Sanjay Kumar Gupta · Anushree Malik
Faizal Bux *Editors*

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Preface

Algae are the first and most basic photosynthetic life-forms, and have more than 20 times higher growth rates than conventional crops. Microalgae, the simplest and tiniest form of plants, hold amazing potential for the sequestration of various nutrients from water to carbon dioxide from air. Algal biomass can be used for food, bioremediation, biofuels, and a number of speciality chemicals. These simplest of organisms hold great potential desperately required for sustainable and renewable management of food, fodder, and fuels, if managed in a scientific manner.

Over the past few decades, tremendous developments have been made in the field of algal technologies. It include enhancement of algae cultivation techniques for biomass production, innovations in harvesting techniques, and development of efficient techniques for conversion of biomass to various biofuels. Simultaneously, efforts have been made to take advantage of multiple avenues of exploitation such as remediation of contaminated water and sequestration of atmospheric CO_2 for the production of algal biomass for biofuels and other products. Laboratory-scale cultivation, biomass production, and subsequent biofuel conversion are promisingly impressive; however, the pilot-scale production of biofuels is still in its infancy for various reasons.

Recent advances in algal biotechnology, molecular techniques, and genetic, chemical, and mechanical engineering have changed the production landscape for algae from dismal to promising. Algal biofuels are a crucial part of the road map for strategic management of the future crisis of petroleum-based fuels, and could also be a sustainable solution for wastewater treatment and climate change abatement. Though algae will not be the answer that singularly resolves sustainability issues, algal biofuels are poised to provide a sustainable alternative which can meet increasing world demand for food and energy.

The book entitled *Algal Biofuels: Recent Advances and Future Prospects* focuses on the role and potentialities of algae in phycoremediation and biofuel production. This book is based on various scientific viewpoints and field experiences, and shares the fascinating compilation of extraordinary innovations

occurring in the field of algal biofuels. It is comprised of 21 chapters contributed by 70 authors from 9 countries, namely, India, China, the USA, Sweden, South Korea, Japan, Egypt, Oman, and South Africa. All the chapters were selected logically and arranged to provide comprehensive state-of-the-art information on practical aspects of cultivation, harvesting, biomass processing, and biofuel production from algae. Each chapter discusses topics with simplicity and clarity. All the chapters and their contents are supported by extensive citations of available literature, calculations, and assumptions based on realistic facts and figures on the current status of research and development in this field.

Chapter 1 provides detailed information about the state-of-the-art developments and the roles of biotechnological engineering in improving ecophysiology, biomass, and lipid yield of microalgae. Chapter 2 summarizes the screening of various micro and macroalgae species used for the production of biofuels. Chapter 3 deals with the applications, process constraints, and future needs for the development of various types of algal biofuels. Chapter 4 provides insights about algal biofilms. Chapter 5 discusses integrated approaches of environmental management by providing extensive information on how algal technologies can be used for wastewater treatment with the concomitant production of algal biomass for biofuels.

A synthetic ecological engineering approach towards sustainable production of biofuel feedstocks is discussed in Chap. 6. This chapter discusses the exploration of suitable algal consortia which can be used for the production of algal biomass for biofuels. The optimization of the algal biomass production is one of the vital aspects of algal biofuels. A specific topic on the modeling of the effects of operational parameters on algal growth is covered in Chap. 7. Recent developments for improving the ecophysiology of microalgae are discussed in detail in Chap. 8. Previous studies have demonstrated the unique potential of algae for the removal of several heavy metals from water and wastewater. Chapter 9 summarizes such aspects of numerous algal species.

Several microalgal species possess the unique potential of sequestration of various nutrients and chemicals. Such species are widely used nowadays for the treatment of wastewater. Therefore, wastewater treatment is coupled with algal biomass production, another significant aspect of algal technologies. Chapter 10 deals with the critical evaluation of algal biofuel production processes using wastewater.

Due to the very small size of microalgae, the harvesting of microalgal biomass is one of the largest techno-economical hurdles in the present scenario. Chapter 11 provides comprehensive information on various technologies used for the harvesting of microalgae, their pros and cons, as well as the recent advances that have been made in this field. Similarly, Chap. 12 provides overall insights into key issues related to pilot-scale production, harvesting, and processing of algal biomass for biofuels.

Together with the cultivation, production, and harvesting of microalgal biomass, another crucial aspect is pretreatment of algal biomass for the production of biofuels. Therefore, it would be an unjust to the readers of this book if a chapter dedicated to pretreatment had not been included. Chapter 13 summarizes

comprehensive information regarding various techniques used for the pretreatment of algal biomass during the production of biofuels.

Algal biomass can be used for the production of a range of biofuels, such as biomethane, biohydrogen, ethanol, bio-crude oil, syngas, and biodiesel. Chapters 14 to 19, deal with recent technological advancements, environmental and economic sustainability aspects of algal lipid extraction, and its conversion into biodiesel, biomethane, and biohydrogen or its hydrothermal liquefaction as promising pathways for bioenergy production. A chapter (20) dedicated to the challenges and opportunities in the commercialization of algal biofuels is also included. This chapter provides a realistic assessment of various techno-economical aspects of pilot-scale algal biofuel production and its potential for commercialization. The last chapter (21) deals with the life cycle assessment of algal biofuels.

In a summation, this edited volume provides a wealth of information based on realistic evaluations of contemporary developments in algal biofuel research with an emphasis on pilot-scale studies. Prospects for the commercialization of algal biofuels is another highlight of book.

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New Delhi, India — Sanjay Kumar Gupta

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1 Introduction

1.1 Sources of Biofuel

In the last few years, energy consumption has dramatically increased due to the ever increasing world population, and the global energy demand has been estimated to grow by >85% by 2040 (Parsaeimehr et al. 2015). The world's energy requirements are majorly satisfied by the fossil fuels that currently serve as the primary energy source. The depletion of the fossil fuels due to the exponentially increasing energy demand has alarmed the research communities to discover alternative energy sources. In view of the above, biofuels generated from renewable resources could be a more effective and sustainable option. Depending on the source, biofuels have been classified as first generation (produced from edible plant substrates such as oilseeds and grains), second generation (produced from nonedible plants or nonedible parts of the plant such as straw, wood, and biomass) and third generation (biofuels derived from algae) (Mohr and Raman 2013). First- and second-generation biofuels have commercial limitations as they require arable land and add to the food crisis faced by today's society. Third-generation biofuels have emerged as a viable option since they do not require arable land. Recently, fourth-generation biofuels have been characterized that use genetically modified organisms (particularly algae) to attain sustainable production of biofuels. Microalgal lipids have been recognized as a high-energy, low cost, and renewable feedstock for biodiesel production (Borowitzka and Moheimani 2013; Gupta et al. 2014; Guldhe et al. 2014; Ansari et al. 2015).

1.2 Advantages of Algae Biofuel

Algae fuel has been recognized by several energy experts to significantly decrease the dependency on fossil fuels and reduce the greenhouse gases (GHG) emissions. Microalgae are tiny autotrophs that are capable of growing in extreme conditions and produce substantial amount of lipids that can easily be converted into the biofuels by bio-/thermochemical methods. Particularly, the neutral lipids–triacylglycerides (TAGs) which serve as energy storage for microalgae are converted into biodiesel

through transesterification process (Chisti 2008; Chen 2011). Several advantages of algal biofuel have been identified by researchers. Demirbas and Demirbas (2011) reported that as per estimates, 20,000–80,000 L algae oil can be produced per acre which is 30 times higher than oil crops such as palm oil. Parker et al. (2008) suggested that the microalgae are responsible for the global carbon fixation (more than 40%) through the efficient utilization of carbon dioxide. Algae are able to thrive in nutrient-rich waste sources including animal wastes, domestic wastewaters (sewage), and some industrial effluents, which can be exploited to develop an integrated process for the treatment of waste sources with simultaneous production of biomass suitable for biofuels production (Abdel-Raouf et al. 2012). In addition, microalgae biomass can be used in aquaculture, as animal feed (Granados et al. 2012) and for extraction of high-value-added bio-products (Lacerda et al. 2011). Mata et al. (2010) and Cuellar-Bermudez et al. (2014) reported that the microalgal compounds including triglycerides, antioxidants, pigments, beta-carotene, polysaccharides, fatty acids, and vitamins are widely used in different industrial sectors (e.g., biofuels, functional foods, nutraceuticals, pharmaceuticals, cosmetics, aquaculture) as bulk commodities. Moreover, microalgae containing lipids and fatty acids, including omega (ω3 and ω6) families, have received attention due to the health benefits upon consumption (Spolaore et al. 2006). However, commercialization of microalgae-based processes is bound to certain limitations (Chisti 2013). Hannon et al. (2010) also revealed that there are number of challenges in the economic cultivation of algae to enhance the oil extraction and fuel process, so that it can compensate petroleum and consequently mitigate CO_2 release. Other major challenges include strain isolation and selection, resources (i.e., nutrient and water) and utilization, harvesting, fuel extraction, refining, utilization of residual algal biomass and production, and coproduct development and management.

2 Biology and Biochemical Composition of Microalgae

2.1 Major Biochemical Groups and Their Function

Determination of the biochemical composition of microalgae biomass provides an insight of the organisms behavior and its adaptational response to changes in its environment (Chen and Vaidyanathan 2013). Especially, microalgae ecophysiology is very essential to understand and optimize the large-scale biomass production for biofuel generation (Chia et al. 2013). The biochemical components in microalgae primarily include proteins, carbohydrates, fats, and nucleic acids. The quantity of the components varies with the type of species (Table 1) and is significantly influenced by the environmental conditions including light intensity, temperature, pH, and nutrients availability. The values of the components range as follows: proteins (10–50%), carbohydrates (10–40%), and lipids (20–80%). Gatenby et al. (1997) reported that the biochemical composition variation, due to growth stage, can be related to the age

Table 1 Main biomass composition of microalgae expressed on a dry matter basis (Biller and Ross 2014; Priyadarshani and Rath 2012)

Strain	Protein	Carbohydrates	Lipids
Scenedesmus obliquus	11.1	40.7	25.7
Prymnesium parvum	28–45	25–33	22–38
Scenedesmus dimorphus	8–18	21–52	16–40
Pseudochoricystis ellipsoidea	27.5	19.3	45.4
Chlorella vulgaris	10.4	12.7	58.0
Chlorella vulgaris minutissima	10.3	13.9	56.7
Chlorella zofingiensis	11.2	11.5	56.7
Chlamydomonas rheinhardii	48	17	21
Botryococcus braunii	40	2	33
Spriogyra sp.	6–20	33–64	11–21
Chlorella FC2 IITG	10.4	24.5	37.3

of culture and nutrient depletion, particularly if an organism grows in batch culture. Proteins make up a large fraction (sometimes even more than carbohydrates and lipids) of the actively growing microalgae having both structural and metabolic functions. It is also involved in the photosynthesis apparatus, CO_2 fixation, and cell growth machinery. Several algae with high-protein fraction are an ideal source of nutrients for production of functional foods, food additives, and nutraceuticals that have been commercialized in the food and feed markets. Recently, certain amino acid fractions from the algal proteins have been identified as a suitable feedstock for production of higher alcohols (Lan and Liao 2013; Eldalatony et al. 2016). Carbohydrates are the significant products derived from photosynthetic process and the carbon fixation metabolism (Ho et al. 2011). *Chlorella*, *Dunaliella*, *Scenedesmus*, and *Chlamydomonas* have been reported more than 50% of starch accumulated based on their dry cell weight (Ueda et al. 1996). The carbohydrates in green algae mainly include starch (storage component) in chloroplasts and cellulose/polysaccharides (structural components) in the cell walls. Both polysaccharides and starch can be converted into sugars for the consequent bioethanol production through microbial fermentation (Wang et al. 2011; Choi et al. 2011a, b; Jeon et al. 2013). Microalgae lipids can be divided into two categories: (a) the storage lipids (neutral or nonpolar lipids) and (b) structural lipids (membrane or polar lipids). Storage lipids mostly include TAGs which are predominantly saturated fatty acids and some unsaturated fatty acids that can be converted to biodiesel by transesterification, while structural lipids contain maximum content of polyunsaturated fatty acids. These PUFAs are essential for the nutrition of humans and aquatic animals. Sterols and polar lipids are the key structural components of cell membranes, providing the matrix for different metabolic processes. It also acts as key intermediates in cell signaling pathways.

Schorken and Kempers (2009) reported that the different types of fatty acid from triacylglycerols are the main targets for the development of biotechnological products. Glycolipids, phospholipids, sphingolipids, carotenoids, sterols, and other lipid-soluble compounds from algae are being utilized for the production of bioactive molecules for

nutrition, cosmetics, and pharmaceuticals. Kumar et al. (2015) suggested that microalgae biomass with appropriate proportions of unsaturated fatty acids (linoleic (18:2), palmitoleic (16:1), oleic (18:1), and linolenic acid (18:3)) and saturated (stearic (18:0) and palmitic (16:0)) fatty acids could be a suitable biodiesel feedstock.

2.2 Sustainable Energy Sources of Microalgae

The second-generation biomass resources (food crops) are not an appropriate option for biofuel generation due to their inefficiency and unsustainability. On the other hand, microalgae are the most sustainable source of biofuel and positive toward food security and their environmental impact, which encouraged researchers to develop technologies related to microalgal biomass production (Ahmad et al. 2011). Lim et al. (2012) suggested that the microalgal sources are believed to be a sustainable option in biofuel production and the ability of meeting the global demand for sustainable transport fuels. Waltz (2009) also suggested energy density, which can be harvested from microalgae, and it is higher than that of the chief oil-producing crops. Moreover, it does not hold a competing tap into the global food supply chain, and this technology makes it economically viable and feasible for large-scale cultivation and harvesting purposes.

3 Lipid Biochemistry in Microalgae

Microalgae have been characterized as oleaginous, as they are capable of accumulating appreciable quantity of lipids. The algal lipid content is considerably influenced due to environmental conditions of the habitat and has been observed to range between 5 and 70% (Table 2). Membrane bilayer constitutes the major fraction of algal lipids, other than triacylglycerol (TAG), hydrocarbons, wax esters, sterols, and prenyl derivatives (Yu et al. 2011). Recent reviews have well documented the biochemistry of lipid synthesis in algae (De Bhowmick et al. 2015; Bellou et al. 2014) and have been presented in Fig. 1. Photosynthesis converts CO_2 to glycerate-3-phosphate (G3P) that is a starting material of storage compounds including lipids and carbohydrates. Lipid biosynthetic pathway initiates by transformation of G3P to pyruvate and subsequently to acetyl-CoA in the plastid. The pathway that converts polysaccharides to lipids also yields Acetyl-CoA (Bellou et al. 2012), which is usually used for sugar assimilation by the oleaginous heterotrophs (Bellou et al. 2014). The storage polysaccharides are fragmented via glycolysis in the cytosol and further via citric acid cycle in the mitochondrion. Environmental stress conditions, however, interfere with the citric acid cycle and lead to citrate accumulation in the mitochondrion followed by its transfer in the cytosol. Further, the citrate is sequentially converted to oxaloacetate and acetyl-CoA by cytosolic ATP-dependent citrate lyase. Cytosolic acetyl-CoA carboxylase catalyzes the conversion of acetyl-CoA to malonyl-CoA that is

Table 2 Lipid content and productivity range of different microalga species (Adopted from Malcata 2011)

Microalga species	Lipid content (%, w/wDW)	Lipid productivity (mg $L.^{-1}d^{-1}$)	Natural habitat
Botryococcus spp.	25.0–75.0	–	Freshwater
Chaetoceros calcitrans	14.6–39.8	17.6	Freshwater
Chaetoceros muelleri	33.6	21.8	
Chlorella emersonii	25.0–63.0	10.3–50.0	Freshwater
Chlorella protothecoides	14.6–57.8	1214	
Chlorella sorokiniana	19.0–22.0	44.7	
Chlorella vulgaris	5.0–58.0	11.2–40.0	
Chlorella spp.	10.0–57.0	18.7–42.1	
Chlorococcum spp.	19.3	53.7	Freshwater
Dunaliella primolecta	23.1	–	Freshwater
Dunaliella salina	6.0–25.0	116.0	
Dunaliella tertiolecta	16.7–71.0	–	
Dunaliella spp.	17.5–67.0	33.5	
Ellipsoidion spp.	27.4	47.3	Freshwater
Haematococcus pluvialis	25.0	–	Freshwater
Isochrysis galbana	7.0–40.0	–	Seawater
Isochrysis spp.	7.1–33.0	37.8	
Nannochloris spp.	20.0–56.0	60.9–76.5	Seawater
Nannochloropsis oculata	22.7–29.7	84.0–142.0	Seawater
Nannochloropsis spp.	12.0–53.0	60.9–76.5	
Neochloris oleoabundans	29.0–65.0	90.0–134.0	Seawater
Pavlova salina	30.9	49.4	Seawater
Pavlova lutheri	35.5	40.2	
Phaeodactylum tricornutum	18.0–57.0	44.8	Seawater
Scenedesmus obliquus	11.0–55.0	–	Freshwater
Scenedesmus spp.	19.6–21.1	40.8–53.9	

utilized for elongation of fatty acids in the endoplasmic reticulum (ER) membrane (Mühlroth et al. 2013). The aforementioned mechanism was specifically demonstrated in *Nannochloropsis salina* and *Chlorella* sp. (Bellou et al. 2012), but it is perhaps similar in oleaginous strains that can thrive under heterotrophic conditions.

In the plastid, malonyl-CoA:ACP transacetylase facilitates the transfer of malonyl-CoA to the acyl-carrier protein (ACP) of the fatty acid synthase (FAS) complex (Blatti et al. 2012). The 3-ketoacyl-ACP synthase catalyzes the generation of ketobutyryl-ACP by condensing the acetyl group with malonyl-ACP. Further, consecutive reduction–dehydration–reduction reactions converts the ketobutyryl-ACP to butyryl-ACP, and such repeated cycles lead to formation of palmitoyl-ACP. Addition of two carbon skeleton from acetyl-CoA leads to formation of stearoyl-ACP. Desaturation of stearoyl-ACP leads to formation of oleoyl-ACP (Yu et al. 2011; Mühlroth et al. 2013). The fatty acids bound to ACP are released and further activated into acyl-CoA by acyl-CoA synthetase situated in the chloroplast

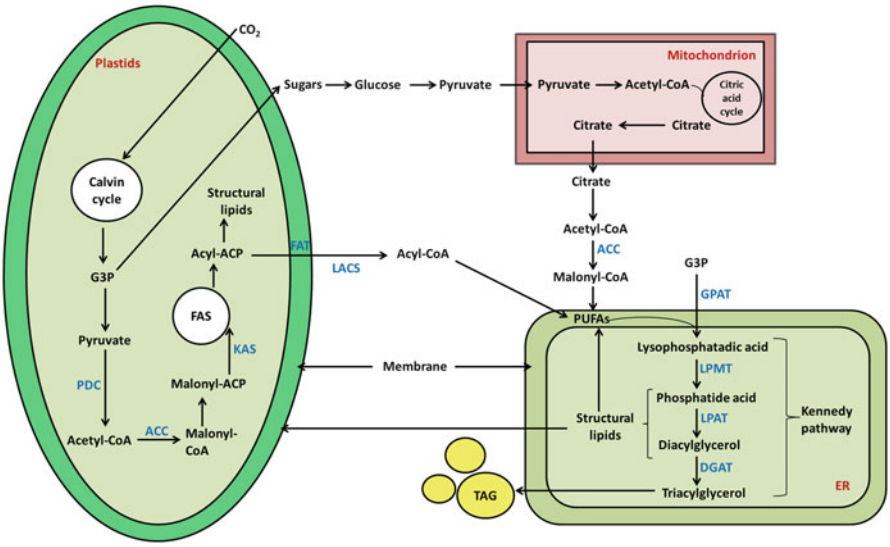

Fig. 1 Outline of lipid synthesis in microalgae (Adopted from Bellou et al. 2014). Abbreviations: *ACC* acetyl-CoA carboxylase, *ACP* acyl-carrier protein, *LACS* long-chain acyl-CoA synthetase, *ATP:CL* ATP-dependent citrate lyase, *CoA* coenzyme A, *DGAT* diacylglycerol acyltransferase, *ER* endoplasmic reticulum, *FAS* fatty acid synthase, *FAT* fatty acyl-ACP thioesterase, *G3P* glycerate-3-phosphate, *GPAT* glycerol-3-phosphate acyltransferase, *KAS* 3-ketoacyl-ACP synthase, *LPAAT* lysophosphatidic acid acyltransferase, *LPCAT* lysophosphatidylcholine acyltransferase, *PDC* pyruvate dehydrogenase complex, *TAG* triacylglycerol

envelope and eventually shifted to the cytosol for lipid synthesis. Polyunsaturated fatty acids (PUFAs) are obtained by esterification of the acyl-CoA chains with the structural phospholipids of the ER. Fatty acids are utilized as precursors for generation of TAG in the ER through Kennedy pathway.

