic viability assessments have been undertaken (Ribeiro and Silva 2013). To provide a snapshot of the current status of biofuel production from microalgae, a summary of these studies is provided in Table 17.3.

17.3.1 Techno-Economic Model Discussion

The summary in Table 17.3 demonstrates that there is a wide range of cost estimates for microalgae biofuel production and that none of them provide a cost estimate that will be economically competitive with current biofuels or fossil fuels (minimum selling prices of cost of production do not include fuel taxes). Further analysis of the studies is provided in the paragraphs below.

The work of Chisti (2007) was released during a time of aggressive investment and development into microalgae biofuels when oil prices were at historical highs. As a result, this work has a number of assumptions that are optimistic, and interestingly, the only analysis that demonstrates that PBR is better than open ponds. The major driver for this discrepancy is the extremely optimistic aerial productivity for PBR (3 times maximum recorded long-term annual averages) and the low

Table 17.3 Techno-economic studies on biofuels from microalgae (all measurements are in US$/L in the respective referenced year)

Description	System	Aerial Productivity	Cost (US$/L)	Reference
Biodiesel production cost from algae grown in PBR and open pond	PBR → Centrifuge → Extract → Transesterification → Anaerobic digestion Open pond → Centrifuge → Extract → Transesterification → Anaerobic digestion	72 g/m^2/d 35 g/m^2/d	$1.34–$2.94 $3.21	Chisti (2007)
Examination of microalgae cultivation using raceways, PBR and fermentation in British Colombia, Canada	Open raceway PBR Fermenter (heterotrophic) After growth stage downstream processing is: Centrifuge → Solvent recovery → Transesterification	9.4–22.9 g/m^2/d 15.3 g/m^2/d 50–100 g/L	$2.65–$14.44 $5.87–$24.60 $0.88–$2.58	Alabi et al. (2009)
Development of algal biomass production model and economic model	PBR inoculation and Open ponds → Centrifuge → Unclear anaerobic digestion	18–37 g/m^2/day	$1.03–$3.98[a]	Williams and Laurens (2010)
Estimated likely cost range for biodiesel production from microalgae considering a range of technologies	Multiple scenarios of equipment options		$2.17–$9.92[b]	Delrue et al. (2012)
US Department of Energy harmonised baseline model for renewable diesel from microalgae	Open ponds Settling → Dissolved Air Flotation → Centrifuge → Wet solvent extraction → Hydrotreating → Anaerobic digestion	13.2–19.1 g/m^2/day	$3.04–$4.90	ANL et al. (2012)

(continued)

Table 17.3 (continued)

Description	System	Aerial Productivity	Cost (US$/L)	Reference
Production of jet fuel from microalgae conducted by industry/ academic consortium in Australian conditions	Open ponds → Spiral plate centrifuge → Sonication → Wet hexane extraction → Degumming → Hydrotreating → Anaerobic digestion	30 g/m^2/day	\$3.27–\$12.27[c]	Klein-Marcuschamer et al. (2013)
Production of biofuels on a large scale in the USA based on the two most promising production pathways	**Advanced lipid upgrading (ALU) pathway** Open ponds → Settling/Dissolved air flotation/Centrifuge → Dilute acid pretreatment of biomass → Fermentation of biomass slurry → Lipid extraction → Hydrotreating of lipids → Anaerobic digestion of waste and cogeneration **Algal hydrothermal liquefaction (AHTL) pathway** Open ponds → Settling/Dissolved air flotation/Centrifuge → Hydrothermal liquefaction → Hydrotreating → Catalytic gasification	13.2–30 g/m^2/day	**ALU** \$1.09–\$3.67 **AHTL** \$1.12–\$3.89	Davis et al. (2014), DOE (2014), Jones et al. (2014)

[a]Density of 0.88 kg/L utilised
[b]0.6 Euro/$
[c]Conversion factor from barrel to dollar used from Williams and Laurens (2010)

capital cost. The major concern with this work is that demonstrable operational issues (such as cleaning and contamination) were treated as trivial matters. Despite these weaknesses and overly positive assumptions, this study shows how challenging the economic production is as it still demonstrates non-competitive production costs.