The Kennedy pathway includes acylation of G3P by glycerol-3-phosphate acyltransferases followed by lysophosphatidic acid acylation by lysophosphatidate acyltranferase to generate phosphatidic acid (PA). Dephosphorylation of PA leads to generation of diacylglycerol (DAG), which is the primary starting material for synthesis of membrane and storage lipids (TAG) occurring in the chloroplast (Mühlroth et al. 2013). The synthesized TAGs are later deposited in the form of lipid droplets in the cytosol (Martin and Parton 2006). Lipid droplets promote the distribution and recirculation of neutral lipids, phospholipids, lysophospholipids, and acyl groups (De Bhowmick et al. 2015). The disclosure and understanding of lipid synthesis mechanism in algae has opened a path for the metabolic engineering to obtain highly potent strains for biodiesel production, which has been discussed in later section.

4 Recent Common Approaches for Enhanced Lipid Production

Commercialization of algae oil-derived biodiesel requires high lipid productivity of the desired and rapidly growing algae. Optimal growth conditions lead to production of huge amounts of algal biomass but with comparatively low lipid contents. Enhancement of microalgal lipids could improve the economics of biodiesel, and considering this fact, lot of efforts have been focused in developing the strategies in order to improve the biomass and lipid contents (Fig. 2). Microalgae biomass and triacylglycerols (TAGs) compete for the photosynthetic assimilate, and modulation of biochemical pathways is needed to improve lipid biosynthesis. Unfavorable growth conditions such as light, temperature, nutrients (nitrogen and phosphorus) limitation, salinity, and heavy metals modulate the lipid biosynthetic pathways in many microalgae species, leading to the generation and accumulation of neutral lipids (20–50% DCW), majorly as TAG, supporting the microalgae to survive under such adverse conditions (Fig. 3). Such ability of algae to overcome unfavorable environmental conditions by modulating their metabolic pathways has been exploited by researchers to obtain high lipid-accumulating strains for biodiesel production.

4.1 Nutrient Limitation

Microalgae growth and lipid composition are significantly affected by the nutrients availability. Under nutrients limitation conditions, the cell division rate gets declined, but active fatty acid biosynthesis is maintained in certain species of algae under sufficient light and CO_2 availability for photosynthesis (Thompson

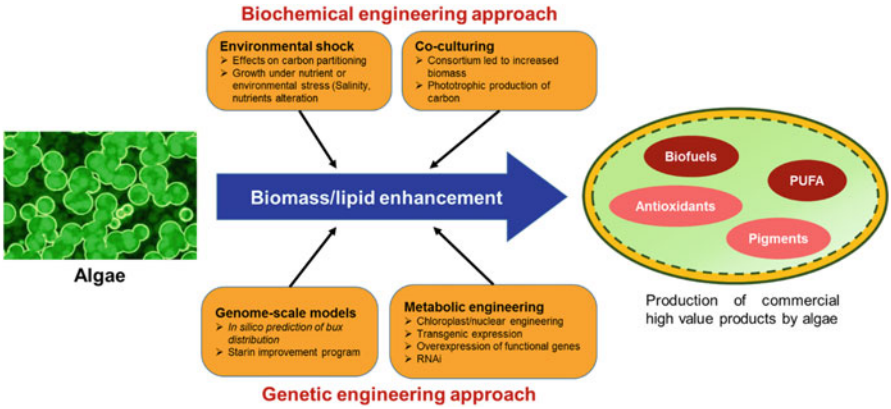

Fig. 2 Various strategic options available to enhance lipid production in algae

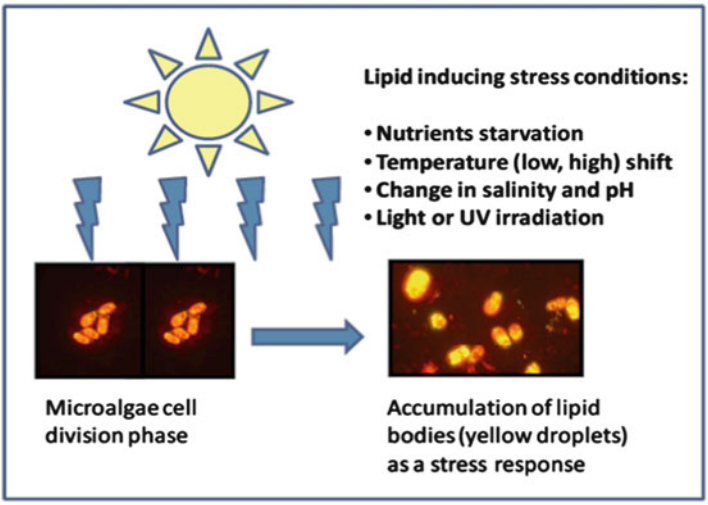

Fig. 3 Induction of algae lipids under unfavorable stress conditions (Adopted from Sharma et al. 2012)

1996). As algal growth declines, synthesis of new membrane compounds is not required, diverting fatty acids into TAG that serve as a defensive mechanism during stress conditions. The ATP and NADPH obtained from photosynthetic reactions are utilized for generation of biomass, regenerating ADP and $NADP^+$ as acceptor molecules for continued photosynthesis under favorable growth conditions. Nutrient limitation conditions lead to depletion of $NADP^+$ pool for photosynthesis due to reduced cell growth and proliferation. Under such conditions, NADPH is consumed in fatty acid biosynthesis, thus regenerating the pool of $NADP^+$ and protecting the cells by continuation of photosynthesis under light conditions (Hu et al. 2008).

Nitrogen is a crucial macronutrient for microalgae, as it influences the growth and lipid metabolism, and is a crucial constituent of the cell organization (Sharma et al. 2012). Nitrogen accounts for 1% to more than 10% of biomass (Costa et al. 2001) and can be used as NO_3^-, NO_2^-, or NH_4^+ and also as N_2. Nitrogen limitation leads to accumulation of lipids in different microalgae species (Table 3). It decreases the cellular proportion of thylakoid membrane, activates acyl hydrolase, and stimulates phospholipid hydrolysis, which together increases the intracellular fraction of fatty acid acyl-CoA. Nitrogen limitation also activates diacylglycerol acyltransferase, which further catalyzes the conversion of the accumulated acyl-CoA to TAG (Xin et al. 2010).

Under favorable conditions, a small amount of TAG is biosynthesized, and carbon can be fixed not only by photosynthesis but also from acetate (Deng et al. 2011) (Fig. 4a). Nitrogen and sulfur are crucial for protein synthesis, and their insufficiency leads to inhibition of the citric acid cycle and photosynthesis due to inadequacy of the proteins that constitute the photosystem reaction center and photosynthetic electron transport. This leads to reduction in photosynthesis and

Table 3 Induction of microalgae lipids under different nutrient starvation stress conditions

Microalgae species/strains	Nutrient stress	Alteration in lipid profile	Reference
Chlorella vulgaris	Nitrogen limitation	Total lipid content was enhanced by 16.41% and TAG accumulation was increased	Converti et al. (2009)
Nannochloropsis oculata	Nitrogen limitation	Total lipid was enhanced by 15.31%	Widjaja et al. (2009)
Phaeodactylum tricornutum	Nitrogen limitation	TAG levels were enhanced from 69 to 75%	Alonso et al. (2000)
Scenedesmus subspicatus	Nitrogen limitation	Increase in total lipids	Dean et al. (2010)
Nannochloropsis salina	Nitrogen limitation	Increase in lipid and TAG contents up to 56.1 and 15.1% of dry weight, respectively	Fakhry and Maghraby (2015)
Chlamydomonas reinhardtii	Nitrogen limitation	Enhanced lipid accumulation	Bono et al. (2013)
Nannochloropsis oculata	Nitrogen limitation	Increase of lipid production and productivity up to 49.7% of dry weight and 41.5 mg $L.^{-1}d^{-1}$	Millan-Oropeza et al. (2015)
Chlorella zofingiensis	Nitrogen limitation	Increase of lipid production and productivity up to 54.5% of dry weight and 22.3 mg $L.^{-1}d^{-1}$	Feng et al. (2011)
Chlorella sp.	Phosphorus limitation	Enhanced lipid accumulation	Liang et al. (2013)
Phaeodactylum tricornutum	Phosphorus limitation	Total lipid content was increased with relatively higher fraction of 16:0 and 18:1 fatty acids	Reitan et al. (1994)
Monodus subterraneus	Phosphorus limitation	Increase in TAG levels	Khozin-Goldberg and Cohen (2006)
Scenedesmus sp.	Phosphorus starvation	Total lipid content increased up to 53%	Xin et al. (2010)
Chlamydomonas reinhardtii	Sulfur limitation	Increase in TAG levels	Matthew et al. (2009)
Chlorella sp., *Parachlorella* sp.	Sulfur limitation	Higher accumulation of lipids	Mizuno et al. (2013)
Cyclotella cryptica	Silicon starvation	Total lipid content was increased from 27.6 to 54.1%	Roessler (1988)

induction of acetate assimilation. Numerous intermediate metabolites formed during the acetate assimilation are pooled toward Kennedy pathway for generation of TAGs (Fig. 4b).

Phosphorus significantly influences the energy transfer and signal transduction mediating cellular metabolic processes, photosynthesis, and respiration. Phosphorus limitation causes defect in cell division, leading to halt of cell growth. The absence of phosphorus also impairs phospholipids synthesis, which promotes the

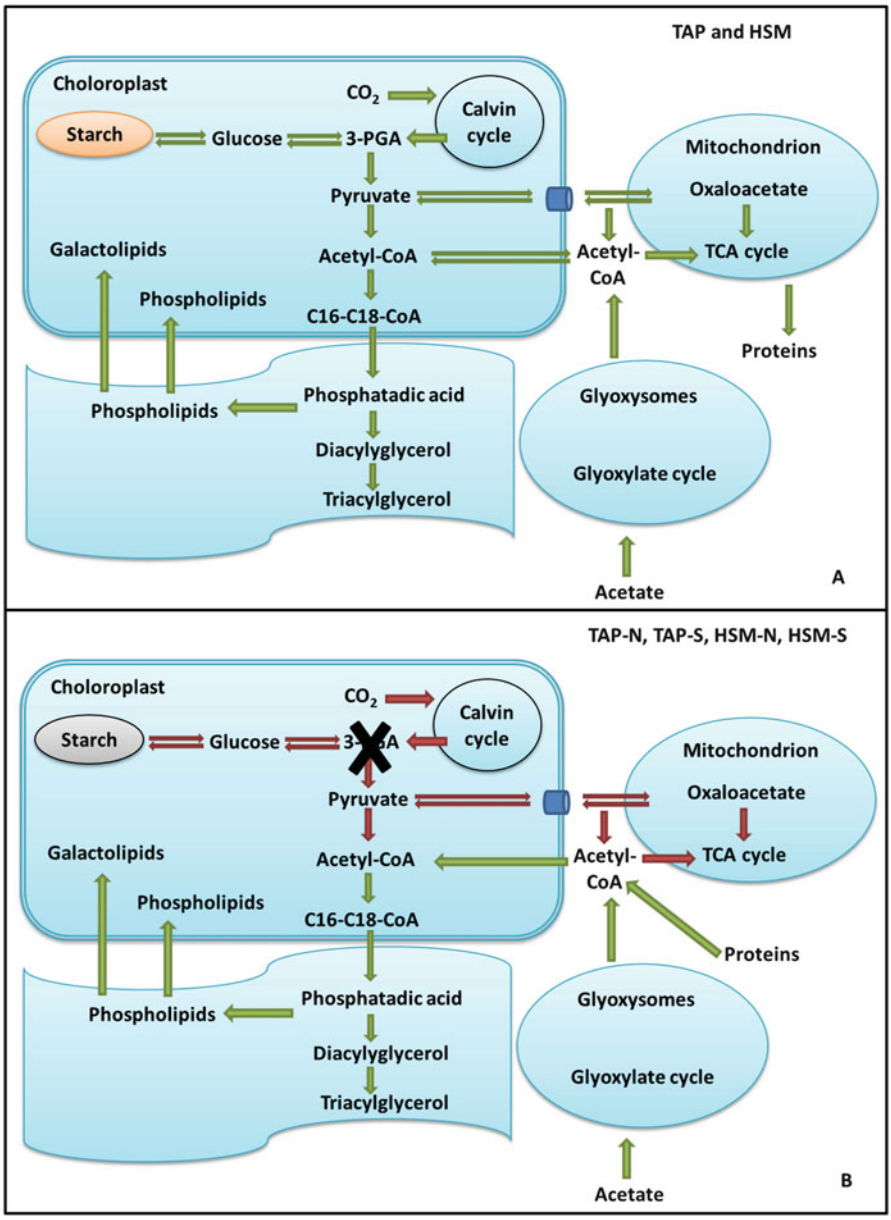

Fig. 4 Synthesis of TAG using (**A**) photosynthesis and acetate assimilation intermediates under nitrogen and sulfur sufficiency, and (**B**) acetate assimilation intermediates under nitrogen and sulfur deficiency (Adapted from Deng et al. 2011)

synthesis of TAGs (Deng et al. 2011). Enhanced accumulation of lipids under phosphorus limitation has been reported in different microalgae species (Table 3). In addition, deprivation of silicon also leads to accumulation of lipids in several algal strains (Table 3).

4.2 Light Irradiation and Temperature Stress

Light is an indispensable factor for the survival and growth of autotrophic organisms. Light intensity affects the algae growth by influencing photosynthesis (Stockenreiter et al. 2013). Algae can grow under varying light intensities and show notable alteration in their gross chemical composition and photosynthetic activity (Hu et al. 2008). The algal growth rate is highest at saturation intensity, and it declines with a shift of light intensity from the saturation (Sorokin and Krauss 1958). The photoadaptation process leads to alteration of the algal cell properties depending on the light intensity, which includes alteration in profiles of pigments, growth rate, and the availability of essential fatty acids (Juneja et al. 2013). The lipid metabolism is modulated under influence of different light intensities leading to alteration of the lipid profile (Table 4). Lower light intensities induce the generation of chloroplast bound membrane polar lipids, while under higher light intensities, the total content of polar lipids gets decreased with a concurrent enhancement in neutral lipids, primarily TAGs (Sharma et al. 2012).

The light cycles and the incident light spectral composition also affect the growth of algae. Light/dark cycles at distinct growth phases significantly alter the algal lipid composition (Table 4). Specific components (wavelengths) of light influence the cellular processes such as chlorophyll synthesis and cell division. The algal growth rate and composition of biochemical contents are also influenced by the wavelength of illuminating light. Specifically, the effect of blue light (400–480 nm), red light (620–750 nm), and UV radiations on the microalgae growth and lipid content has been reported (Table 4).

Algae have the capability to survive and grow under varied temperature (15 to 40 °C), and it is one of the crucial environmental factors that affect the growth rate and composition of biochemical contents in algae. Temperature significantly influences the fatty acid composition of algae with an alteration of fatty acid saturation (membrane lipids) to adapt against the changing environment due to a temperature shift (Table 4). In most microalgae species, fatty acid unsaturation increases with decreasing temperature, while fatty acid saturation increases with increasing temperature.

4.3 Salinity-, pH-, and Metal-Induced Stress

Salinity (salt concentration) is another important parameter that influences algal growth. Algae show different growth rate and biochemical composition under the

Table 4 Induction of lipids under different light irradiation and temperature stresses

Microalgae species/strains	Stress	Alteration in lipid profile	Reference
Scenedesmus sp. 11–1	High light intensity	Lipid content of 41.1% and neutral lipid of 32.9% were achieved	Liu et al. (2012)
Tichocarpus crinitus	Low light intensity	Increased levels of TAG	Khotimchenko and Yakovleva (2005)
Chlorella sp. and *Monoraphidium* sp.	High light intensity	Three times more neutral lipids than under low light intensity	He et al. (2015)
Pavlova lutheri	High light intensities	Increased total lipid content	Carvalho and Malcata (2005)
Selenastrum capricornutum	Dark treatment	Increase in linoleate fatty acid	McLarnon-Riches et al. (1998)
Isochrysis galbana	Shorter light period	Increase in PUFA	Bandarra et al. (2003)
Dunaliella viridis	No light	Increase in total lipid content	Gordillo et al. (1998)
Phaeocystis antarctica	Low UV-B	Increase in PUFA	Jiang and Chen (1999)
Chaetoceros simplex	High UV-B	Increase in total lipids	Jiang and Chen (1999)
Phaeodactylum tricornutum	UV radiation	Increased PUFA	Liang et al. (2006)
Chlorella vulgaris	Blue light	Increase in lipid fraction	Miyachi and Kamiya (1978)
Micractinium pusillum, *Ourococcus multisporus*	Red light	Enhanced lipid content and lipid productivity	Kumar et al. (2014)
Botryococcus braunii	Red light	Increased lipid production	Baba et al. (2012)
Chlorella ellipsoidea	Lowering temperature	Unsaturated FA content was enhanced by 2-fold	Joh et al. (1993)
Dunaliella salina	Shift from 30°C to 12°C	Increase in unsaturated lipids	Thompson (1996)
Spirulina platensis, *Chlorella vulgaris*, *Botryococcus braunii*	Increase in temperature	Level of saturated FAs was increased	Sushchik et al. (2003)
Monoraphidium sp. SB2	Grown at 30 °C	Lipid content was increased	Wu et al. (2013)

presence of salt concentration other than their natural/adapted concentration in the growth medium (Table 5). Gradual rise in initial NaCl concentration from 0.5 M to 2.0 M during cultivation of *Dunaliella tertiolecta* enhanced the lipid content (intracellular) and level of TAG (Takagi 2006). In another study, increase in saturated and

Table 5 Induction of lipids under different salinity, pH, and metal stress

Microalgae species/strains	Stress	Alteration in lipid profile	Reference
Dunaliella	Increase of NaCl from 0.5 to 1 M	Increased intracellular lipid content (67%)	Takagi (2006)
Nannochloropsis salina	Salinity of 34 PSU	Lipid content increased up to of 36% dry tissue mass	Bartley et al. (2013)
Chlamydomonas mexicana	Salinity of 25 mM	Total lipid content was increased up to 36%	Salama et al. (2014a)
Scenedesmus obliquus	Salinity of 17.5 mM	Total lipid content was increased up to 36%	Kaewkannetra et al. (2012)
Unidentified *Chlamydomonas* sp.	Low pH	Increase in saturated FAs	Tatsuzawa et al. (1996)
Chlorella sp.	Alkaline pH	Increase in TAG	Guckert and Cooksey (1990)
Chlorella	pH 5.0	Enhanced TAG accumulation	Zhang et al. (2014)
Chlorella minutissima	Cd or Cu	Increased lipid content and lipid productivity	Yang et al. (2015)
Nannochloropsis sp.	As(III)	Increased lipid content and fatty acid saturation	Sun et al. (2015)
Chlorella vulgaris	Fe^{3+}	Increase in total lipids to 56.6% of biomass	Liu et al. (2008)
Chlorella vulgaris	TiO_2	Increased production of fatty acids	Kang et al. (2014)

monounsaturated fatty acids of *Dunaliella* was observed in response to increase in NaCl concentration from 0.4 to 4 M (Xu and Beardall 1997). The growth rate and lipid content of freshwater alga *Botryococcus braunii* was increased with increasing NaCl levels in the culture medium (Ben-Amotz et al. 1985).

Medium pH significantly influences algal growth because it regulates the solubility and availability of nutrients and CO_2 (Juneja et al. 2013). Alteration in lipid composition of microalgae has been reported with fluctuations of the medium pH (Table 5). Incrementally adjusted pH during the growth promoted accumulation of lipids compared to constant pH in five species of *Chlorellaceae* (Skrupski et al. 2014). *Chlorella* cultivated under alkaline pH stress conditions showed an increased TAG level with a decrease of membrane lipids (Guckert and Cooksey 1990). In another study, the TAG content was enhanced to 63% in *Chlorella* at initial pH of 5.0 (Zhang et al. 2014).

Metal ions also enhance the lipid content in several microalgae species (Table 5). Exposure of *Euglena gracilis* to low chromium (Cr^{6+}) concentration increased the lipid content under photoautotrophic or mixotrophic growth conditions (Rocchetta et al. 2006). Zerovalent iron nanoparticles increased the lipid productivity of *Arthrospira maxima* and *Parachlorella kessleri* by 40 and 66%, respectively (Padrova et al. 2015). Fatty acid saturation was increased in *Dunaliella salina* and

Nannochloropsis salina cells by nickel (Mohammady and Fathy 2007). Arsenic [As (III)] exposure enhanced the cell lipid content in *Nannochloropsis* sp. with a decreased fraction of polyunsaturated fatty acids and increased fractions of short-chain saturated (C16:0, C18:0) and monounsaturated (C16:1, C18:1) fatty acids (Sun et al. 2015). Thus, metal stress can be used to modulate the fatty acid profile of microalgae and obtain biodiesel with desired properties (Miazek et al. 2015).

4.4 Supplementation of CO_2 and Phytohormones

Carbon constitutes around 50% of microalgae biomass on a dry weight basis, which majorly comes from the photosynthetically fixed carbon dioxide. Photosynthesis includes light and dark reactions, with dark reaction as one of the rate-limiting steps because of the insufficient availability of CO_2. Carbon fixation in microalgae is initiated by sequestration of CO_2 into Calvin cycle, and low concentrations of CO_2 in air become a key limiting factor. Thus, external supply of CO_2 can overcome substrate limitation and enhance the photosynthetic efficiency, subsequently improving the biomass and constitutes including carbohydrates and lipids (Sun et al. 2016). Supplementation of 15% CO_2 increased the biomass concentration and total lipid content of *Nannochloropsis* sp. from 0.71 to 2.23 g L^{-1} and 33.8–59.9%, respectively (Jiang et al. 2011). The highest specific lipid productivity of 0.164 g-lipids g-cell^{-1} day^{-1}and oleic acid content of 44% was obtained in *C. vulgaris* with 15% CO_2 after 7 days of cultivation (Ji et al. 2013). The oleic and linoleic fatty acid levels were increased in *Scenedesmus* sp. and *Chlorococcum* sp. on supplementation of 5% CO_2 (Prabakaran and Ravindran 2013).

Plant hormones (phytohormones) increase the microalgae growth by modulating the intrinsic biochemical pathways (Hunt et al. 2011). Phytohormones are chemical messengers which regulate the plant growth and developmental processes. Phytohormones, including auxins, brassinosteroids, cytokinins, jasmonides, gibberellins, ethylene, abscisic acid, polyamines, , salicylates, and signal peptides, have been identified in various algae species (Tarakhovskaya et al. 2007; Raposo and Morais 2013). Supplementation of phytohormones for enhanced microalgae biomass and metabolite production has been extensively studied (Hunt et al. 2011; Bajguz and Piotrowska-Niczyporuk 2013; Tate et al. 2013; Raposo and Morais 2013; Czerpak and Bajguz 1997). A newly discovered phytohormones diethyl aminoethyl hexanoate enhanced the growth by 2.5-fold and the total fatty acid content up to 100 mg g^{-1} DCW with *Scenedesmus obliquus* (Salama et al. 2014b). The cell number of *Chlorella vulgaris* was increased with supplementation of indole-3-acetic acid (IAA) at 0.1 μM by 53%, indole-3-n-butyric (IBA) at 0.1 μM by 46%, phenylacetic acid (PAA) at 1 μM by 34%, and naphthyl-3-acetic acid (NAA) at 1 μM by 24% compared to control after 48 h of cultivation (Piotrowska-Niczyporuk and Bajguz 2014). The levels of photosynthetic pigments, soluble proteins, and monosaccharides were also enhanced at the respective phytohormones concentrations. The biomass production of *Chlamydomonas reinhardtii* was enhanced

between 61 and 69% with supplementation of IAA, gibberellic acid (GA3), and kinetin (KIN) (Park et al. 2013).

Strategies involving nutrient deprivation, salinity, light, temperature, CO_2, and phytohormones have been extensively utilized for enhancing the lipid content of microalgae, but such approaches have limitations to increase the feasibility of the overall process. However, the knowledge of the biochemical mechanisms and the molecular insights for lipid accumulation influenced by such stress environments in microalgae cells could be useful in inventing new strains, improving known strains and methods for greater lipid productivities. Thus, metabolic engineering approach has been recently initiated to develop highly efficient and potent strains to enable the algae-based biodiesel production feasible.

5 Molecular and Genetic Engineering Tools for the Improvement in Microalgal Lipids

A significant improvement in the strategies to improve the microalga biomass is needed in order to achieve a good quality biodiesel. The use of genetic and metabolic engineering approach to develop microalgal strains with high lipid-accumulating capability is a good approach for strain improvement (Larkum et al. 2012; Singh et al. 2016). The key genes coded for lipid synthesis pathways have been recently identified, and full genome of several microalgal strains have been deciphered (Tabatabaei et al. 2011). Environmental risk assessment and long-term viability of genetically engineered microalgal strains in open ponds are the major challenges.

5.1 Strain Improvement Using Mutagenesis Approach

Specific algal strains are utilized for a particular purpose. For example, fast-growing strains are used for biomass production, whereas other strains are utilized for biomass production of astaxanthin, eicosapentaenoic acid (EPA), and oils (Pulz and Gross 2004; Trentacoste et al. 2013). UV irradiation, reactive oxygen species, and changes in genetic material result in transformation of wild-type strain into mutants, which causes genetic variability with potential for evolution (Hlavova et al. 2015; Eyre-Walker and Keightley 2007). Different types of mutagens can be used to generate the mutants. The mutation frequency depends upon the intensity of mutagenic compounds used for mutations. Mutagenic effect is very specific; hence, to maximize and cover the mutation in an entire genome, several thousands of independent mutants should be produced. A desired phenotype can be selected from this mutant population. Although mutant population generation is a simple task, the selection of mutants with desired phenotypes through mutational screening is

Table 6 Different mutagens, their mode of action, and mutations caused (Adopted from Hlavova et al. 2015)

Mutagen	Mode of action	Most common mutation caused
EMS, MNNG	Alkylation of DNA base, particularly guanine	Point mutations
UV irradiation	Photochemical reaction leading to cyclobutane ring	Point mutations, deletions
Gamma irradiation	Ionization leading to double-stranded break	Deletions
Heavy ion beams	Ionization leading to double-stranded break	Chromosome breaks and exchanges
T-DNA, antibiotics resistance gene	DNA fragment insertion	Insertions, deletions

extremely challenging, creating a major hurdle of any mutagenic screen (Hlavova et al. 2015). Specific screening protocols to screen the desired phenotypes based on the mutant properties such as improved growth, increased cell size, improved productivity of a biochemical content, or resistance to different compounds.

5.1.1 Available Chemical and Physical Treatment Methods for Mutagenesis

Chemical and physical treatments to generate mutations are most favorite options among the researchers due to simplicity in their application, and their mutagenic capabilities are well described (Table 6). Alkylating agents, for example, methylnitronitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS), are most extensively used chemical mutagens for algal cells. For the first time, Chaturvedi and Fujita (2006) utilized these agents in mutagenic screenings in order to increase EPA production in *Nannochloropsis oculata* and to enhance growth of *Chlorella* species (Ong et al. 2010). Irradiations (UV, gamma rays, and heavy ion beams) are used as typical physical mutagens. Mutagenesis by UV is very easy to perform and do not require specialized equipment or chemicals. The mutagenic potential and mode of action of each type of radiation on cells depend on its energy. The simplicity and potential makes this method very popular, both in basic research with certain specifications and in applied science to generate engineered strains, which can synthesize higher amount of oils (Neupert et al. 2009; de Jaeger et al. 2014; Vigeolas et al. 2012). Improved production of astaxanthin using gamma irradiation is also evident (Najafi et al. 2011). Despite their tremendous capability, irradiation techniques are not very commonly used because it requires specialized equipment making these procedures highly expensive.

Point mutations can be used to isolation of essential gene mutants through alter the activity of gene product without its inactivation. Extra precautions are needed to

ensure the survival of desired gene mutants. Conditional mutants show the phenotype under specific and restrictive conditions, whereas under permissive conditions, they behave as wild type. Temperature-sensitive mutants are the most commonly used type of mutants. Temperature-sensitive mutants have mutations in cell cycle regulators.

These mutants grow and divide normally at a permissive (usually lower) temperature, whereas their growth and cell division is completed inhibited at restrictive (higher) temperatures (Harper et al. 1995; Hartwell et al. 1974; Nurse et al. 1976; Thuriaux et al. 1978). These types of mutants were verified for lipid production at a restrictive temperature in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* (Yao et al. 2012), which assisted the production of neutral lipids by 20%. Some of the mutants showed differences in lipid composition with temperature shift. As the main consumer of the cell's energy reserves is blocked, the mutants can show variable amounts of starch along with lipids. The temperature-sensitive mutants could possibly produce lipid or starch with temperature increase serving as a convenient switch. However, such a temperature switch can be very costly in real-scale algal bioreactors, where temperature controller adds additional costs. Physical and chemical mutagens yield strains having enhanced properties, but these are not considered as GMOs. The spectrum of products obtained by this approach is limited by the natural properties of algal species (Hlavova et al. 2015).

5.2 *Genetic Engineering of Microalgae and Its Technical Progress*

Even though our Mother Nature is very diverse in terms of various algal species (approximately 10,000 species), only a few thousand are collected, several hundred are explored for biochemical characteristics, and just few are cultivated for industrial application (Spolaore et al. 2006; Parmar et al. 2011). Although lot of research was dedicated to the commercial cultivation of some limited algal species, metabolic engineering of algae is equally important to gain enhanced yield of biomass as well as their biochemical content and to optimize their growth and harvesting. GM strains are usually associated with accidental consequences to environment and public health. These problems need to be taken into consideration for designing a high-scale reactor for mass cultivation of genetically modified strains. The large-scale cultivation deals with serious risk of escape of genetically modified strains and contamination of the natural strains (Parmar et al. 2011). Modified strains have a great chance of release in air and transported over far distances and persist in diverse harsh environmental conditions. Despite these consequences, researchers are continuously developing transgenic algal strains to boost up recombinant protein expression, enhanced metabolism, and enhanced photosynthetic activities, which helps to boost the future of engineered microalgae (Rosenberg et al. 2008).

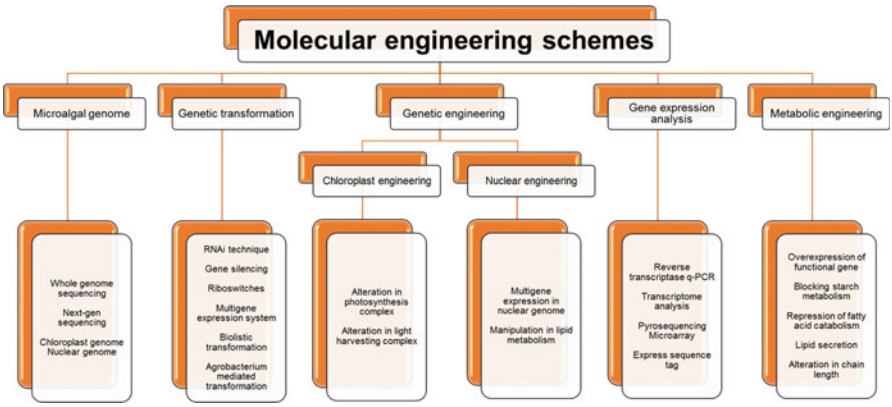

Fig. 5 Molecular schemes for enhancing accumulation of lipids in microalgae (Adapted from Singh et al. 2016)

The idea of increasing valuable compounds in microalgae using genetic engineering approach is very attractive. The most impressive strategies in implementation of molecular tools for the enhancement of microalgal lipids are summarized in Fig. 5. The absence of cell differentiation and allelic genes due to their haploid nature of most vegetative stages of microalgae makes their genetic manipulations much simpler than higher plants (Pulz and Gross 2004). In the last decade, there is a significant advancement in the development for microalgal transformation methods. Genetic modifications in a variety of more than 30 algal species such as *Chlorophyta*, *Rhodophyta*, *Phaeophyta*, diatoms, euglenoids, and dinoflagellates have been successfully conducted to date using molecular tools (Radakovits et al. 2010). Most of the researchers studied the genetic modification of *Chlamydomonas* genome, because stable genetic transformation is reported for these species (Boynton et al. 1988; Fernandez et al. 1989; Merchant et al. 2012; O'Neill et al. 2012; Singh et al. 2016). Nevertheless, in recent past, whole genome sequencing for many lipid-containing microalgal strains was conducted which includes *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *Nannochloropsis*, *Coccomyxa* sp., *Micromonas*, *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Volvox carteri*, and *Thalassiosira pseudonana* (O'Neill et al. 2012; Merchant et al. 2012; Singh et al. 2016).

5.3 *Tools and Techniques of Genetic Transformations in Microalgae*

Varieties of transformation methods are available to transfer particular DNA into microalgal cells such as agitation in the presence of DNA, particle bombardment and silicon carbide whiskers, agitation of a cell suspension along with DNA and glass beads, electroporation, artificial transposons, *Agrobacterium* infection,

viruses, and *Agrobacterium*-mediated transformation. The most important steps for the successful transformation are insertion of foreign DNA molecules into the host cell and maintaining its viability for long term. The first successful genetic transformation was achieved in *Chlamydomonas reinhardtii* by agitating its cell suspension in the presence of DNA, polyethylene glycol (PEG), and glass beads (Kindle 1990). A few years later, this method was successfully applied for gene transformation in some other microalgae such as *Amphidium* and *Sybiodium* (Wijffels et al. 2013). Nevertheless, the major weakness of this method is the requirement of cell wall-deficient host strain. Therefore, this method cannot be used for microalgal strains having thick and complex cell wall structures, viz., *Scenedesmus* and *Chlorella* (Misra et al. 2014; Voigt et al. 2014).

The more advanced method such as electroporation is more suitable in these circumstances, as this technique can easily disrupt the lipid bilayers of the cell wall creating a channel for the efficient transport of genetic material through the plasma membrane by means of electric current. This method was used for transformation in *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Chlorella eliopdodeia*, *Chlorella* sp., *Phaeodactylum*, *Dunaliella salina*, and *Nannochloropsis oculata* (Singh et al. 2016). Diacylglycerol acyltransferase (BnDGAT2) gene from *Brassica* was successfully transformed in *Chlamydomonas reinhardtii* to improve its lipid accumulation using electroporation method (Ahmad et al. 2015). However, its efficiency depends on several factors such as pulse length, temperature, field strength, membrane characteristics, and medium composition and concentration of DNA (Kumar et al. 2004). Particle bombardment is the widely used method for chloroplast and nuclear genome transformation to manipulate metabolic pathways such as fatty acid biosynthesis and TAG synthesis. The multiple copies of recombinant DNA can be delivered through cellular as well as chloroplast membranes using this method, resulting in increased chances of successful mixing regime (Leon-Banares et al. 2004). This method has been successfully applied for stable chloroplast and nuclear transformation of *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Chlorella kessleri*, *Chlorella sorokiniana*, and diatom *Phaeodactylum tricornutum* (Niu et al. 2012; Singh et al. 2016). Several researchers have demonstrated the high lipid content of these microalgal strains, which can be further enhanced by this transformation method. Electroporation and particle bombardment methods usually used for eukaryotic microalgae provided highest transformation rate and enables lipid enhancement (Tabatabaei et al. 2011).

Transformation conducted using *Agrobacterium tumefaciens* is the most widely used technique for plant cells (Kumar et al. 2004) due to its natural ability to transfer inter-kingdom DNA transfer. Microalgal lipids content can be improved through *Agrobacterium tumefaciens*-mediated transformation by expressing exogenous genes coded for lipid metabolism. Cheng et al. (2012) transformed (gfp gene) *Schizochytrium* using this method. The challenge in transformation of algae is the

Table 7 Molecular approaches to enhance lipid accumulation in microalgae (Adapted from Singh et al. 2016)

Molecular approach	Microalgae	Targeted trait/ pathways	Result
Chloroplast engineering (RNAi technology)	*Chlamydomonas reinhardtii*	Light harvesting complex	Increase in biomass productivity
Metabolic engineering Overexpression of functional genes Acylglycerol acyltransferases (DGAT)	*Chlamydomonas reinhardtii*	Kennedy pathway	Increase in mRNA level (7–29.1 times). No effect on lipid accumulation
Malic enzyme (ME)	*Phaeodactylum tricornutum*	Pyruvate metabolism	Increase in expression and enzyme activity 2.5-fold increase in total lipid content
Glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), diacylglycerol acyltransferase (DGAT)	*Chlorella minutissima*	Kennedy pathway	Twofold increase in lipid content
Blocking competitive pathways Starchless mutant	*Scenedesmus obliquus*	Starch metabolism	51% increase in mutant strain as compared to wild type
ADP-glucose pyrophosphorylase	*Chlamydomonas*	Starch metabolism	Tenfold increase in lipid content
Knockdown of enzymes lipase/phospholipase/ acyltransferase	*Thalassiosira pseudonana*	Lipid catabolism	3.5-fold increase in lipid content
Alteration in fatty acid chain length Acyl-ACP thioesterases	*Phaeodactylum tricornutum*	Fatty acid chain termination (TE)	Increase in the quantity of short-chain length lauric and myristic fatty acids
Fatty acid secretion Overproduction of free fatty acids Deletion of cyanophycin synthesis gene Phosphotransacetylase gene deletion	*Synechocystis* sp.	Fatty acid pathway Cyanophycin pathway	Fatty acids secretion into the medium Increase in production of fatty acids

application, and efficiency of transformation method is not uniform for all the algal strains; therefore, a lot of efforts have been dedicated to develop the transformation methods (Rosenberg et al. 2008; Singh et al. 2016). A different molecular approaches overview is presented in Table 7.

5.4 Genetic Engineering in Selective Organelles of Microalgae

5.4.1 Chloroplast and Nuclear Engineering

Chloroplast is an attractive choice for genetic manipulation that results in high-level expression of foreign genes (O'Neill et al. 2012; Singh et al. 2016). Therefore, chloroplast is the best choice for the manipulation to enhance biomass, lipid, and pigment production (Napier et al. 2014). Such approach was successfully implemented previously in microalgal strains such as *Haematococcus pluvialis*, *Chlamydomonas reinhardtii*, *Dunaliella* sp., and *Scenedesmus* sp., which are reported for their high lipid-producing capacities (Guo et al. 2013; Gutierrez et al. 2012; Potvin and Zhang 2010). Photosynthetic activity in *Chlamydomonas reinhardtii* was improved after modifications in light-harvesting complexes (LHC) using RNAi technology (Wobbe and Remacle 2015). The genetically engineered *Chlamydomonas reinhardtii* showed reduction in photo inhibition, thus improving the biomass yields. Chloroplast engineering can enhance lipid accumulation and biomass production simultaneously leading to increase in the overall volumetric lipid productivity in order to make biodiesel production an economical process.

Alteration in microalgal nucleus may provide a great chance to enhance the lipid accumulation as well as the quality of microalgal biodiesel. The presence of lipid biosynthesis genes in different cell organelles has already been revealed by whole genome analyses of microalgal strains (Wang et al. 2014; Misra et al. 2012). The nuclear genome of microalgae contains approximately 6% of total genes responsible for lipid biosynthesis (Misra et al. 2012). Most of these genes are coded for membrane lipid synthesis, TAG synthesis (DGAT), and fatty acid chain termination; therefore, manipulation in these genes can improve quality as well as quantity of the microalgal lipids in genetically engineered strains. Microalgal strains such as *Phaeodactylum tricornutum*, *Nannochloropsis oceanica* CCMP1779, and *Fistulifera* sp. have been successfully used for nuclear transformations (Muto et al. 2013; Singh et al. 2016; Vieler et al. 2012). Most of the studies reported single gene insertion for microalgal nuclear transformations. Noor-Mohammadi et al. (2014) developed a novel technique involving multigene expression, in which multigene pathway in yeast was constructed, integrated it in nuclear genome of *Chlamydomonas reinhardtii* for the co-expression of three reporter proteins (Ble, AphVIII, and GFP). The multigene expression technique can also be used to express functional genes of biomass generation and lipid synthesis pathway to improve lipid productivity. The multigene expression studies have a great potential, which can be suitably exploited to improve both quality of fatty acid synthesis and quantity of TAG synthesis.

5.5 *Expression Analysis of Genes Involved in Lipid Biosynthesis*

The microalgal lipid biosynthesis pathway has been intensively studied and well understood now (Cao et al. 2014; Radakovits et al. 2011; Purton et al. 2013) (Fig. 2). Lipid biosynthesis is a multistep reaction, catalyzed by fatty acid synthase (an acyl-carrier protein) (Harwood and Guschina 2009). Acyltransferases of the Kennedy pathway such as acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA:diacylglycerol acyltransferase (DGAT), and acyl-CoA:lysophosphatidic acyltransferase (LPAAT) are the key enzymes in the formation of fatty acid patterns of TAGs (De Bhowmick et al. 2015). High microalgal growth rates and biomass production under favorable conditions (optimum nutrients and cultivation parameters) are the outcome of increased translation and transcription processes (Merchant et al. 2012; Singh et al. 2016). Fan et al. (2014) examined the consequence of nutrient stress (phosphorus, nitrogen, and iron) on *Chlorella pyrenoidosa*, whereas the upregulation in expression of accD and rbcl genes was observed at higher concentrations of iron leading to high lipid productivity in *Chlorella sorokiniana* (Wan et al. 2014). Such studies provide the in-depth mechanism of lipid accumulation in the microalgae due to changes in cultivation conditions (Jusoh et al. 2015; Fan et al. 2014). The recent, most advanced molecular methods including microarray analysis, transcriptome analysis, and full-length overexpressed sequence tag (EST) transcript sequencing can reveal the mechanism of lipid biosynthesis under different stress conditions and give deep understanding of the key genes involved in triggering lipid accumulation (Trentacoste et al. 2013; Shin et al. 2015). Gene expression analysis can disclose the major functional genes involved in lipid biosynthesis, and therefore existing stress strategies can be improved further to achieve better lipid yields in microalgae.