The work of Alabi (2009) provides a comprehensive evaluation of microalgae produced phototrophically in open ponds and PBR as well as heterotrophically in fermenters in a Canadian context. This study utilised realistic capital costs and operating data from industry and long-term research studies. The high boundary for fuel costs was due to low productivity (low solar irradiation in Canada and no production in winter), high capital costs (lined ponds) and low oil content (15 %). The low boundary for fuel costs occurred at high productivity (high solar irradiation), low capital costs (no pond lining) and higher oil content (30 %). Heterotrophic solutions were deemed to be unsustainable due to the need for an organic feedstock which is typically derived from terrestrial crops and therefore not suitable for mass production.

The potential of EPA (Omega 3) to provide an economically viable solution was also investigated. This demonstrated that at optimistic conditions, microalgae could economically be grown for the purpose of EPA production; however, in these cases, biofuels were a minor by-product. Although a viable option and currently being pursued by commercial companies (Cellena and Aurora), there are concerns over market saturation at biofuel production levels (Benemann et al. 2012).

Williams and Laurens (2010) developed a model for algal biomass production from first principles that utilised solar irradiation levels and typical lipid/protein/carbohydrate ratios to predict biomass productivity. Although this model provides indications of upper limits of aerial productivity, the economic assessment is somewhat optimistic due to the following assumptions:

- Unproven high productivities (annual average)
- Optimistic and unproven harvesting costs
- High protein prices especially considering there is no market for algae protein
- 100 % nutrient recycle from anaerobic digestion and dewatering operations

Unlike other studies, Delrue et al. (2012) used an innovative approach to address the high level of uncertainty in regard to the use of different technologies on the overall economics of microalgae biofuels. In this work, Monte Carlo simulation techniques were used to generate thousands of different scenarios based on different technologies and performance levels. Table 17.4 summarises the output of this simulation with each row representing a scenario in which one technology was kept constant and all the others changed. The minimum and maximum costs represent the lower and upper limits of the confidence level or range of the estimates, with a 50 % probability that the ultimate cost of biofuels with this technology choice will lie in between these boundaries.

Table 17.4 Unit biofuel cost using different technologies (US$/L) (Delrue et al. 2012)

Scenario	Lower	Upper
All technologies	3.67	6.97
Growth technologies		
Open pond	2.17	4.22
Photo-bioreactor	3.82	7.23
Harvesting/drying technology		
Centrifuge	3.45	5.93
Belt filter press	3.40	5.82
Solar drying	6.13	9.92
Bed drying	3.28	5.60
Extraction technology		
n-hexane	3.83	6.95
Dimethyl ether	3.65	6.97
Conversion technology		
Transesterification	3.75	7.00
Hydrotreating	3.63	6.88
Energy and nutrient cycle		
Anaerobic digestion	3.65	6.88
Gasification	3.75	7.07

The 'all technologies' scenario represents the overall projected price range of biofuels based on all available technologies. As an observation, these values are considered optimistic as the study assumed the flue gas could be utilised as the carbon source and wastewater would provide a source of nutrients (N and P). Unfortunately, there are very few locations that have ideal growth conditions, that is high solar irradiance and stable temperatures, large plots of low-cost non-arable land, access to waste water and close proximity to high volume and concentrated sources of CO_2 (Benemann et al. 2012; Lundquist et al. 2010). This work again indicates that biofuels from microalgae are unlikely to be viable at the current levels of productivity with the current technologies.

In an attempt to develop a baseline model for techno-economic assessment, lifecycle assessment (LCA) and resource assessment of microalgae to biofuel processes, the Department of Energy (DOE) worked with the three American national laboratories to develop a harmonised model of renewable diesel production from microalgae (ANL et al. 2012). This is an extensive work that builds upon the modelling and research of multiple groups and industry representatives over a 50-year period. The fundamental data in this model are used as a reference for most other studies including those referenced in the bottom two rows of Table 17.3. The process modelled is shown in the block diagram in Fig. 17.3.