5.6 *Overexpression of Lipid Biosynthesis Enzymes*

5.6.1 Acetyl-CoA Carboxylase (ACC)

Lipid metabolism is a complex process, which involves a number of chemical conversion processes catalyzed by different enzymes. Acetyl-CoA carboxylase (ACC) strongly control the metabolic flux of fatty acid synthesis in plants, and hence, its overexpression is studied in several species in order to enhance the generation of lipids. Overexpression of ACCase could be one of the most successfully implemented approaches for the improvement in fatty acid synthesis in microalgae. It has been well established that overexpression of ACCase can enhance accessibility of malonyl-CoA in chloroplast which subsequently trigger increase in fatty acid biosynthesis (Blatti et al. 2013; Liang and Jiang 2013; Singh et al. 2016). A one- to twofold rise in activity of plastid ACC along with 6%

increase in fatty acid content was observed when the cytosolic ACC from *Arabidopsis* was overexpressed in *Brassica napus* plastid (Roesler et al. 1997). Four ACC genes of *E. coli* BL21 were cloned and overexpressed in the same strain by Davis et al. (2000). It showed an enhanced ACC enzymatic activity which subsequently increased the intracellular malonyl-CoA pool. A sixfold increase in the rate of fatty acid synthesis was observed after co-expressing thioesterase I (encoded by the tesA gene) and ACCase (encoded by accA, accB, accC, accD). It confirmed that the committing step catalyzed by ACC was certainly the rate-limiting step for fatty acid biosynthesis in this strain. Nevertheless, enhancement in lipid production was not highly significant, suggesting that effective transformation of fatty acids to lipids was prevented by a secondary rate-limiting step after fatty acid formation in *E. coli*. ACC isolated from microalgae was also reported to be overexpressed in diatoms (*N. saprophila* and *C. cryptica*) (Roessler 1990). Similar to *E. coli*, the transgenic diatoms also resulted in insignificant increase of lipid accumulation (Dunahay et al. 1995, 1996). The expression of ACCase leads to an increase in microalgae under certain nutrient-limited cultivation conditions, however, not necessarily associated with higher lipid yields (Fan et al. 2014). Sheehan et al. (1998) concluded that enhancement in the whole lipid biosynthesis pathway in diatoms may not be solely dependent on the overexpression of ACC enzyme alone. In support to this conclusion, there are very rare reports mentioning the increase in the relevant enzymes with subsequent enhancement in lipid accumulation. It can be stated on a conclusive note that ACC does not catalyze the rate-limiting step alone, and a secondary rate-limiting step emerged when ACC was overexpressed in a particular species.

5.6.2 Fatty Acid Synthetase (FAS)

KAS subunit of FAS in *E. coli* was overexpressed by Subrahmanyam and Cronan (1998) to facilitate the C2 concatenation which was a failed trial due to extreme toxicity for the cell. In another study, overexpressed *E. coli* KAS III showed major alterations in the fatty acid composition of rapeseed with the significant changes in 18:1 fatty acids and short-chain fatty acids (14:0) (Verwoert et al. 1995). Likewise, KAS III from spinach *Spinacia oleracea* was overexpressed in cress *Arabidopsis*, tobacco *Nicotiana tabacum*, and rapeseed, which led to increase of fatty acids (16:0) along with the decline of the lipid synthesis rate (Dehesh et al. 2001). Targeting subunits of FAS for manipulation to enhance metabolism of fatty acid are challenging because the differences in multipoint controls among different species create critical complications in heterologous expression of multienzymatic complexes (Courchesne et al. 2009).

5.6.3 Acyl-CoA:Diacylglycerol Acyltransferase (DGAT)

DGAT is associated with last stage of TAG formation for the formation of triacylglycerol from fatty acyl-CoA and diacylglycerol. The insertion of *Arabidopsis* DGAT in yeast and tobacco showed increase of DGAT activity by 200–600-fold, and TAGs accumulation increased by three- to ninefold in the transformed yeast, whereas in the transformed tobacco, TAG content amplified to sevenfold (Bouvier-Nave et al. 2000). The overexpression of DGAT gene in plant *Arabidopsis* has also enhanced the oil content by 10–70% due to positive influence of DGAT activity (Jako et al. 2001). Overexpression of DGAT would force the conversion of diacylglycerol to TAG instead of phospholipid formation. Another study conducted by Thelen and Ohlrogge (2002) reported that formation of fatty acid can be stimulated by enhancing TAG synthesis rate in plants through overexpression of DGAT. These results suggest DGAT is definitely engaged in rate-limiting step of lipid biosynthesis. However, overexpression of DGAT in microalgae is hardly reported until today.

5.6.4 Lysophosphatidate Acyltransferase (LPAT)

Lysophosphatidate acyltransferase (LPAT) is one of the enzyme engaged in TAG formation, and its overexpression can enhance lipid accumulation. Zou et al. (1997) for the first time attempted the conversion of rapeseed with a putative sn-2 acyltransferase gene from the *Saccharomyces cerevisiae*. They overexpressed lysophosphatidate acyltransferase (LPAT) activity in rapeseed and observed 8–48% increase in oil content. However, the increasing activity of LPAT in developing seeds may disturb the steady-state level of diacylglycerol. Some of the enzymes including acetyl-CoA synthase (ACS), ATP:citrate lyase (ACL), and malic enzyme (ME), which are not related to lipid metabolism can also increase the pool of essential metabolites for lipid biosynthesis via influencing the rate of lipid accumulation.

5.6.5 Acetyl-CoA Synthase (ACS)

ACS is known to be involved in the formation of acetyl-CoA using acetate as substrate. In the presence of acetate, bacterial strains overexpress ACS with subsequent enhancement in fatty acid synthesis rate (Lin et al. 2006). For instance, overexpression of ACS gene in *E. coli* led to a ninefold increase in ACS activity, subsequently increasing the utilization of acetate from the medium, which can contribute to lipid biosynthesis. Brown et al. (1977) reported similar observations of enhanced lipid biosynthesis.

5.6.6 Malic Enzyme (ME)

Malic enzyme ME can convert malate into pyruvate along with reduction of a $NADP^+$ into NADPH (Wynn et al. 1999). It was reported that ME with its increased activity can enhance the pool of cytosolic NADPH, providing additional reducing energy to lipogenic enzymes including ACL, ACC, and FAS. A metabolon could be formed between ME and FAS to create a channeling of NADPH. These are formed by ME toward the FAS active sites. Zhang et al. (2007) investigated overexpression of ME in *Mucor circinelloides* to process lipogenesis without energy restriction to achieve high lipid accumulation. The genes encoding ME from *Mortierella alpine* (malEMc) and *M. circinelloides* (malEMt) were overexpressed in *M. circinelloides* which led to three- and twofold increase of ME activity for the transgenic malEMc and malEMt strains, respectively. A faster lipid accumulation for the transgenic malEMt and malEMc strains (2.5- and 2.4-fold higher, respectively) was predicted because of the ME activity increase in both cases.

5.6.7 ATP:Citrate Lyase (ACL)

ACL provides source of acetyl-CoA for fatty acid biosynthesis by catalyzing the conversion of citrate into oxaloacetate and acetyl-CoA. ACL is one of the major enzymes in lipid accumulation regulation in mammals, oleaginous yeast, and fungi. Rangasamy and Ratledge (2000) constructed a gene that encoded for a fusion protein of the rat liver ACL. These are with the leader peptide for the small subunit of ribulose bisphosphate carboxylase and inserted into the genome of tobacco. Overexpression of this gene enhanced the total ACL activity by fourfold, subsequently increasing the quantity of fatty acids by 16%, however, without any major changes in fatty acid profile.

5.7 Inhibiting the Competitive Pathways

Blocking the pathways (e.g., carbohydrate and lipid catabolism), which are considered competitive for the desired product, is an effective strategy for improving microalgal lipid accumulation (Blatti et al. 2012; Liu and Benning 2013; Radakovits et al. 2010). Carbohydrate metabolic pathways are essential for accumulation and storage of carbon in the form of starch (Gonzalez-Fernandez and Ballesteros 2012). Therefore, suppressing the carbohydrate metabolism can divert the carbon flow toward lipids biosynthesis. A mutant of *Scenedesmus obliquus* showed up to 51% increase in TAG accumulation (0.217 g mol^{-1}) over the wild type (0.144 g mol^{-1}) under similar conditions (Breuer et al. 2014). Moreover, there was no alteration in photosynthetic behavior of both the wild type and mutants. This genetic manipulation only affected the carbohydrate metabolism and not

photosynthetic performance. In another study, TAG accumulation was increased by ten times in mutant strain of *Chlamydomonas*. It was believed that deactivation of ADP-glucose pyrophosphorylase catalyzed the committing step in metabolism of starch (Li et al. 2010). These breakthrough investigations provided a future direction for lipid enrichment by redirecting "C" pool from synthesis of starch toward accumulation of lipids by knocking down the key genes involved in carbohydrate synthesis. However, it should be noted that interruption in synthesis of starch might result in reduced microalgal growth, which would ultimately have worse effect on the final productivity of lipids. Instead, suppression of lipid catabolism is one of the effective tactics employed to enhance the microalgae lipid accumulation. Such trials have been conducted in a mutant strain of *Thalassiosira pseudonana* and shown 3.5-fold increase in lipid accumulation after knocking down the regulation of multifunctional enzymes lipase/phospholipase/acyltransferase in the lipid catabolism (Trentacoste et al. 2013) (Table 5). These strategies can be employed for microalgae to enhance lipid accumulation without compromising microalgal growth.

5.8 Modification in Fatty Acid Chain Length for the Improvements in Lipid Quality

The properties of produced biodiesel depend upon the microalgal lipid's composition. Therefore, enhancing the lipid accumulation in microalgae is not enough, and development of approaches for the advancement of lipids quality in microalgae is crucial to meet the standard specifications for biodiesel (Parsaeimehr et al. 2015). The most desirable fatty acids for biodiesel production are monounsaturated and saturated fatty acids (12:0, 14:0, 16:0, 16:1, 18:0, and 18:1). Acyl-ACP thioesterase releases the fatty acid polymer from fatty acid synthase and thereby controls the chain length of fatty acid. These enzymes can enhance the composition of the fatty acids which is useful to achieve anticipated fuel properties. The transformation of two shorter chain length fatty acid acyl-ACP thioesterases from *Umbellularia californica* and *Cinnamomum camphora* into *Phaeodactylum tricornutum* significantly improved the percent composition of myristic (C14:0) and lauric (C12:0) acids in overall fatty acid profile (Radakovits et al. 2011). The strategies involving the alteration of fatty acid chain length using molecular approaches to improve the microalgal lipid quality with desired compositions have significant potential for biodiesel generation in the near future.

6 Conclusion and Future Outlooks

Economical production of biodiesel from microalgae is a bottleneck in biorefinery industries. One of the most possible approaches to achieve this goal is by assuring high lipid accumulation in microalgal cells. This chapter describes the recent

advancements in lipid enhancement approaches. Several novel approaches conducted to enhance biolipids in the recent past have been discussed. The strategies such as altering the light intensity and nutrient composition of the medium; inducing stress conditions such as salinity and temperature; and adding certain chemicals and phytohormones can be successfully combined with the application of wastewater as nutrients in order to make the biomass generation a cost-effective process. These innovative strategies have ensured bright future in microalgal biotechnology for successful enhancement in biomass and lipid productivity. Despite of their great potential, the traditional biochemical approaches still need a lot of significant improvements for enhanced lipid accumulation so as to fulfill the need for biodiesel commercialization. Therefore, strain improvements through mutations metabolic engineering approaches and synthetic biology strategies can possibly provide us the necessary developments in microalgal biotechnology so that microalgae can be used as a feedstock for commercial lipid production. Particularly in biocatalyst engineering, attention should be given on collection of novel genetic properties of microalgae, including genome sequencing to explore the accessibility of appropriate hosts and gene libraries, development of novel methods of nuclear transformation and controlled overexpression of lipid metabolites, and blocking competitive pathways. A rise in both technological applicability and fundamental knowledge is required to report the current bottlenecks in the developments of microalgal biodiesel and to make microalgal biodiesel production "a fully competitive process." Employment of genetic engineering including overexpression of enzymes, inducible promoters, and redirection flux transcription factor regulation of key metabolites involved in lipid biosynthesis pathway can enhance lipid accumulation. These approaches will provide a potential breakthrough in increasing lipid accumulation as well as it can achieve the desired quality for standard biodiesel production. These advancements and innovative strategies are certainly moving toward the economical and sustainable biodiesel production.

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Comprehensive Screening of Micro-and Macroalgal Species for Bioenergy

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1 Introduction

Algae represent a much diversified photosynthetic organism characterized by many different phyla and different physiological attributes. They accumulate lipids, carbohydrates, proteins and other high-value biomolecules during their growth that can be converted into different kinds of renewable bioenergies (Dasgupta et al. 2015). The unicellular or grouped microscopic microalgae are attractive candidate in biofuel research (Chisti 2007). The multicellular macroalgae (including seaweeds) contain higher amount of carbohydrates and are considered for biogas and bioethanol production (John et al. 2011). Thermochemical conversion methods can convert all types of algal biomass directly into crude oil and biogases (including methane) so there is no need to screen their potentiality (Amin 2009). However, for production of particular biofuel such as biodiesel, bioethanol, butanol and biohydrogen, they need to be screened for the accumulation of substantial amount of lipid, carbohydrates and other biomolecules. Some strain of microalgae has capabilities to divert the metabolic flux towards enhanced triglyceride (TAG) production under nutritional stress (Dasgupta et al. 2015). Studies have shown that microalgae have the potential to produce lipid up to 80% of their dry weight (Chisti 2007). Some microalgae are reported for their higher lipid productivity and favourable biodiesel properties such as *Chlorella*, *Scenedesmus* and *Selenastrum* (Song et al. 2013). After the lipid extraction, the residual biomass can be used to produce butanol. The macroalgae contains higher amount of carbohydrate (at least 50%) and very low amount of lipid. Thereby, they could produce biogases, bioethanol and butanol by fermentation and are poor in biodiesel production (Wei et al. 2013). Some macroalgal species such as *Porphyridium*, *Laminaria* and *Ulva* contain high amount of carbohydrates (Murdock and Wetzel 2009).

Hydrogen (H_2) evolution in algae is identified as a consequence of anaerobiosis or nutrient deprivation (Melis and Happe 2001). Some genus of green algae such as *Chlamydomonas*, *Scenedesmus*, *Chlorella*, etc. and blue-green algae could generate H_2 in the absence of oxygen (Melis and Happe 2001). Microalgae *Botryococcus braunii* has the ability to produce triterpenic hydrocarbons in high amount which can be converted into fuels (Niehaus et al. 2011).

The algal growth dynamics and biomolecule accumulation pattern tightly linked to environmental factors include light intensity, amount of CO_2, temperature, amount and type of nutrients, pH, etc. (Juneja et al. 2013). Tropical or subtropical regions of earth are suitable for algal growth due to abundant availability of solar radiation throughout the year and saline water (Michanek 1979). They are found in different natural habitats and grow in different seasons and are best adapted to those specific local conditions (Michanek 1979). Therefore, locally isolated strains would be the best for large-scale cultivation and biofuel production. A proper screening and selection process to identify the most suitable local strains is essential for the successful development of microalgal biofuels. For large number of algal sample easy, less expensive and rapid screening is required as an alternative to traditional gravimetric-based quantification protocols, which takes only few days and needs less amount of biomass. Rapid screening methods include microscopic analysis of biomolecules using fluorescent dye, electron

microscopy, mass-spectrophotometric analysis, spectroscopic analysis and different assays. Conventional gravimetric methods are extraction, production and quantification of biomolecules using chromatographic methods.

2 Microscopic Screening of Lipid Molecules

2.1 Light Microscopy

Microscopic quantification of biomolecules mostly for the neutral lipids has been shown to be quite useful for screening of oleaginous microalgae. Fluorometric determination of lipid using the dyes is a rapid, easy and nondestructive method. Confocal fluorescence microscopy is used as a tool to quantify the lipid bodies per cell. Some lipophilic dyes such as Nile blue (Smith 1908), Nile red (Kimura et al. 2004; Chen et al. 2009), BODIPY 505/515 (Brennan et al. 2012) and Sudan Black B (Ru-rong et al. 2011) are popularly used for fluorescence microscopic analysis of lipids.

2.1.1 Nile Red Staining

Nile blue as a histochemical stain was introduced by Smith to distinguish blue-stained neutral lipids and red-stained acid lipids (Smith 1908). Later on, Nile red (9-(diethylamino)-5H benzo [α] phenoxazin- 5-one), photostable and highly fluorescent in non-polar hydrophobic environments (lipid bodies), was synthesized from Nile blue oxidation (Fowler and Greenspan 1985). The intensity of fluorescence depends on concentrations of the dyes increasing up to an optimal limit (Huang et al. 2009; Chen et al. 2009). The optimal concentration is algae specific and varies considerably (0.01–100 μg mL^{-1}) (Huang et al. 2009; Chen et al. 2009). The temperature of staining ranges from 37°C to 40°C (Chen et al. 2009), and the time of treatment would be 2–7 mins (Cooksey et al. 1987; Pick and Rachutin-Zalogin 2012).

Nile red dye has several advantages over the other lipophilic dyes:

- Due to its metachromatic properties, interestingly it has different emission spectrum (when excited at 488 nm) according to the types of lipid; neutral lipids show yellow emission (560–640 nm) and polar lipids, orange/red emission (greater than 650 nm) (Elsey et al. 2007). Thereby the great advantage to use this dye is to easy quantification of lipid of interest (Diaz et al. 2008; Guzmán 2012).
- The amount of neutral lipids present is indicated by degree of fluoresces, and it is highly correlated with lipid measured by gravimetric method for *Chlorella* sp. (Huang et al. 2009; Chen et al. 2009), *Tetraselmis suecica* (Guzmán 2010) and *Nannochloropsis gaditana* (Simionato et al. 2011). However, sometimes, it is difficult to address the absolute quantification.
- Despite of screening of different algal species, it could anticipate the lipid production in different growth stages and different culture conditions with nutrient

stress (Dean et al. 2010). The reduction or complete removal of nitrogen sources from microalgae culture medium has been shown to be effective in increasing the concentration of lipids in *Scenedesmus subspicatus*, *Chlamydomonas reinhardtii* and several other species (Greenspan et al. 1985).

The major limitations of the Nile red staining method are as follows:

- Restricted permeation and uneven staining due to cell wall (Chen et al. 2009; Doan and Obbard 2011; Pick and Rachutin-Zalogin 2012).
- High chlorophyll content (1–4% of dry weight) interferes and increases the background fluorescence preventing reliable lipid quantification (Chen et al. 2009).
- Dilution of Nile red with DMSO (range from 5 to 20%) could improve permeability of Nile red staining in microalgae (Chen et al. 2009; Doan and Obbard 2011). However higher concentration of DMSO could affect the cell survival (Pick and Rachutin-Zalogin 2012). Another drawback is DMSO also used for extraction of lipid from cell.
- Sometimes, percentages of staining of cell are very poor only 25–30% (Doan and Obbard 2011). Thereby, it affects the correlation between Nile red staining and gravimetric methods (Chen et al. 2009).
- Absolute quantification of lipid can be done using lipid standard such as triolein, a symmetrical triglyceride (Massart et al. 2010).

Several chemical and physical strategies have been adopted to improve the Nile red staining.

- In some studies, glycerol has been used to transport Nile red that has no known cell growth inhibition (Doan and Obbard 2011).
- Green algae also contain solvent- and acid-resistant polymer sporopollenin that requires an additional physical treatment. Chen et al. (2011) proposed the use of microwave-assisted pretreatment for penetration of dye to achieve homogeneous and efficient staining.
- An electric field could improve the transport of the dye as studied in *Chlorella vulgaris* and *Spirulina* sp. (Azencott et al. 2007; Su et al. 2012).
- Lyophilized algae could be another option for improvement of staining as observed in seven *Chlorella* strains (Huang et al. 2009).
- Combining Nile red staining with flow cytometry and single-cell sorting further improve Nile red staining of live cells (Chen et al. 2009).

2.1.2 BODIPY 505/515 Staining

BODIPY 505/515 (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene) strong ultraviolet-absorbing molecules could successfully stain lipid vesicles (Cooper et al. 2010; Govender et al. 2012). This dye shows green peak (515–530 nm) emission when excited with a blue laser (450–490 nm) (Govender et al. 2012; Brennan et al. 2012; Xu et al. 2013):

- Good correlation was observed for measurements between BODIPY 505/515 fluorescence and gravimetric analysis in *Tetraselmis subcordiformis* lipid vesicle (Xu et al. 2013).
- This dye when bound to lipids shows green fluorescence and with chloroplasts shows red fluorescence (Cooper et al. 2010; Brennan et al. 2012).
- The environmental polarity of cells does not affect this staining procedure unlike the other staining methods like Nile red (Cirulis et al. 2012).
- Acetone (1–2%) is more often used for solution preparation to maintain the cell integrity (Cirulis et al. 2012).
- Unlike Nile red, this stain easily incorporates into cells of all microalgal cell wall such as Chlorophyceae, Xanthophyceae, Haptophyceae and even those with thick silica due to its high lipid/water partition coefficient (Cooper et al. 2010). Fast permeation was observed in different algae such as *Nannochloropsis atomus*, *Nannochloropsis oculata*, *Tetraselmis suecica* and *Dunaliella tertiolecta*. Maximum fluorescence was attained within 1 min (Brennan et al. 2012).