The model was built around multiple 4850 ha sites located in areas with suitable climate and water availability to achieve a total production volume of 5 billion gallons/year. The sites were chosen according to previous geographic information service (GIS)-based resource assessment modelling. The comprehensive nature of

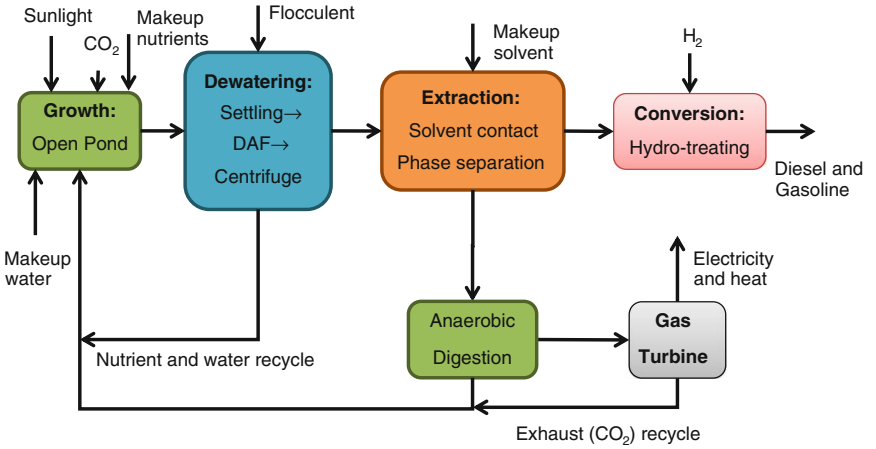

Fig. 17.3 DOE baseline model

this work provides an excellent insight into the major factors affecting the economics of large-scale biofuel production from microalgae. Key observations are as follows:

- At the baseline yield (annual average of 13.2 g/m^2/day productivity and 25 % lipid content), capital costs represent over 70 % of the final diesel selling price. Due to the high capital burden, the break-even price of diesel production is \$2.46/L, while the selling price to achieve a 10 % rate of return on capital investment is \$4.90/L (All further costs mentioned per below are based on a 10 % rate of capital return).
- The addition of pond liners significantly increases the capital cost and therefore the unit cost of renewable diesel production, and at the baseline yield, the price is increased by \$1.38/L. Alternative pond design or suitable ground would negate the need for pond liners, significantly reducing the capital cost.
- Higher yields (e.g. the baseline uses an annual average of 13.2 g/m^2/day productivity and 25 % lipid content) significantly reduce cost to (\$3.03/L) as the pond area, and therefore, capital investment significantly reduces. At lower yields (<20 g/m^2/day), pond and liner costs dominate the capital expenditure; however, at higher yields, the other system elements have a much more significant effect.
- In a best case scenario with harvesting/extraction costs halved (due to technology advancement), pond installation costs reduced by 30 % (due to optimised construction approach), and with the removal of liners, the selling price was approximately \$2.50/L less at the low yield of 12.5 g/m^2/day. If the yields were increased to 50 g/m^2/day via strain improvement (selection or genetic modification), then prices would further decrease to <\$1.25/L. However, at increasing yields, the capital costs of the non-growth elements and the operating costs start to dominate the cost.

- Selling the spent algal biomass as fish protein ($350/tonne) had a limited effect on the economic analysis as this was approximately equivalent to the value provided by the AD plant. To have a greater effect, the selling price of the protein would need to be in excess of $350/tonne.

As an observation, the break-even cost of production in the base case scenario of this model was $2.46/L, while under the same conditions, the minimum selling price was $4.90/L. This increase is required to deliver a rate of return on equity of 10 % (IRR). A key high-level observation from this study is that the capital cost of the growth system is the major impediment to cost competitive microalgae-based biofuels. At low productivities (<20 g/m^2/day), the growth system dominates the capital expenditure, especially when liners are required. In some situations, liners can be done away with (typically resulting in a halving of pond construction cost); however, there is little opportunity to reduce the cost of pond construction due to earthmoving being a very developed field. The most effective way to reduce capital expenditure is to increase the productivity of the microalgae so that less pond area is required, from the studies considered in this work 30 g/m^2/day seems to be a minimum for competitiveness. Higher yields can only come through optimisation of growth conditions, selective breeding and ultimately from genetic modification to limit photo-inhibition.