2.1.3 Sudan Black B Staining

Sudan Black B ($C_{29}H_{24}N_6$) is a nonfluorescent, dark brown-to-black powder, relatively thermostable fat-soluble diazo dye used for staining of neutral triglycerides and lipids (Lison 1934). Sudan Black B has maximum absorbance (A645) at a wavelength of 645 nm as observed in microalgae (Ru-rong et al. 2011). However, this method has limitations to demonstrate fat globules present in marine algae (Ru-rong et al. 2011).

2.2 Electron Microscopy

The conventional electron microscope reveals the ultrastructure and internal cell organization. In addition with cytochemical techniques, molecule of interest in cell structure can also be located (Angermuller and Fahimi 1982). Under conventional transmission electron microscopy, lipid bodies are observed as slightly electron-dense rounded structures with different sizes and shapes and no visible internal structure (Waltermann and Steinbuchel 2006).

3 Spectroscopic Screening of Biomolecules

3.1 Raman Spectroscopy

Raman spectroscopy allows rapid characterization of algae regarding its biochemical, molecular composition and the degree of unsaturation of lipids (Huang et al. 2010; Samek et al. 2011). Algal cell contains different biomolecules including

lipids, carbohydrates, proteins, nucleic acids and pigments, and each has its characteristic Raman spectrum (Huang et al. 2010; Samek et al. 2011). This technique has also been used to identify algal species (Wood et al. 2005). Advancement in instrumentation such as resonance Raman spectroscopy (RRS), tip-enhanced Raman spectroscopy (TERS), surface-enhanced Raman spectroscopy (SERS), laser tweezers Raman spectroscopy (LTRS) and coherent anti-Stokes Raman spectroscopy (CARS) resulted in more resolution to detect real-time changes in algae cells. Resonance Raman spectroscopy is associated with particular molecule specific which enhanced due to resonance effect (Brahma et al. 1983). The in vivo lipid profiling and quantitative determination of the degree of unsaturation and iodine value (IV) of storage lipid has been detected using single-cell laser-trapping Raman spectroscopy (Wu et al. 2011; Samek et al. 2011). The ratio of unsaturated to saturated carbon-carbon bonds of the fatty acids can be detected by spectra at 1656 cm^{-1} (cis-C=C stretching mode) and 1445 cm^{-1} (CH_2 scissoring mode) (Samek et al. 2010, 2011). Estimations can be further validated by gas chromatography and mass spectroscopy analysis.

Major advantages of Raman spectroscopy are as follows:

- Biological system is a wet system, but water gives very weak signal (Parker 1983).
- Analysis of in vivo molecules is possible by Raman spectroscopy. It does not require elaborate preparation for sample and signal processing like other infrared (IR) spectroscopy (Heraud et al. 2007).
- Identification of algae by characteristic peaks of species-specific biomolecules in the cells has been reported in literature (Wu et al. 1998; Parab and Tomar 2012). It could also be applied for differentiating non-toxic and toxic algal strains (Wu et al. 1998).
- It is handy and portable for the outdoor experiments in natural habitat (Wood et al. 2005).

Major limitations are:

- Strong fluorescence of pigments that might interfere and obscure the characteristic Raman spectral features (Parab and Tomar 2012).

3.2 *Nuclear Magnetic Resonance (NMR) Spectroscopy*

NMR is considered a useful diagnostic method that is complementary to other analytical tools for TAG identification in algal cells (Gao et al. 2008; Beal et al. 2010). A liquid-state NMR has potential in the detection of TAG formation in cells of *Neochloris oleoabundans* induced by nitrogen starvation. The lipid molecules have relatively rapid molecular motion compared to the other biomolecules within the cell. Thereby they are selectively detected by the NMR method (Davey et al. 2012).

3.3 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a simple and inexpensive determination of chemical information about proteins, lipids and carbohydrates and can be validated by comparing to the traditional measurements. Giordano et al. (2001) first reported the application of FTIR spectroscopy to whole microalgal cells. Afterwards, this methodology becomes popular for chemical, physiological and ecophysiological studies (Murdock and Wetzel 2009; Dean et al. 2010). It can provide information on different functional groups such as C = O, =C – H, –CH2, –CH3, C – O – C, O – H, N – H and >P = O (Dean et al. 2010; Duygu et al. 2012). Thereby, it can discriminate the diversity of strain and difference in chemical composition (Sigee et al. 2002). The wavelength region 1590–1484 cm^{-1} characterizes a protein spectrum. Because this peak is exclusively due to the combination of C = O, N - H and C - N, stretching vibrations in amide complexes and also the peak region of BSA support this wave region. Lipid peak is observed at the wavelength region 1778–1706 cm^{-1} by strong vibrations of the C = O. Carbohydrate absorption bands due to C - O - C of polysaccharides have been identified at the wavelength region 1216–925 cm^{-1}. The other factor that determines the peak intensity is the concentration of molecules in the sample (Smith 1908). According to Beer's law, the absorbance is directly proportional to the concentration (Eq. 1). Thereby, the peak height/area increases as the protein, lipid and carbohydrate compositions are more in a certain alga than others.

$$A = \varepsilon lc (A = \text{absorbance}, \varepsilon = \text{absorptivity}, l = \text{pathlength}, c = \text{concentration}) \quad (1)$$

Laurens and Wolfrum (2011) showed the use of NIR and FTIR spectroscopic fingerprinting of algal biomass to predict lipid content and composition. Identification of bands can also be done by the published algal FTIR spectra in relation to specific molecular groups (Sigee et al. 2002; Duygu et al. 2012). Recently, 16 freshwater microalgae have been screened for protein, carbohydrates and lipids at cellular level during the stationary phase using FTIR (Dasgupta et al. 2015) (Fig. 1).

Advantages of FTIR are as follows:

- It is a very sensitive, quick method and no need of external calibration.
- Very small concentration of molecules can be determined by FTIR.
- This spectroscopy provides better signal to noise ratio as it has single light beam compared to the other dispersive instrument which generally has double light beam.

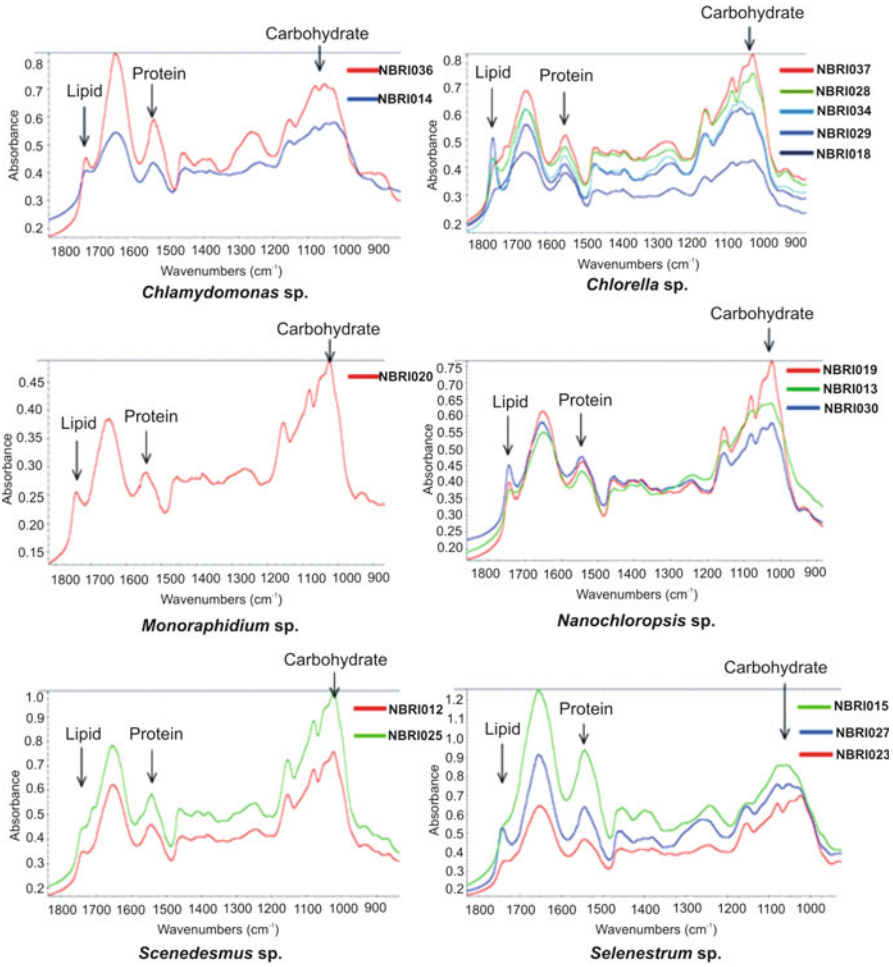

Fig. 1 FTIR spectra of 16 different algal strains. *Selenastrum* (027), *Chlorella* (029) and *Selenastrum* (015) are found to be more promising strains for protein, lipid and carbohydrate content, respectively

4 Spectrophotometric Screening

4.1 Screening of Growth Potential

Spectrophotometric techniques are used to determine the concentration of cells in media by measuring the amount of light absorbed. This technique has been widely used to evaluate algal growth potential, an important factor for biofuel production. Algal growth has been measured as optical density in particular wavelength with respect to the days of culture (Fig. 2). Algae growth potential can be screened in uniform growth condition and also in different growth conditions.

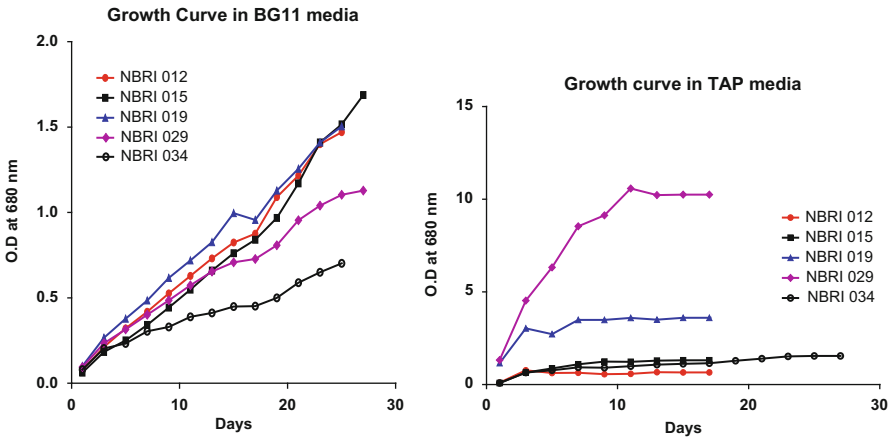

Fig. 2 Graphical representation of optical density (680 nm) with respect to the days of the five algal cultures showing their growth pattern in different growth condition (in BG11 and TAP media)

This spectroscopic analysis has been substantiated by dry weight measurements and calculation of the biomass productivity. Biomass content (BC) can be measured by the total weight of the dry biomass (g)/L of culture.

Biomass productivity (BP) can be calculated by the following formula (Griffiths and Harrison 2009)(Eq. 2):

$$\mathrm{BP}\,(\mathrm{mg/L/d}) = (B_2 - B_1)\,(\mathrm{mg/L})/(t_2 - t_1)\,(d) \tag{2}$$

B_1 and B_2 are biomass concentrations and t_1 and t_2 are the two sampling points, respectively.

4.2 Colorimetric Quantification of Carbohydrates

The dinitrosalicylic acid assay (DNSA) (Miller 1959), phenol-sulfuric acid assay (Dubois et al. 1956) and anthrone method (Hedge and Hofreiter 1962) are generally used for the determination of all types of carbohydrates. DNSA reagent detects reducing ends of carbohydrates and gives red colour. The absorbance is determined at 540 nm. In phenol-sulfuric acid assay, carbohydrate reacts with concentrated sulfuric acid generating furfural which again reacts with phenol to generate yellow-gold colour (480 nm). In anthrone method, furfural reacts with anthrone to form a green colour complex (620 nm). D-Glucose is commonly used for calibration curve. Carbohydrate (TC) % in dry biomass was calculated by the following formula (Eqs. 3 and 4):

$$\text{TC} = \text{OD(optical density)} \times \text{slope value of calibration curve} \times 100 \quad (3)$$

Carbohydrate content (CC) was calculated by the following formula:

$$\text{CC} = \text{BC} \times \text{TC} \quad (4)$$

5 Extraction and Quantification of Lipids

Different solvent extraction methods are adopted for complete extraction and quantification of lipids. Folch method (Folch et al. 1957) is rapid and easier. The combination of chloroform and methanol (2:1 v/v) is used for the extraction of lipid. The large amount of biomass can be extracted by this method. Another similar method is Bligh and Dyer method (Bligh and Dyer 1959) where chloroform/methanol ratio is different (1:2 v/v). Other than these basic methods, several solvents with different combination have been used such as ethanol, isopropanol, butanol, hexane, etc. (Sheng et al. 2011). Levine et al. (2010) reported processing of wet algal biomass by in situ lipid hydrolysis and supercritical in situ transesterification (SC-IST/E) method.

Other than solvent extraction, the mechanical extraction methods are also widely used. Some include beading (Richmond 2004), expeller press (Ramesh 2013), microwave-assisted pyrolysis extraction (Du et al. 2011), ultrasound-assisted extractions, pulsed electric field and hydrothermal liquefaction (Brown et al. 2010). Osmotic pressure is considered a cost-effective way of extraction (Adam et al. 2012) and used for different microalgae such as *Chlamydomonas*, *Scenedesmus*, *Chlorella* and *Botryococcus* (Lee et al. 2010, Yoo et al. 2012). Addition of organic carbon source (acetic acid) would help increase the biomass and lipid productivity of algae (Fig. 3).

Percentage of total lipid (TL) in dry biomass can be determined by the following formula (Eqs. 5–7):

$$\text{TL (Percentage of total lipid)} = \text{weight(extracted lipid)}/\text{weight (biomass taken)} \times 100 \quad (5)$$

The lipid content (LC) was calculated by the following formula:

$$\text{LC} = \text{TL}/100 \times \text{BC} \quad (6)$$

The lipid productivity (LP) (mg/L/d) was calculated by the following formula (Griffiths and Harrison 2009):

$$\text{LP} = \text{BP} \times \text{TL}/100 \quad (7)$$

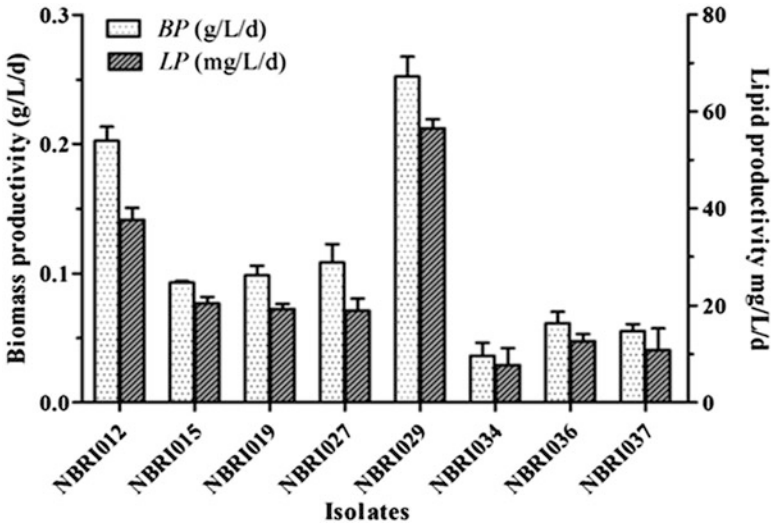

Fig. 3 Screening of eight microalgal isolates for biomass and lipid productivity during stationary phase of growth cycle in TAP media. Among them, only two isolates, NBRI029 and NBRI012, show higher biomass and lipid productivity (Dasgupta et al. 2015)

6 Gas Chromatographic Analysis of Fatty Acids

Algal lipids are composed of triglycerides, which form fatty acid methyl ester (FAME) when reacted with methanol. This process is called transesterification of lipid. The fuel properties of algal biodiesel are determined by the fatty acid (FA) profile. The gas chromatography is the most frequently used for the analysis of FAs. Indeed, for the quantification of individual FA in any lipids, GC must be adopted. FA profile of lipid obtained from different microalgae has different percentages of FA of C16 and C18, a key factor required for screening of algae to produce biodiesel (Dasgupta et al. 2015) (Fig. 4).

7 Assessment of Biodiesel Properties

Investigation of biodiesel properties of different algal FAME is an important factor for using the oil as biodiesel (Table 1). The key properties include iodine value (IV), degree of unsaturation (DU), saponification value (SV), cetane number, etc. The unsaturation of FAs is represented by iodine value (IV) and depends on the origin of oil (Ramos et al. 2009). The maximum limit of IV is 120 according to European standard. Higher IV means higher unsaturation which resulted in polymerization of FAs and engine deposits during heating (Ramos et al. 2009; Francisco et al. 2010). The degree of unsaturation (DU) influences the oxidative stability and represents

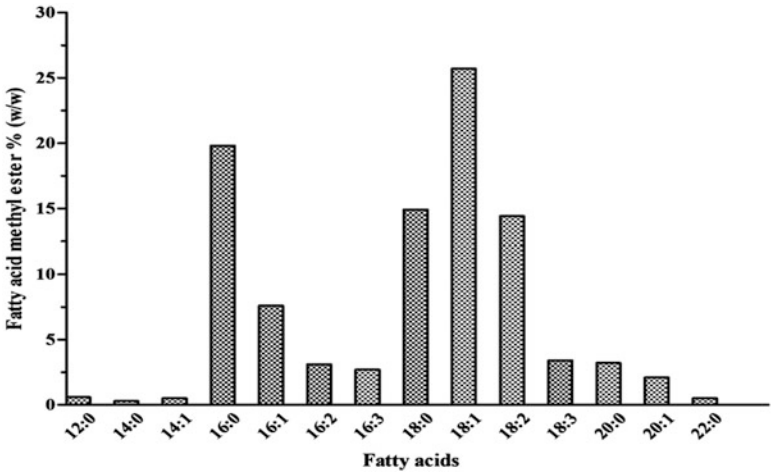

Fig. 4 Fatty acid methyl ester (FAME) profile of *Scenedesmus* sp. NBRI012 shows the percentage of FA

Table 1 Comparison of biodiesel properties

	DU (%)	CN[a]	IV[b]	SV	References
Scenedesmus	83.1	54.6	82.8	202.7	Dasgupta et al. (2015)
Chlorella sp.	74.1	56.7	65	217.8	Francisco et al. (2010)
Peanut	113.1	53	97	–	Ramos et al. (2009)

DU degree of unsaturation, *CN* cetane number, *IV* iodine value, *SV* saponification value
[a]Minimum limit of CV in Indian standard (IS 15607) and European standards (EN 14214) is 51
[b]Maximum limit of IV of European standards (EN 14214) is 120 g I_2 100 g^{-1}

the sum of the masses of mono- and polyunsaturated acids (Francisco et al. 2010; Dasgupta et al. 2015). Saponification value (SV) represents the potassium hydroxide (KOH) required to completely saponify one gram of diesel. Cetane number represents the combustion quality and ignition delay time (Ramos et al. 2009; Knothe 2012). The minimum value of CN Indian standard (IS 15607), European standard (EN14214), and Australian standard is 51; the ASTM D6751 and the Brazilian National Petroleum Agency (ANP255) provided the minimum values of CN 47 and 45, respectively. The values of saponification value (SV), iodine value (IV), cetane number (CN), and degree of unsaturation (DU) can be calculated using empirical (Eqs. 8–11)

$$\mathrm{SV} = \Sigma(560 \times \mathrm{F})/\mathrm{M} \tag{8}$$

$$\mathrm{IV} = \Sigma(254 \times \mathrm{F} \times \mathrm{D})/\mathrm{M} \tag{9}$$

$$\mathrm{CN} = (46.3 + 5458/\mathrm{SV}) - (0.225 \times \mathrm{IV}) \tag{10}$$

$$\mathrm{DU} = (\mathrm{MUFA}, \mathrm{wt}\%) + (2 \times \mathrm{PUFA}, \mathrm{wt}\%) \tag{11}$$

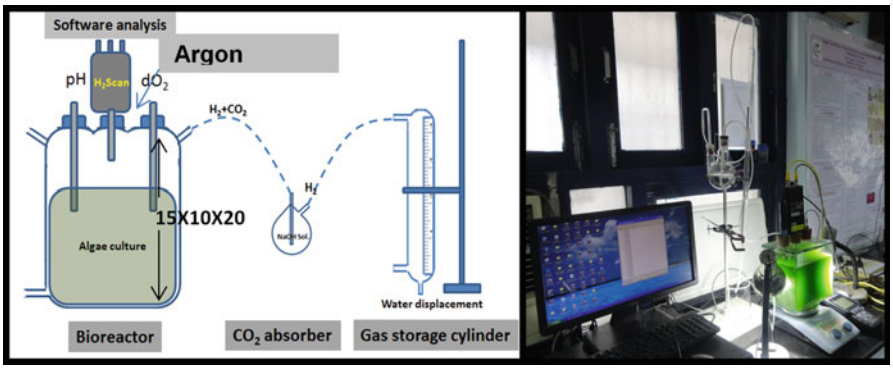

Fig. 5 Algal culture in S-deprived TAP media in fabricated photobioreactor with H_2 sensor, dO_2 and pH probe

where F is the percentage of each fatty acid, M is the molecular mass of each fatty acid, D is the number of double bonds, MUFA is monounsaturated fatty acid and PUFA is polyunsaturated fatty acid in wt %.