The work of Klein-Marcuschamer et al. (2013) evaluated three different paths to biomass-derived jet fuel, including microalgae, perennial oil crop and heterotrophic fermentation. Their techno-economic analysis focused on algae and processes that had sufficient data available. The major anomaly with this work is the use of centrifuges to concentrate the microalgae from harvest concentration (0.05 % ash free dry weight) to 25 %. Their motivation for this is that this has been demonstrated through the use of Evodos centrifuges. The consequence of this is that harvesting capital and operating costs (power) represent 70 % of the final cost.

This is clearly not going to be viable, with options such as settling or DAF (with or without flocculation) required as a pre-settling stage. Interestingly, however, this demonstrates another challenge associated with large-scale microalgae production—low-cost options exist; however, they are often problematic or not proven at scale.

As microalgae research and commercial R&D projects have matured, the US Department of Energy (DOE) has identified two production pathways as shown in the final row of Table 17.3[1]. In the ALU methodology, the microalgae production system is treated as a biorefinery with the microalgae constituents (lipids, carbohydrates and protein) being converted into green diesel, ethanol and animal feed or AD feedstock, respectively. In the latter, the whole microalgae biomass is converted to green crude via hydrothermal liquefaction. The high costs in Table 17.3 indicate the current state of the art, while the lower cost represents the proposed achievable

[1]The information is summarised in DOE (2014); however, process economics data are drawn from Davis et al. (2014) for the ALU process and Jones et al. (2014) for the AHLT process. Harvest and dewatering numbers were taken from further work conducted on the original harmonisation report (ANL et al. 2012).

future cost. The major savings are associated with greater yield (30 g/m^2/day), no plastic liners, dewatering costs halved and high efficiencies in all processing operations.

17.3.2 Techno-Economic Conclusions

Evaluating the available techno-economic studies for the production of biofuels from microalgae leads to the inventible conclusion that using existing technologies, the biofuel production is at least 2 but more likely 4–5 times more expensive than current fossil fuels and biofuels (final costs are dependent on the expected yields, growth system design and harvesting system employed). The major roadblocks to economic fuel production include:

1. **High yields**: Regardless of the system, it seems a minimum annual average productivity of 30 g/m^2/day is required with at least 30 % lipid content. Although highly possible, this has yet to be demonstrated and will require continued strain development either through traditional methods or genetic modification.
2. **Low Capital costs**: At low productivities (<25 g/m^2/day), growth system capital costs dominate and in the case of open ponds, liners are cost prohibitive. With this limitation, it is difficult to see how PBR's could ever be cost effective, as they typically do not increase aerial productivity rates (Benemann et al. 2012) relative to open ponds. Furthermore, capital and operating costs for dewatering must be lowered through the use of innovative concentrating steps (self- or electro-flocculation followed by settling or DAF). Using centrifuges for complete harvesting is not viable (Klein-Marcuschamer et al. 2013).
3. **Co-products**: In production systems that produce a protein for animal feed, selling prices will typically need to be in excess of $350/tonne to compete with on-site anaerobic digestion of wastes (ANL et al. 2012).

In addition to these challenges, the climate in most locations causes significant seasonal variation in microalgae productivity (5–10 times in the American context), resulting in underutilised capital for significant periods of the year. Furthermore, assumptions associated with low-cost water availability and CO_2 availability are very optimistic as very few sites around the world have these resources in combination with the correct climate.

With this in mind, two observations can be made as follows:

1. Biofuels from microalgae will be viable as long as significant (disruptive) improvements are made in the growth/harvesting stages.
2. There are a handful of sites around the world where climate, land availability, CO_2 and water availability align to transform this potential viability into commercial reality.

Considering the current pace of development and intense interest in this space, the former is likely to occur in the next 10–20 years. In the meantime, microalgae will continue to be exploited commercially for high-value products.