8 Real-Time H_2 Measurement and Gas Chromatographic Estimation of Biohydrogen Production

In real time, H_2 production (percentage of H_2 v/v in total evolved gas) can be measured with an H_2 sensor (HY-OPTIMA-700 H2scan, Valencia, CA) fit with the photobioreactor, which determines the online percentage of H_2 in total evolved gas in the headspace of photobioreactor by the hyper-terminal in the computer (Dasgupta et al. 2015) (Fig. 5). Hydrogen can also be measured by thermal conductivity detectors (TCD) which has been used in packed-column gas chromatography (GC) where most often helium (He) has been used as carrier gas.

9 Gas Chromatographic Estimation of Ethanol Production

Algae are the potential source of carbohydrate which can be converted to bioethanol by ethanologenic microorganisms. The conversion includes the following steps:

(i) Pretreatment of biomass
(ii) Hydrolysis of cellulosic materials to fermentable sugars
(iii) Fermentation of the hydrolysate by ethanologenic microorganisms such as yeast

Gas chromatography (GC-FID) quantification of ethanol is inexpensive and offers wide range detection (1–30% v/v) (Ellis et al. 2012). Some algae are promising candidates for ethanol production such as some brown macroalgae (Enquist-Newman et al. 2014), *Ulva* (Saqib et al. 2013), *Sargassum* (Borines et al. 2013), red algae *Gracilaria* (Kumar et al. 2013), etc.

10 Future Prospective

Finding the best organism using high-throughput techniques is not always an economic and feasible option. Genetically modified organism could definitely serve better for further improvement of the strain. However, some countries that genetically modified organisms remain unwelcomed. Several companies worldwide are attempting for commercialization of the algae fuel. However, the major bottleneck is the high production cost. The fuel cost is more than 50% of fossil fuel. Though the bioenergy research has been started 30 years back, but due to low cost of fossil fuel, it was always ignored. Recently, the vision has been changed for bioenergy research, and in the near future desperately we need the alternative to fossil fuel. In long-term projects in coming decades, the gasification of biomass and crude oil extraction will be expected to serve the energy needs. In addition, for low-cost production of the fuel, the use of wastewater, use of inexpensive bioreactors such as disposable plastic bags, sequestration of CO_2 and 'algal biorefinery' concept need to be explored for economic viability of the process.

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Microalgae for Biofuels: Applications, Process Constraints and Future Needs

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1 Introduction

Microalgae are eukaryotic (e.g. green algae, diatoms) photosynthetic organisms capable of utilizing carbon dioxide and light for the synthesis of carbohydrates as energy compounds. They have been known since many years, but their large-scale cultivation has started a few decades ago. They have the potential to grow in open systems such as raceway ponds, circular ponds and lakes and also in controlled condition like closed photobioreactors. Microalgae are advantageous considering their higher productivity than terrestrial oilseed plants and ease of cultivation in wastewater and saline water. Microalgae do not compete with agricultural land for cultivation. They have dual role such as utilization of CO_2 from atmosphere as well as remediation of wastewater by utilizing nutrients from wastewater to grow into biomass. Microalgae contain different types of major metabolites and high-value products such as proteins, lipids, carbohydrates, vitamins, pigments, antioxidants, minerals, etc. (Gupta et al. 2016; Mata et al. 2010; Rawat et al. 2011; Shriwastav et al. 2014; Francavilla et al. 2015). Their major metabolites are rich in essential amino acids and essential fatty acids, e.g. omega-3 fatty acids. Productivity of these major metabolites can be increased through mode of cultivation and nutrient limitation/stresses. Commonly, the lipids from microalgae are converted into biodiesel by the process of transesterification. After lipid extraction, a huge amount of residual biomass is left that is known as lipid-extracted algae (LEA). LEA still contains the high-value metabolites like proteins and carbohydrates in residual biomass (Ansari et al. 2015; Ju et al. 2012). Lipid-extracted algae can also serve as a good resource for biomethane, bioethanol and syngas production. In addition, protein fraction of LEA has promising potential as food and feed additive for animal and aquaculture. LEA biomass due to rich nitrogen content can also be employed as a fertilizer. Therefore, considering the rich chemical composition of microalgae, it can be considered as a good feedstock for the biorefinery.

2 Biochemical Composition of Microalgae

Proteins, lipids, carbohydrates, pigments, vitamins and minerals comprise the biochemical constituents of microalgae. Among all, lipids, proteins and carbohydrates are the major constituents. The microalgal proteins (6–52%) are rich in essential amino acids, and their yield percentage depends upon the mode of cultivation and nutrient limitation. Microalgal lipids are very suitable for biodiesel production via fatty acid esterification to produce fatty acid methyl ester (FAME). The lipids are also a good source of essential unsaturated fatty acids such as alpha-linolenic acid (ALA, C18:3), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), so it has potential application to be used as a food and feed ingredient. Microalgae are also a good source of carbohydrates mainly in the form of starch, cellulose, sugar and other polysaccharides, and the

biomass carbohydrate contents highly depend on types of species, cultivation condition and environmental factors. The overall microalgae carbohydrates have good digestibility.

2.1 Proteins

Microalgae abundantly are considered as a feedstock for biofuel production especially utilizing lipid, but besides lipid, they also contain many other valuable components. Proteins are the major primary metabolites in living organisms including the microalgae. Amino acids are the basic constituents of proteins which define the nutritional quality or value of protein on the basis of essential amino acid content, proportion and availability. Most of microalgal proteins are rich in essential amino acids. The proteins and amino acid profile of microalgae have been compared to different sources of food proteins and their proportion in which algal protein composition is nutritionally more favourable (Becker 2007). Recently, in different cases, microalgae proteins have recommended as a replacement protein source, due to their high nutrient quality and balanced content of essential amino acids (Romero Garcia et al. 2012). High protein yield directly depends upon cultivation condition and rich nitrogen source medium. In nitrogen limitation/starvation, fixed carbon produced by photosynthesis switches the metabolic pathway from protein to lipids or carbohydrates subsequently decreasing the protein yield (Singh et al. 2016).

2.2 Carbohydrates

Among the three major metabolites, carbohydrates are least rich in energy (15.7 kJ/g) (Wilhelm and Jakob 2011). The carbohydrates such as starch, cellulose and other polysaccharides are found in the form of storage products or the structural component of the cell wall. Microalgae cell lacks the lignin which makes them a good feedstock for food/feed ingredients since it requires no energy-intensive pretreatment. Although less in energy, microalgal carbohydrates have potential to become preferable feedstock for the production of biohydrogen, bioethanol, biobutanol and biomethane through integrating with biotechnological conversion technologies. The carbohydrate content in marine and freshwater microalgae varies significantly; microalgae *P. cruentum* and *P. tricornutum* contained 34.5 and 19.7% carbohydrates, respectively. While in fresh microalgae *Scenedesmus* sp., carbohydrate content was noted to be 23.3%. In nitrogen limitation, *C. vulgaris* accumulates 38.41%, *Tetraselmis cordiformis* accumulates 35%, *Spirulina maxima* accumulates 35% and *Spirulina platensis* accumulates 55–65% carbohydrates (Markou et al. 2012). It is also known that light energy is one of the most important energy sources for microalgae which affect the carbohydrate accumulation. High light intensity

(200–400 μmol m^{-2} $s^{-1.}$) resulted in high carbohydrates; *Porphyridium* sp. and *Spirulina maxima* were noted for threefold increase in carbohydrates upon enhanced light intensity (Markou et al. 2012).

2.3 Lipids

Lipids are one of the major primary metabolites of microalgae. The content of lipids varies between 15 and 60% on dry cell weight basis. Based on their polarity, microalgal lipids are generally classified into polar (structural) and non-polar or neutral (storage) lipids. Polar lipids are further subdivided into phospholipids and glycolipids. The function of the non-polar lipids, predominantly found in the form of TAG, is to store energy. These stored lipids are transesterified to produce biodiesel. Polar lipids form bilayer cell membrane and typically have high amount of PUFA; those have high potential for use in food/feed. Lipids in microalgae and their composition vary species to species such as some microalgae contain high amount of neutral lipid than others (Lv et al. 2010). Under starvation/nutrient limitation condition, microalga changes the metabolic pathway towards the storage of neutral lipids primarily in the form of TAG. For nutritional value of microalgae lipid, the controlled cultivation is very important that produces saturated and unsaturated fatty acids (PUFAs). Polyunsaturated fatty acids contained essential fatty acids such ALA, EPA and DHA which are used in feed and food for animals and humans.

2.4 Pigments

Microalgae colour is one of the most important characteristics which are determined by their pigments. These colour substances known as natural pigment have predominant role in the photosynthetic metabolism (D'Alessandro and Antoniosi Filho 2016). Apart from being photosynthetic components, they also have biological activities and act as antioxidants, anti-inflammatory agents, etc. Microalgae pigments are differentiated into three major classes: (a) carotenoids, (b) chlorophyll and (c) phycobiliproteins.

2.5 Carotenoids

These are the fat-soluble pigments, and their colour varies from brown, red, orange and yellow. The average carotenoid content in microalgae ranges in 0.1–0.2% which can go up to 14% on dry weight basis. Due to solubility in fat, they enter

in blood circulation and get attached to different lipoprotein. The human body cannot synthesize these essential pigments, so it is important to supplement these in diets. Based on chemical structure, carotenoids are divided into two groups, carotenes including beta-carotene and lycopene and xanthophylls including astaxanthin, lutein and canthaxanthin. On the basis of involvement in photosynthesis, carotenoids are subdivided into primary and secondary carotenoids. In primary carotenoids, only those carotenoids are included which are directly involved in photosynthesis, e.g. beta-carotene and lutein. Both these carotenoids function in light-harvesting and photoprotective action. Secondary carotenoid, e.g. astaxanthin and canthaxanthin, is not involved in photosynthesis process. *Haematococcus pluvialis* is known as a prime source of natural astaxanthin. It can produce more astaxanthin under nutrient limitation and contains 0.2–3% astaxanthin on dry weight basis (Batista et al. 2013). Beta-carotene is orange-yellow in colour. It has a large demand as a natural pigment or nutritional supplementation, and it is also the precursor for vitamin A. *Dunaliella salina* is used at industrial scale to produce beta-carotene (14%) (Spolaore et al. 2006). *Dunaliella salina* is the first microalgae used for the commercial production of the high-value product (beta-carotene) from microalgae. The world market of carotenoid is growing by 2.3% annually; in 2010, it had the market of 1.2 billion USD which is expected to reach 1.4 billion USD by 2018 (BCC-Research, The Global Market for Carotenoids 2011).

2.6 Chlorophyll

It is green-coloured, fat-soluble pigment with porphyrin ring in its structure and is ubiquitously found in nature. These are responsible for photosynthesis by converting solar energy into chemical energy. Chlorophyll is tetrapyrrole in structure in which magnesium ion is centrally placed. On the basis of light absorption spectra of microalgae, chlorophyll has been grouped in many types, e.g. chlorophyll a, b, c, d and f. Chlorophyll a has a blue-green colour, chlorophyll b is a brilliant green, chlorophyll c is yellow green, chlorophyll d is a brilliant/forest green and chlorophyll f is emerald green. Most of the microalgae have chlorophyll a and c as the dominant chlorophylls in which chlorophyll a is the major light-harvesting complex and contains chlorophyll in the range of 0.5–1.0% on dry cell weight basis. The commercial application of chlorophyll is observed in food and feed industries, cosmetics and pharmaceuticals.

2.7 Phycobiliproteins

It is water-soluble pigments, made up of cell protein and reasonably easy to isolate and purify. Phycobiliprotein content varies from 2 to 8% on dry cell weight basis. It

is water soluble, made up of protein and covalently bound with amino sulphur-containing amino acid—cysteine. Phycobiliprotein functions to accumulate light during photosynthesis. Phycobiliprotein, viz. phycocyanin and phycoerythrin, is commonly produced on commercial level from *Spirulina* spp. and *Porphyridium* spp., respectively. The phycobiliprotein is well known as the natural food colourant in pudding and as an antioxidant in immunology laboratories. Annual market for phycocyanin is around 5–10 million USD (Sekar and Chandramohan 2007).

3 Microalgae Cultivation

High-density cultivation of microalgae biomass for value-added product (VAP) extraction is still challenging mainly due to unavailability of water, land area requirement and inefficient illumination area, limitations of gas-liquid mass transfer, operational complications and contamination and production cost. Low density of microalgae biomass and small size of the microalgal cells add up more challenges to handle the culture for harvesting.

The commercial and cost-effective production of biofuels and other VAPs like food and feed ingredients from microalgae requires economic production of large quantity of algal biomass (Chisti 2007; Griffiths and Harrison 2009). Practically, suitable large-scale microalgae cultivation can be achieved via (i) open pond cultivation and (ii) closed photobioreactors (Carvalho et al. 2006). Figure 1 shows a generalized schematic representation of algae cultivation and biofuel production.

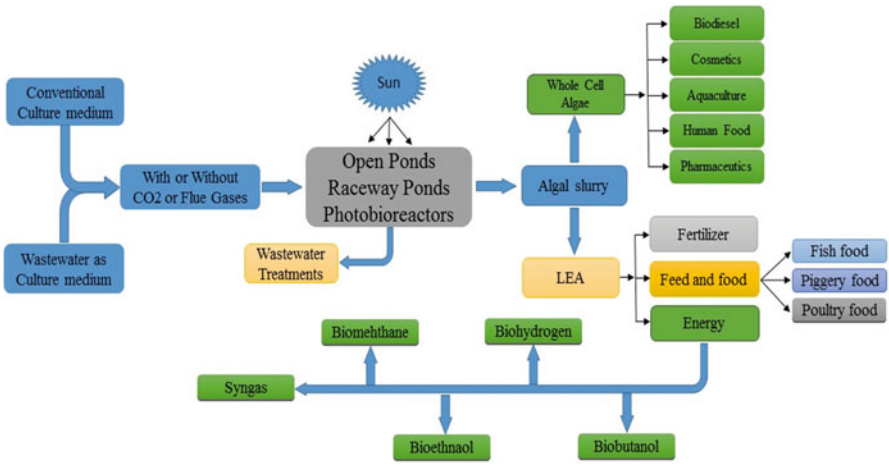

Fig. 1. Schematic representation of algae cultivation and biofuel production

3.1 Open Ponds and Raceways

Open raceway ponds are open systems that are most widely used for outdoor microalgae cultivation using solar irradiations. The open ponds are generally constructed using concrete, and since their shape resembles with racetrack, these ponds are called as raceway ponds. The open raceway ponds are easy to construct, operate and maintain. The depth of the open raceway ponds varies from one region to another depending on the intensity of the available sunlight. The optimized depth can be in the range of 15–30 cm from the surface. Depth is set such that the shadowing effect of the microalgae culture can be avoided to enhance the biomass productivity. The microalgae culture in the pond requires continuous stirring for mixing and recirculation of both culture and the nutrients. The mixing helps to avoid the formation of concentration gradients, also provides homogenous illumination and overcomes the shadowing effect if caused. Generally, stirring is provided by the use of paddle wheels.

There are many advantages and disadvantages of open raceway pond over closed photobioreactor. The open raceway pond is directly affected by both biotic and abiotic factors. The main disadvantages are lower productivity than closed photobioreactor. It is mainly due to low atmospheric CO_2 concentration and low gas-liquid mass transfer of CO_2 from the atmosphere resulting in lower dissolved carbon in algal culture medium which remains insufficient to meet the needs of photosynthesis. To overcome this challenge, an external chemical source of carbon such as carbonates or direct injection of CO_2 is done. Open raceway pond cultivation is also affected by water evaporation, fluctuations in temperature and variations in photoperiod. In addition, open raceway ponds, since being open to the environment, face contamination by other competing microalgal species making it a major challenge to maintain the monoalgal culture of a selected microalga. Therefore, extremophiles like *Spirulina* and *Dunaliella salina* are found to grow with lesser issues of contamination.

Apart from disadvantages, open raceway ponds have several advantages which include lower construction, operation and maintenance cost. Cleaning is less energy consuming than closed photobioreactors. These ponds can be constructed in deserts and nonarable lands. The net input energy is less than what is required for closed photobioreactor (Brennan and Owende 2010).

The cost of per kilogram of oil from algae grown in open raceway pond (7.64 USD) is cheaper than algae grown in closed photobioreactor (24.60 USD). The price of per kilogram algal biomass cultivated in open raceway pond (1.54 USD) is lesser than biomass obtained from photobioreactor (7.32 USD) (Rashid et al. 2014).

3.2 Photobioreactors

Basically, the photobioreactors allow monoalgal/axenic microalgae cultivation under controlled conditions to obtain high biomass for various food-, feed- and fuel-based applications. Several types of photobioreactors exist for cultivation of microalgae biomass. Biomass productivity, lipid content and lipid productivity of selected microalgae species in closed photobioreactors and the open ponds reported from various authors are summarized in Table 1. These include widely used tubular photobioreactors, plate reactors, bubble column reactors and not so frequently used semi-hollow spheres. Vertical-column photobioreactors are characterized by high mass transfer and good mixing with low shear stress. It has low energy consumption and can potentially be scaled up. It has reduced photoinhibition and photo-oxidation. In addition, it is advantageous for immobilization of microalgae and can be readily tempered (Ugwu et al. 2008). Flat panel photobioreactors on the other hand provide large illumination surface area and are noted for high biomass productivities (Ugwu et al. 2008). These are relatively cheap and easy to clean up and cause low oxygen build up. Tubular photobioreactor is considered for having large illumination area and is also suitable for outdoor cultivation. However, the vertical-column photobioreactors have limited/small illumination surface and are not considered worthy for scale up. Possible hydrodynamic stress is a challenge in

Table 1 Biomass productivity, lipid content and lipid productivity of selected microalgae species in closed photobioreactors and the open ponds

Microalgae	Cultivation condition	biomass productivity ($gL^{-1}day^{-1}$)	Lipid Productivity ($gL^{-1}day^{-1}$)	Lipid (%)	Reference
C. vulgaris	PBR	84.8 mg L^{-1} day^{-1}	10.3 mg L^{-1} day^{-1}	22.8	Frumento et al. (2013)
A. faculatus	Flask	–	74.07 mg L^{-1} day^{-1}	59.6	Singh et al. (2015)
N. atomus	HBR indoors	12.9 g m^{-2} day^{-1}	–	–	Dogaris et al. (2015)
N. atomus	HBR outdoors	18.2 g m^{-2} day^{-1}	–	–	Dogaris et al. (2015)
N. oculata	PBR	0.296–0.497	0.084–0.151	22.7–41.2	Chiu et al. (2009)
Chlorella saccharophila	Flask	23 mg/L^{-1} day^{-1}	4.16 mg/L^{-1} day^{-1}	18.1	Chinnasamy et al. (2010)
Nannochloropsis sp.	PBR	0.300–0.360	–	32.0–60.0	Briassoulis et al. (2010)
Mix culture	Flask	276 mg L^{-1} day^{-1}	–	23.62	Hena et al. (2015)
Porphyridium cruentum	–	0.37	9.5 mg L^{-1} day^{-1}	34.8	Ahmad et al. (2011)

(continued)

Table 1 (continued)

Microalgae	Cultivation condition	biomass productivity ($gL^{-1}day^{-1}$)	Lipid Productivity ($gL^{-1}day^{-1}$)	Lipid (%)	Reference
S. quadricauda	–	0.19	35.1	18.4	Ahmad et al. (2011)
Skeletonema sp. CS 252	–	0.09	27.3	31.8	Ahmad et al. (2011)
Scenedesmus sp. DM	–	0.26	53.9	21.1	Ahmad et al. (2011)
Pavlova salina CS 49	–	0.16	49.4	30.9	Ahmad et al. (2011)
Anabaena sp.	Open pond	–	0.24	–	Milano et al. (2016)
C. sorokiniana	Inclined tubular	–	1.47	–	Milano et al. (2016)
Tetraselmis	Column	–	0.42	–	Milano et al. (2016)
Scenedesmus sp.	Jar	–	0.07	–	Milano et al. (2016)
Chlorella	Flat plate	–	3.2–3.8	–	Milano et al. (2016)
C. vulgaris TISTR 8580	Bottle	–	12.9	28.1	Tongprawhan et al. (2014)
C. protothecoides TISTR 8243	Bottle	–	13.3	22.9	Tongprawhan et al. (2014)
Chlorococcum sp. TISTR 8416	Bottle	–	15.4	31.8	Tongprawhan et al. (2014)
Chlorella sp. TISTR 8263	Bottle	–	13.9	25.7	Tongprawhan et al. (2014)
S. armatus TISTR 8653	Bottle	–	10.7	21.4	Tongprawhan et al. (2014)
Marine *Chlorella* sp.	Bottle	–	21.3	28.2	Tongprawhan et al. (2014)

flat-panel photobioreactors. Tubular photobioreactors suffer with the disadvantage of forming gradients of pH, dissolved oxygen and CO2 along the reactor tubes. The major limitation with all these reactor systems is that they are costly to set up and operate (Lam and Lee 2012).

4 Applications of Microalgae Biomass for Biofuels

Microalgal biomass is rich in lipids which are suitable to produce biodiesel by fatty acid methyl ester (FAME). The biodiesel production from microalgae lipid can be integrated with the other energy-producing processes that could make the biodiesel

an economical and sustainable product. Apart from biodiesel, microalgae biomass can also be used to produce bioethanol by fermentation, biomethane by anaerobic digestion, biobutanol and syngas. Biofuel production capacities of various microalgal strains are summarized in Table 2.

Table 2 Biofuel production capacities of various microalgal strains

Microalgae	Whole cell/ LEA	Pretreatment	Target product	Yield	Reference
Mixed culture	Whole	Acid	Biobutanol	3.74 g/L	Castro et al. (2015)
C. vulgaris JSC-6	Whole	Acid + alkali	Biobutanol	13.1 g/L	Wang et al. (2016)
S. almeriensis	LEA	800°C	Syngas	94%	Beneroso et al. (2013)
C. vulgaris	Whole	Catalytic pyrolysis	Syngas	89.21%	Hu et al. (2014)
N. oculata	Whole	*N*-Methylmorpholine-N-oxide	Biomethane	339 mL_{CH4}/g_{vs}	Caporgno et al. (2016)
Tetraselmis sp.	LEA	AD with waste sludge	Biomethane	236 mL CH4/g VS_{added}	Hernandez et al. (2014)
–	–	Milling	Biomethane	0.304–0.557 L CH4/g VS	Zhao et al. (2014)
Tetraselmis spp.	LEA	Sonication	Biomethane	248 mL/g VS	Ward and Lewis (2015)
S. abundans PKUAC 12	Whole	Diluted acid	Bioethanol	0.103 g of ethanol/g DCW	Guo et al. (2013)
C. vulgaris FSP-E	Whole	Acid	Bioethanol	11.7 g/L	Ho et al. (2013)
Chlorella sp. KR-1	LEA	Diluted acid	Bioethanol	0.16 g/g LEA DCW	Lee et al. (2015)
S. obliquus CNW-N	Whole	–	Bioethanol	0.195 g EtOH/g biomass	Ho et al. (2013)
S. obliquus CNW-N	Whole	–	Bioethanol	0.202 g EtOH/g biomass	Ho et al. (2013)
S. obliquus CNW-N	Whole	–	Bioethanol	0.128 g EtOH/g biomass	Ho et al. (2013)
S. obliquus	Whole	*Aspergillus niger* whole cell lipase	Biodiesel	90.82	Guldhe et al. (2016)
C. sorokiniana	Whole	–	Biodiesel	91	Misra et al. (2014)
Scenedesmus sp.	LEA	Alkali and thermal	Biohydrogen	45.54 mL/g-volatile	Yang et al. (2010)

(continued)

Table 2 (continued)

Microalgae	Whole cell/ LEA	Pretreatment	Target product	Yield	Reference
Scenedesmus sp.	LEA	Thermal	Biohydrogen	40.27 mL/g VS	Yang et al. (2011)
Mixed culture	Whole	–	Biohydrogen	5.22 mmol	Chandra and Venkata Mohan (2011)
C. vulgaris	Whole	HCl Hydrolysis	Biohydrogen	0.94 mol/mol sugar	Liu et al. (2013)

4.1 Biodiesel

Microalgae are known as renewable feedstocks for biodiesel production due to ability to accumulate high amount of lipids. Among all major metabolites (lipids, proteins and carbohydrates) of microalgae, lipids have gained significant amount of interest to overcome fossil fuel crisis. The lipid content depends on species, and biomass condition such as lipids in lyophilized biomass of *Chlorella pyrenoidosa* (47%), dried biomass of *Nannochloropsis oculata* (26.8%), wet biomass of *C. vulgaris* ESP-31 (14–63%), algal cake of *C. vulgaris* ESP-31 (26.3%) and dried biomass of *C. pyrenoidosa (56.3%)* varies from species to species (Cao et al. 2013; Li et al. 2011; Tran et al. 2013). In normal cultivation condition, the capacity of lipid accumulation of various microalgae is low which hampers the biodiesel production cost. To surpass these challenges, many strategies have been developed such as cultivation in nutrient limitation/starvation, use of mixed culture, reactor design (open pond, closed photobioreactor, etc.) and supplementation of chemicals and hormones. Among all the lipid-enhancing strategies, the nutrient (nitrogen) limitation is widely used. Cultivation of *Chlorococcum nivale* and *Scenedesmus deserticola* in nitrogen starvation condition significantly enhanced lipid yield from 31.6 to 40.7% and 48 to 54%, respectively (Singh et al. 2016). In another study, Gao et al. (2013) found that cultivation of *Chaetoceros muelleri* under nitrogen limitation caused twofold increase in lipid yield (23–46%) and decrease in biomass productivity (19–12 mg $L^{-1}day^{-1}$). In chemical conversion of microalgal lipid to biodiesel via transesterification, lipid reacts with alcohol (e.g. methanol) in the existence of catalyst (e.g. acidic, alkaline or enzymes) and results in FAME and glycerol. There are two methods of transesterification, i.e. two-stage method in which biomass drying, lipid extraction and purification steps are involved, while in in situ transesterification (direct), lipid extraction and transesterification occur concomitantly. Johnson and Wen (2009) applied both methods of transesterification for *S. limacinum* biomass, and they obtained crude biodiesel (57%) and FAME (66.37%) by two-stage method and 66% of crude

biodiesel and 63.46% of FAME by direct transesterification (Johnson and Wen 2009). Guldhe et al. (2016) achieved 90.87% of yield and 80.97% of biodiesel conversion from *Scenedesmus obliquus* by using whole cell lipase enzyme of *Aspergillus niger* as catalyst (Guldhe et al. 2016).

4.2 Biomethane

Lipid extraction for current liquid biofuel from microalgae leaves approximately 60–70% of residual biomass as byproduct. Anaerobic digestion of LEA biomass is used as a substrate for the production of methane and the release of nutrients such as soluble nitrogen, phosphorus, etc. Anaerobic digestion is a series of process in which microorganisms break down the biodegradable substance in the absence of oxygen. The four key steps involved in anaerobic digestion are hydrolysis, acidogenesis, acetogenesis and methanogenesis. In hydrolysis process, large or complex organic molecule (carbohydrate, proteins, lipids) is broken down in the small constituents (e.g. sugar, amino acids and fatty acids) by microorganism. In acidogenesis, microorganisms further break down the remaining complex molecules into ammonia, CO_2 and H_2S. In acetogenesis, acetoacetate, CO_2 and H_2 are formed. In methanogenesis, methanogenic bacteria utilized intermediate product of other steps and transform it into methane, CO_2, and H_2O. Among all four steps, hydrolysis is a rate-limiting step; the whole process depends on hydrolysis. LEA biomass used as a substrate and fermentative bacteria is used as an inoculum that converts carbohydrates and proteins into biomethane. Several factors are involved and influence biomethane production like upstream (cultivation, harvesting and lipid extraction) and downstream processing (biomass pretreatment, C/N ratio and inoculum). LEA biomass which has low C/N ratio is not suitable for biomethane production (Rashid et al. 2014). To overcome low C/N ratio, in many cases, rich carbon waste (e.g. biodiesel byproduct glycerol) is utilized to improve the biomethane production. Widely, C/N ratio of microalgal biomass varies from 4.16 to 7.82, and when this ratio is lesser than 20, it is unsuitable for microorganism. It has been observed that C/N ratio lower than 15 shows detrimental effect and produces ammonia nitrogen (Ehimen et al. 2013; Ward et al. 2014). Pretreatment is the vital step for methane production; pretreatment increases the surface area, makes the substrate more digestible and improves the fluidity in the reactor. Different types of treatment like mechanical, ultrasound, microwave, thermal, chemical treatment, biological and combined pretreatments are used; however, heat treatment is the most efficient and is widely used for biomethane production. Thermal pretreatment of microalgal biomass (at 50–250°C) enhances solubilization, sanitizes the feedstock and produces high yield of methane (Rodriguez et al. 2015). Thermal treatment on whole and LEA biomass of *Nannochloropsis gaditana* shows that methane production has been enhanced by 40 and 15% by whole and LEA biomass, respectively (Alzate et al. 2014). Thermal pretreatment (150–170°C) on whole *N. salina* increased the methane yield by 40% (0.31 L/gVS) (Bohutskyi

et al. 2015). Hernandez et al. (2014) used supercritical CO_2 extraction ($SCCO_2$) techniques for lipid extraction from *Tetraselmis* sp. and found that LEA biomass has more potential (236 mL CH4/g VS_{added}) than whole algae. Lipid-extracting solvent (hexane, chloroform, etc.) system also affects the methane production (Choi et al. 2010; Yun et al. 2014).

4.3 Bioethanol

Bioethanol production from food crops (sugar cane and corn) can directly impact on food prices and deforestation. Second-generation feedstock for bioethanol production has a lot of challenges. Saccharification of lignocellulose is one of the major challenges because of resistance due to high content of lignin (Guo et al. 2013). In addition, these feedstocks are inexpensive than sugar, but lignocellulosic feedstock requires strong pretreatment prior to fermentation. Whole microalgae biomass as well as LEA biomass has potential to be used as an economical and sustainable feedstock for the production of bioethanol. Polysaccharide-rich microalgae biomass does not have lignin and therefore is easy and less resistant to conversion in fermentable sugar. Microalgal species like *C. vulgaris* and *C. reinhardtii* UTEX 90 stored starch as energy source; these species easily hydrolyze in glucose with chemical or enzymatic process (Brányiková et al. 2011; Choi et al. 2010). The combination of diluted acid and enzyme (cellulase) pretreatment method employed by Guo et al. (2013) for *S. abundans* PKUAC 12 biomass yielded 0.103 g of ethanol/g of dry weight algae. Among all pretreatment (chemical, enzymatical, combination of chemical and enzymatic, etc.) methods, dilute acid pretreatment is widely used. Chemical and enzymatic pretreatment was used for *C. vulgaris* with 51% carbohydrates, which resulted in 93.6% and 90.4% glucose yield, respectively (Ho et al. 2013). Hernández et al. (2015) compared the acid and enzymatic pretreatment of *Chlorella sorokiniana* and *Nannochloropsis gaditana* that caused monosaccharide yield of 128 and 129 mg/g DW, respectively. In case of *Scenedesmus almeriensis* under acid hydrolysis (for 60 min at 121°C), the yield of monosaccharides was 88 mg/g. Harun and Danquah (2011) used diluted acid (1% H_2SO4 v/v at 140°C for 30 min) hydrolysis as pretreatment of *Chlorococcum humicola*, and 7.20 g/L bioethanol was obtained when15 g/L of microalgae were used for pretreatment. The cost of pretreatment can be minimized by using carbohydrate-rich microalgal species.

4.4 Biobutanol

Carbohydrates are one of the major primary metabolites of microalgae, and its contents depend on the type of species and mode of cultivation. In microalgae, most knocked primary metabolite is lipids for biodiesel production which leaves the

defatted biomass after lipid extraction. Hence, whole and LEA biomass which contains carbohydrates can also be used to produce biobutanol. Butanol is one of the most plentiful biofuels produced by acetone-butanol-ethanol (ABE) fermentation process in which microorganism converts carbohydrate residues into acetone, butanol and ethanol via anaerobic process. Butanol is environmentally friendly, and it has potential to direct use in vehicles, and it is better than ethanol because of its greater energy content, better immiscible and lower volatility, corrodibility and hygroscopicity (Castro et al. 2015; Srirangan et al. 2012). In ABE fermentation process, the *Clostridium* species and *Clostridium acetobutylicum* are predominantly used for biobutanol production, and the ratio of the three products (acetate, butanol and ethanol) in ABE fermentation is 3:6:1 (Cheng et al. 2015). Pretreatment is crucial to increase the surface area of microalgal biomass and to make it more susceptible for microorganisms for biobutanol production. Dilute acid hydrolysed microalgal biomass produced the lower ABE (2.74 g/L), while combination of acid and enzymatic hydrolysed biomass yielded highest ABE (9.74 g/L) (Kumar and Gayen 2011). Castro et al. (2015) optimized the acid hydrolysis of microalgal biomass, and they found that 1.0 M acid concentration at 80–90°C for 120 min is optimum to get the sugar yield of 166.1 g/kg of dry algae and 3.74 g/L butanol production. Cheng et al. (2015) used LEA biomass as a substrate and *C. acetobutylicum* as a model microorganism and achieved butanol yield of 0.13 g/g carbohydrates.

4.5 Syngas

Microalgae are a feedstock for renewable energy production, but most of their energy-forming processes are time consuming and energy intensive and required chemicals and enzymes for the process. Hence, it is very important to select an appropriate method that can make biofuel economically viable. Many conversion strategies have been utilized for biofuel production, and among all, the pyrolysis is a more explored technology in which microalgal biomass gets transformed into solid, liquid and gaseous products (Shie et al. 2010). Syngas, also known as synthetic gas, is a mixture of different gases such as CO_2, CO and H_2. The syngas is produced by gasification in which microalgal biomass undergoes the heat treatment and biomass breaks down and produces gases (synthetic natural gas and to create ammonia or methanol) as primary product and char tars as byproducts. Syngas production involves many reactions such as oxidation reaction, water gas reaction, methanation reaction, water-gas shift reaction, etc. The production of syngas also depends on microalgal biomass quality, instrument used for gasification and process parameters such as temperature and catalyst used for gasification (Raheem et al. 2015). In production of syngas, temperature is a vital parameter. Syngas yield increases from 28 to 57% when temperature is increased from 552°C to 952°C (Raheem et al. 2015). For production of syngas, Hiranoa et al. (1998) partially oxidized the *Spirulina* sp. (at 850–1000°C) to find out theoretical

biomethanol production, and the findings showed that the microalgae biomass at 1000°C has optimum theoretical yield (0.64 g) of methanol per gram of biomass. Beneroso et al. (2013) carried out microwave-assisted pyrolysis to examine whole and extracted residues of *Scenedesmus almeriensis* at 400–800°C. The high yield of syngas (c.a. 94 vol%) was obtained at 800°C after pyrolysis of residues.

5 Process Constraints and Future Needs

Microalgae are the third-generation feedstock for biodiesel production. It has promising potential for biofuel production and also offers many other valuable products. Apart from CO_2 sequestration, microalgae also carry out phytoremediation. The biggest process constraint is that high biomass is not achieved in microalgae cultivation. Low biomass production and single-product strategy are one of the bottlenecks in developing economical and sustainable microalgae industry. The requirement of huge volumes of water always remains a big challenge in microalgae cultivation. Economical and effective biomass harvesting technology is still in demand. The cost of biomass production remains high in closed photobioreactor, and open raceway ponds suffer from low biomass productivity and contamination issues. Multiproduct development strategy from microalgae biomass can make the microalgae biotechnology processes the viable and economical one. Integration of microalgae production with simultaneous wastewater treatment has the potential for sustainable biomass generation for biofuel and feed-/fertilizer-related products.

5.1 Factors Limiting Growth and Biomass Production

For large-scale commercial production of microalgae biomass, closed photobioreactor and open raceway systems are widely used. The choice of cultivation system depends on the final product; closed photobioreactors were always preferred for high-value product synthesis from microalgae. Both cultivation systems have their own advantages and disadvantages. Microalgae are photosynthetic organism, so light is one of the limiting factors for growth and biomass production. Light does not penetrate in the dense microalgae culture. In an open system, it is very hard to control and supply optimum light condition for optimum growth and biomass production. The other evaporation of water causes changes in ionic composition and pH of the medium. Seasonal variation also negatively affects photoperiod hours and biological clock of microalgae. Large-scale open system always has high-risk contamination. The unwanted microorganism such as protozoa, zooplankton and other undesirable microalgae species competes for nutrients. These unwanted microorganisms are known as grazer that grazes microalgae in 2–3 days. Zooplankton can reduce 90% of the microalgae cell density in 48 h,

while *Daphnia* could bring massive change of over 99% in a few days (Rawat et al. 2013). In large-scale microalgae cultivation, mechanical failure in the system cannot be ignored. Therefore, the high biomass production remains one of the challenges in microalgae biotechnology.

5.2 Environmental Sustainability of Algal Biodiesel

Microalgae are the photosynthetic unicellular organism. It requires solar light and CO_2 from environment to fix and grow into biomass. Biodiesel production from microalgae is eco-friendly because it releases low levels of NO_x and SO_x after combustion. Most importantly microalgal biodiesel is compatible with existing combustion engines without any further modifications (Rashid et al. 2014). Microalgae biodiesel also has similar fuel properties (density, viscosity, flash point, cold flow and heating value) like petrodiesel. Around the globe, climate change is one of the most debatable topics. In climate change, CO_2 which is emitted by anthropogenic activities plays an important role. For production of one ton of microalgae biomass, microalgae consume 1.83 tons of CO_2 (Chisti 2007). The microalgae cultivation could be integrated with industry such as cement factory to provide CO_2 in proper utilization. It is very important to determine the carbon footprint. Carbon footprint of microalgal biodiesel is lower than the petroleum fuel. Microalgae water footprint (WF) is the water required for cultivation and media preparation. WF is predominantly based on evaporation rate, hydraulic retention time and photosynthesis rate. Evaporation rate highly depends upon local climate from 0.48 $m^3\ m^{-2}\ year^{-1}$ to 2.28 $m^3\ m^{-2}\ year^{-1}$ in arid regions (Usher et al. 2014). The average annual WF of microalgae biodiesel grown in open raceway pond and closed photobioreactor is 14–87 and 1–2 m^3/GJ significantly lower than biodiesel produced from soybean (287 m^3/GJ) (Usher et al. 2014). The carbon footprint is acceptable if it is lower than the petroleum fuel or equal on energy basis (Chisti 2013). Microalgae cultivation does not require freshwater; it can grow in domestic wastewater, municipal wastewater, industrial wastewater and marine water. Integration of wastewater treatment and microalgae cultivation can make biodiesel production a sustainable process (Gupta et al. 2016; Rawat et al. 2011; Shriwastav et al. 2014).

5.3 Economic Sustainability of Algal Biofuels

The price of microalgal biomass cultivated in open raceway pond and closed photobioreactor is $7.32 and $1.54, respectively, while the price of microalgae oil per kilogram grown on raceway and photobioreactor is $7.64 and $24.60, respectively. The cost of microalgal biodiesel is very high, and it must be reduced to make it commercially viable. The price of microalgae biodiesel per barrel is US

$300–2600 in oil market (Rashid et al. 2014). It is also found in many studies that algal biofuel price is double of petrol fuel. According to Chisti et al., the price of microalgae oil without transport charge and taxes is $2.80 per litre. In current time, the price of crude oil is less than $60 per barrel. To replace 1% of annual US petroleum consumption, a huge amount (~31 million tons) of biomass with 40% oil (w/v) is required (Chisti 2013). To make microalgae economical and sustainable, low-cost microalgae cultivation, widely accepted harvesting process and green technology to extract high oil yield are required. Integration of microalgal biofuel technology to other technologies is very important to further reduce the overall biodiesel production cost. Integration like the use of treated wastewater for microalgal cultivation, use biomass for aquaculture feed and LEA for other applications will help to reduce the overall cost of microalgae products. The use of wet biomass directly to extract oil and transesterification for biodiesel production can also be one of the strategies. The use of residual biomass in aquaculture feed, piggery feed, poultry feed and animal feed could be alternative and novel idea. The high content of carbon in residual biomass can be used for biomethane, bioethanol, biobutanol and syngas production. It can also be used as a conventional fertilizer to enhance the crop productivity.

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Algal Biofilm Systems: An Answer to Algal Biofuel Dilemma

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Contents

1 Introduction

Among all energy feedstock, microalgae are promising candidates due to their ability to adapt and grow in diverse environments (natural and engineered) and cultivation types (open ponds and photobioreactors). In addition, its ability to grow on a range of wastewater streams makes it more economically and environmentally feasible than other terrestrial feedstock for biofuel production (Choudhary et al. 2016a). Also, microalgae have great biodiversity in biomass and consequent variability in their biochemical composition which varies with type of nutrient medium

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(Prajapati et al. 2013). High lipid-containing wastewater-grown algal biomass can be used to produce biodiesel substantially higher than existing oilseed crops (Drira et al. 2016). Algal biomass can be directly fed in an anaerobic digester for biomethane production (Prajapati et al. 2014) or fermented to produce bioethanol (Hwang et al. 2016). Residual biomass from these processes can further be utilized as a livestock or fish feed and fertilizer (Mulbry et al. 2008a; Roeselers et al. 2008). However, the production of biofuels and bioproducts from algal biomass is still in infant stage due to lack of any reliable and cost-effective harvesting method (Choudhary et al. 2015).