17.4 Energy

In addition to being economically viable, biofuels from microalgae must also meet life cycle targets to provide quantitative improvements to current fuels. The key elements typically considered in life cycle assessment of biofuels include:

- **Energy**—Usually the net energy ratio (NER), that is, does it require more energy to produce the fuel than is available in the fuel.
- **Greenhouse gas**—Are the net greenhouse gas emissions lower than fossil fuels or current biofuels.
- **Water use**—How many litres of water are consumed to produce a litre of biofuel.

The key motivation for asking these questions is whether or not the proposed process is sustainable. A range of LCA is reviewed in de Boer et al. (2012) with a focus on energy consumption. These are provided in Table 17.5.

The motivation for analysing energy is that the energetic viability is very closely linked with the economic viability. That is, it is almost impossible to have a process which is economically viable when the process uses more energy than it produces. The review of the LCA studies shown in Table 17.5 leads to the following conclusions:

- IF PBR's are used, then cultivation is typically the major energy user
- If raceway ponds are used, the major energy user is either dewatering, cell disruption or solvent extraction.

These conclusions suggest that an energetically viable process must use raceway ponds, process wet biomass (avoid drying), minimise energy required for cell disruption and minimise solvent recovery. Evaluation of different approaches by de Boer et al. (2012) indicated that hydrothermal liquefaction and wet processing methods with limited cell disruption were energetically feasible. This aligns with the processing focuses of the American laboratories as they process wet (fermentation and hydrothermal liquefaction) and do not use cell disruption.

As a final note, it is important to continually evaluate the key life cycle criteria (greenhouse gas emissions, net energy ratio and specific water consumption) in addition to the techno-economic analysis. This is simply because the lowest costs solution is not always the most sustainable.

Table 17.5 Major energy consumption components in life cycle analysis studies (de Boer et al. 2012)

Paper	Growth	Dewatering	Extraction	Conversion	Major energy consumption components
Batan et al. (2010)	PBR	Centrifugation	Solvent	Transesterification	PBR and solvent extraction
Brentner et al. (2011)	Flat plate PBR	Flocculation (Floc.)	Supercritical methanol transesterification of wet biomass		PBR
Lardon et al. (2009)	ORP	Floc., rotary press and drying	Solvent	Transesterification	Lipid extraction (90 % of energy dry, 70 % wet)
Razon and Tan (2011)	Flat plate PBR + ORP	Gravity and microfiltration	Bead mill and decanter	Transesterification	PBR and Bead mill
	ORP	Floc., thickener and drying (belt dryer)	Solvent	Transesterification	Drying
Sander and Murthy (2010)	PBR + ORP	Filter press or centrifuge and drying	Solvent extraction	Transesterification	Dewatering and drying
Xu et al. (2011)	ORP	Floc., centrifuge, mechanical dehydration	Cell disruption, drying solvent	Transesterification	Dewatering and drying
			Solvent (Bligh and dyer)	Hydrotreating	Solvent extraction
Stephenson et al. (2010)	PBR	Floc.	Homogenisation and solvent extraction	Transesterification	Cultivation in PBR
	ORP	Floc. and centrifugation	Homogenisation and solvent extraction	Transesterification	Cultivation

17.5 Conclusion

This work provides a review of the economic and energy attributes of microalgae biofuels. The clear outcome of this analysis is that the economics of microalgae biofuels need to improve substantially before they can compete with current fuels. In addition to this, the challenges faced in reaching the metrics are productivity and capital cost and there are other barriers, including:

- The instability of algae monocultures and exposure to pests and viruses.
- The limited number of sites with access to the optimum climate, available land, low-cost CO_2 source and abundant water.
- Competition from other options including biofuels from terrestrial crops, electric cars and unconventional fossil fuels.

Despite these substantial challenges, there is a very strong chance that microalgae will become a source of liquid fuels into the future; however, this will be at a time when oil prices are higher, further development has driven costs down to suitable levels and there is strong markets for co-products. That is, microalgae biofuels are likely to form part of a solution to liquid transport fuels rather than being the ultimate solution.

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Index

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