The two major challenges to the implementation of an integrated algal system include the harvesting and processing of biomass in a way that allows for downstream processing to produce biofuels and other valuable bioproducts. The only practicable methods of large-scale production of microalgae are open raceways and closed photobioreactors of various designs in which microalgae are cultivated in suspended form. Raceways are of low construction and operational cost but are of low biomass productivity as compared to a photobioreactor which is of high operating costs. In spite of the benefits or limitations of open and closed suspended algal cultivation methods, substantial challenges are present like biomass harvesting that can account for up to 30–40% of total operating costs (Lee and Ahn 2014). The small size of algal cells (2–40 μm) in suspended open pond systems makes the harvest of biomass difficult and requires energy-intensive harvesting methods such as filtration, flotation, flocculation, sedimentation, and centrifugation. Recently, algal biofilm systems have drawn interest as an alternative to suspension-based culture systems. In these systems microalgae grow as biofilm on material surface rather than in suspension. The algae can be harvested simply by scraping and has a solid content of 10–20% which is higher than that obtained by centrifugation (Gross 2013). As compared to suspended growth, attached microalgae are advantageous for the mass cultivation of algae because of their ability to concentrate biomass naturally in a relatively small footprint area and are more readily harvestable (Lin et al. 2003). The high potential of algal biofilm to convert light energy into suitable biomass (as food or energy precursor) shifted the focus toward engineering the algal biofilm systems (Heimann 2016; Shen et al. 2016).

2 Algal Biofilm Development and Dynamics

Depending on the cultivation systems, microalgae can be either phytoplankton growing in suspension condition or benthic growing on substrata/support in association with other microorganisms. The benthic microalgae, either found naturally (aquatic, soil, and on tissues of plants and animals) or in some artificial systems (porous materials, plumbing systems, pipelines, and separation membranes), are termed as microalgae biofilms (Irving 2010). They are defined as autotrophic population of microalgae and bacteria embedded in a gel-like matrix of extracellular polymeric substances (EPS) which colonize the substrate in aquatic

environments as visible film (Nobre et al. 2013). According to Berner et al. (2015), the formation of microalgal biofilm starts with initial attachment of microalgae to a surface due to interaction between cells and attachment surface or with other cells already colonized to the surface (Di Pippo et al. 2009). With increasing attachment, microalgal cells start forming microcolonies and secrete a sticky matrix of EPS. The EPS secreted by various microorganisms, such as bacteria and microalgae, consists of high-molecular-weight compounds such as proteins, nucleic acids, lipids, polysaccharides, and humic acids. The EPS matrix not only acts as a diffusion barrier and adsorbent but also provides stability and facilitates a suitable microenvironment for microbial development and interactions (Riding 2000). Gradually, the microalgal biofilm along with EPS matrix grows into heterogeneous cluster of cells and voids which form irregular three-dimensional networks (Berner et al. 2015). The different organisms in the multispecies biofilm colonize the favorable zones and form heterogeneous structure with distinct patterns (De Beer et al. 1997; Kesaano and Sims 2014).

The EPS plays an important role in initial adhesion of cells to the surface and is influenced by multiple factors like biofilm age, nutrient availability, species composition, response to stress, and also indirectly linked to temperature and light via algal photosynthesis (Berner et al. 2015; Kesaano and Sims 2014). For designing and developing algal biofilm systems, the deep understanding of all parameters is required for controlled formation and survival of algal biofilms. Di Pippo et al. (2009) showed direct correlation of EPS produced between quality and quantity of biofilm w.r.t biomass. Wolfstein and Stal (2002) studied simultaneous effect of irradiance and temperature on EPS and showed that with increasing temperature and light intensity, the amount of EPS is enhanced. The process of EPS production is shown to be a complicated process, which is affected by many physiochemical parameters (Roeselers et al. 2008). Hence, more extensive research on structures of biofilms by techniques like confocal laser scanning microscopy are less practical in a production context and mostly limited to small-scale reactors. Moreover, it is also considered that the mechanisms of biofilm development process vary from species to species (Roeselers et al. 2008), so it is difficult to conclude a single possible mechanism feasible for all kinds of biofilm systems.

Once the algal biofilm is established, it is the interaction between different microorganisms (autotrophic and heterotrophic) in the EPS connected network which makes it functional (Roeselers et al. 2007). Romaní and Sabater (2000) have shown that extracellular enzymes from EPS that contribute to degradation of organic matter reinforce the interdependence between biofilm structural components (algae and bacteria) and their activities. Éva et al. (2007) did some bacteriological and algological investigations to identify changes in the element contents of biofilms. Authors demonstrated that strong interactions between algae and bacteria in biofilm communities exist in initial colonization process. The authors concluded that the higher the bacterial diversity and abundance, the more carbon sources were available to algae, and the more algae attached to the surface, the higher surface area for bacterial attachment. Bacteria were said to provide inorganic carbon to algae, which in turn supplied organic carbon and oxygen to bacteria. The

algal-bacterial dynamics and interactions within a biofilm are schematically diagrammed in Fig. 2. In another study conducted by Irving and Allen (2011), it was observed that *Chlorella vulgaris* shifted from suspended to attached growth in the presence of other species (non-sterile conditions). Results showed that higher percentage of total attached biomass was found in non-sterile (79.8% attachment) than in sterile (23.7% attachment) conditions. Many authors have also confirmed that the initial colonization phase of the algal-bacterial biofilms is faster on surfaces precolonized by heterotrophic bacteria (Roeselers et al. 2007; Romaní and Sabater 2000). The role of bacteria in the development and physiology of algal biofilm was evaluated by Rivas et al. (2010), and results showed that the ability of bacteria to produce QS signals is mainly responsible for enhanced growth of both bacteria and algae.

The major groups of microorganisms detected in algal biofilms are algae, cyanobacteria, and heterotrophic bacteria, while protozoa are also frequently present. Apart from green algae, many large colony-forming cyanobacterium species like *Phormidium*, *Microcystis*, *Oscillatoria*, *Closterium* sp., *Anabaena*, and *Aphanizomenon* have received particular attention, because they often dominate the biofilm in eutrophic lakes (Ahn et al. 2013). The species composition analysis of biofilms from the three water sources by Ahn et al. (2013) showed dominance of diverse species of *Bacillariophyceae* (*Asterionella* sp., *Nitzschia* sp., *Fragilaria* sp.) and *Cyanophyceae* (*Phormidium* sp. and *Microcycstis* sp.). *Phormidium*, a filamentous cyanophyte, is being reported to dominate systems with high temperatures and nutrient concentrations with low depths (Boelee et al. 2014a; Talbot 1993). In engineered biofilm cultivation systems, it is crucial to understand that the initial colonization depends on the biofilm composition and attachment surface but also on the growth medium and flow conditions (Czaczyk and Myszka 2007). The flow velocity is also critical for biofilm development because it determines the rate of supply of nutrients and discharge of waste materials. If the flow is turbulent, detachment of cells from surface occurs and hence decreases the thickness of biofilm and will consequently reduce system productivity (Boelee et al. 2014b) Moreover, strong flow increases the shear stress on biofilm structure and causes loss of harvestable biomass (Berner et al. 2015). Zippel et al. (2007) used a flow-lane incubator that allows simultaneous control of irradiance, temperature, and flow velocity and concluded that a low flow rate can improve cell attachment at the beginning of biofilm growth, while a higher flow is desirable for further development for algal biofilm. Water velocities higher than critical values may cause physical disruption and displacement of cells and hence reduce the biofilm thickness and productivity (Berner et al. 2015).

Species distribution and dominance are also affected by flow velocity of cultivation medium and temperature. Liu et al. (2016) observed the effect of flow rate on algal biofilm community of an outdoor Algal Turf Scrubber (ATS). The abundance of 66–73% for cyanobacterium *Stigeoclonium* in the beginning was decreased sharply to 8 % at high flow rate (8 L min^{-1}), while its abundance remained 46 % at medium flow rate (2 L min^{-1}) and 20–31 % at lower flow rates (4–6 L min^{-1}). For unicellular algae *Desmodesmus*, the abundance was greatly increased from 5 %

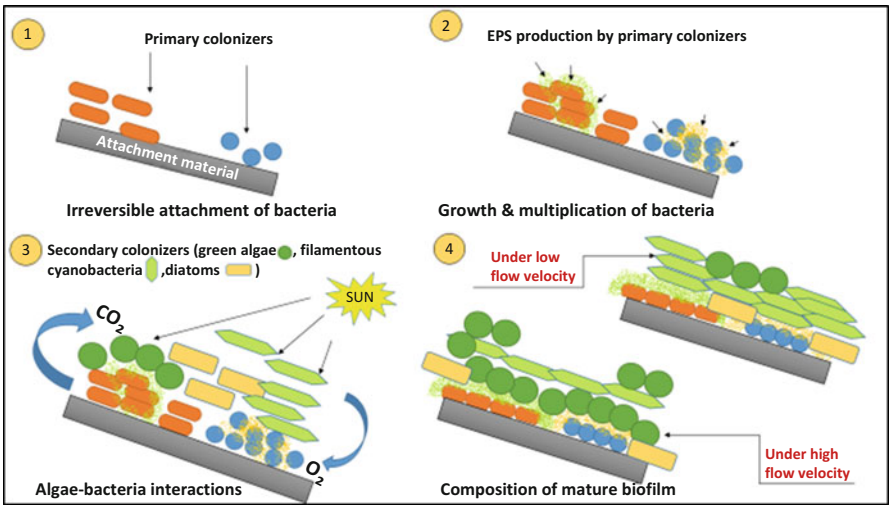

Fig. 1 Schematic representing algal biofilm formation process and its dynamics

in the beginning to 71 % when flow rate was increased to 8 L min^{-1}. In summary, lower flow rates induce dominance of filamentous algae in the biofilm, and higher flow rates enhance colonization of green algae (Fig. 1). Temperature is another important factor that determines the biofilm composition. The increase in temperature might enhance the enzymatic activities for the degradation of available organic matter by bacteria and enhanced photosynthetic, respiration, and growth rates of algae. The study conducted by Villanueva et al. (2011) showed greater bacterial growth rate and earlier bacterial colonization at the higher temperature. It was hypothesized that only initial colonization process is affected by temperature, but at the end of the biofilm formation, the process might be buffered by the unique structural properties of the biofilms and hence remain unchanged with respect to temperature.

3 Biofilm Cultivation System: An Alternative Platform for Biomass Production with In Situ Harvesting

Microalgae biofilm cultivation systems are considered as most suitable culture technique when high-volume biomass is required for biofuel purposes. Depending on the application, different biofilm systems with varying designs, geometry configurations, and attachment materials have been developed (Hoh et al. 2016). The schematic of major categories of biofilm systems is being represented in Fig. 2. The efficiency of biofilm systems is directly related to the attachment material type, surface area, and design of reactor. On the basis of movement of biofilm support

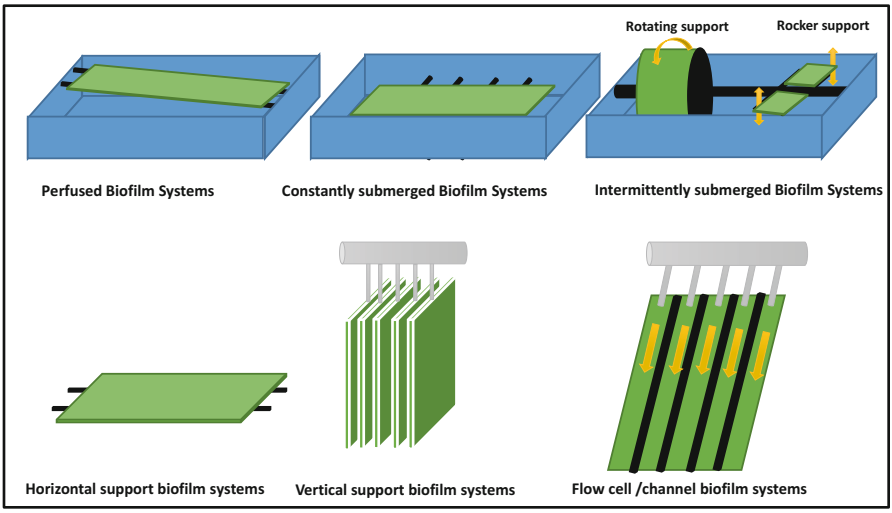

Fig. 2 Schematic of different biofilm cultivation systems

relative to culture media, it can be categorized as stationary and rotating. Based on the position of biofilm support, stationary systems can be further categorized into horizontal, vertical, and flow cell (Hoh et al. 2016). Another categorization can be based on the relative movement of cultivation medium and biofilm support. When cultivation medium is allowed to flow over stationary nutrient permeable support surface, it is called perfused biofilm systems (Heimann 2016). On the other hand, the support surface can be immersed in cultivation medium either all the times as in completely submerged systems or for some time as in intermittently submerged systems (Berner et al. 2015).

Though horizontal biofilm reactors have the advantage of effective absorption for photosynthesis, requirement of large surface limits its application in areas of space limitations (Ozkan et al. 2012; Posadas et al. 2013). The horizontal biofilm reactor developed by Ozkan et al. (2012) used concrete as a cultivation surface and provided 0.5–3.1 g m^{-2} day^{-1} biomass productivity using a microalgae consortium and 0.71 g m^{-2} day^{-1} biomass productivity using *B. braunii*. In a similar study, Posadas et al. (2013) used an algal-bacterial reactor and showed that algal-bacterial biofilm exhibited twice the biomass as compared to the bacterial biofilm reactors.

In contrast to the horizontal biofilm reactors, vertical systems contain vertically positioned attachment surface which may consist of either single-layer or multi-layer support systems (Gross et al. 2015a). The vertical plate reactors have advantages of small system footprints and spacing for effective light dilution. Liu et al. (2013) introduced a multiple layer vertical biofilm reactor where more than one algal films were joined in a manner to dilute the high intensity of sunlight which may cause photoinhibition. Results showed that the concept of multiple films facilitated high photosynthetic efficiency and resulted in high biomass

productivity up to 15 g m^{-2} day^{-1} for *Scenedesmus obliquus*. In a similar study, Shi et al. (2014) used a twin-layer approach, based on a prototype of Naumann et al. (2013) to separate the biomass from the liquid medium with a provision for intermittent contact. The authors achieved mean areal biomass productivity of 6.3 g DW m^{-2} day^{-1}.

The constantly submerged systems are usually designed as flow cells or channels in which microalgae grow on a solid surface which remain embedded persistently in a layer of growth medium. To recirculate the medium over growth surface, pumping costs are higher for horizontal systems and comparatively lower for inclined systems (Berner et al. 2015). Zippel et al. (2007) developed a four-lane flow cell incubator for cultivation of phototrophic biofilms and reported the productivity in the range of 1–50 g m^{-2} under different irradiance and flow rates when grown on polycarbonate for 21 days. With another flow cell reactor, Guzzon et al. (2008) produced biomass productivity of 2.9 g m^{-2} day^{-1} using polycarbonate slides as biofilm support. In a recent study, Irving and Allen (2011) showed higher biomass productivity of 5.5 g m^{-2} day^{-1} in flow cell utilizing *Scenedesmus obliquus* and *Chlorella vulgaris*. Apart from high volumetric productivity, the reactor facilitated easy monitoring of biofilm development due to extremely small path length. Another recent study utilizing biofilm of mixed microalgae cells grown on PVC plastic sheet showed productivity of 2.1–7.7 g m^{-2} day^{-1} which was highest for constantly submerged system (Boelee et al. 2011). The resulted higher biomass productivity and photosynthetic efficiency of biofilm systems may be due to the improved light and nutrient availability due to direct contact of cells and light or air.

In intermittently submerged systems, three main types have been identified depending on configuration of biofilm support systems: Algal Turf Scrubbers, rocking cultivation system, and rotating biofilm reactor (Shen et al. 2016). The advantage of these systems is that biofilm is restored by fresh nutrients while exposed directly to air and light (Berner et al. 2015; Hoh et al. 2016). One of the first systems in this category was Algal Turf Scrubbers (ATS) and their derivatives, similar to flow cells or channels, and it is also the most developed and popular cultivation type for algal biomass production under pilot scales (Adey et al. 2011; Liu et al. 2016). A number of authors (Adey et al. 2011; Kebede-Westhead et al. 2003; Mulbry et al. 2008b; Pizarro et al. 2006; Wilkie and Mulbry 2002) have used ATS for algal cultivation coupled with wastewater treatment. Mulbry and Wilkie (2001) used laboratory scale ATS for biomass cultivation of wastewater consortium grown on polyethylene and reported productivity of 5 g m^{-2} day^{-1}. Further assessment of pilot-scale ATS under outdoor conditions showed higher biomass productivity of 25 g m^{-2} d^{-1} (Mulbry et al. 2008b).

The second type is rocking cultivation system which is designed to mimic the wave action existing in natural aquatic systems. Johnson and Wen (2010) were first to introduce this type of system, to investigate microalgal biofilms especially for biomass production. Using this laboratory system, *Chlorella* sp. showed biomass productivity of 2.57 g m^{-2} day^{-1}. With another rocker system based on the same

approach, Shen et al. (2014b) used *Chlorococcum* sp. as the best-performing algae in terms of adhesion and produced 0.53–1.47 g m^{-2} day^{-1} under optimized growth conditions.

The third type, i.e., rotating biological contractors (RBC), is the most recently developed algal biofilm systems. The first bench-scale rotating algal biofilm reactor, designed by Christenson and Sims (2012), consisted of a rotating wheel wrapped in a rope, with the rope acting as attachment material. It coupled harvesting by continuously running the rope through an apparatus which scrape off the biofilm from the rope automatically. Apart from in situ harvesting, the system showed high productivity of 14 g m^{-2} day^{-1}. Orandi et al. (2012) used a modified form of RBC consisting of several vertical disks rotating in a bioreactor containing nutrient medium and achieved 0.74 g m^{-2} day^{-1}. A recent study by Blanken et al. (2014) used Algadisk RBC which was of similar design but with only a single disk rather than multiple disks and showed very high productivity of 20.1 g (DW) m^{-2} day^{-1} utilizing *Chlorella sorokiniana*. Gross et al. (2013, 2015b) have reported another configuration of an RBC system based on a vertical conveyor belt design where a sheet of attachment material is rotated through liquid with a plurality of drive shafts. Although the system achieved a productivity of 3.5 g m^{-2} d^{-1}, it could be mechanically complicated in context to harvesting.

The different biofilm systems are categorically represented in Table 1. In summary, not a single system is being identified as the best system efficient under all physical (control/outdoor) and nutrient (standard media/wastewater) conditions. Hence, there is a need to develop an inexpensive, efficient, and scalable biofilm system that can drastically and efficiently reduce the energy and water requirements of the process.

4 Design Considerations/Requirements of Algal Biofilm System

Most of the documented studies on algal biofilm are focused on nutrient removal at bench- and pilot-scale levels. However, limited research is done on utilization of algal biofilm systems for biomass production and further conversion into biofuels. For engineered biofilm systems, the knowledge of factors affecting the biomass production efficiency of developed reactor is important for successful application. Considering the application of algal biofilm system under outdoor conditions, the effect of environmental factors like light and temperature is not included in this section. For long-term outdoor operation of algal biofilm, the choice of attachment material and selection of suitable harvesting frequency are most important design parameters to be considered.

Table 1 Categorization of biofilm systems (Modified from Berner et al. 2015)

Biofilm cultivation system with types	Attachment material	Species	Productivity ($g\ m^{-2}\ day^{-1}$)	Reference
Perfused system with vertical support				
Twin-layer PBR	Polycarbonate membranes	*Halochlorella rubescens*	1.7–6.6	Schultze et al. (2015)
Multiple-layer photobioreactor	Filter paper	*Botryococcus braunii*	2.9	Wang et al. (2015)
Twin-layer PBR	Nylon filter	*Halochlorella rubescens*	1.2	Shi et al. (2014)
Pilot-scale phototrophic biofilm reactor	Polyethylene woven geotextile	Wastewater consortium	2.7–4.5	Boelee et al. (2014a, b)
Twin-layer PBR	Paper filter	*Isochrysis* sp. TISO	0.6	Naumann et al. (2013)
		Nannochloropsis sp.	0.8	
		Phaeodactylum tricornutum	1.5	
		Tetraselmis suecia	1.8	
Attached cultivation reactor	Cellulose acetate/ nitrate filter	*Scenedesmus obliquus*	7.1	Liu et al. (2013)
Attached cultivation bioreactor	Cellulose acetate/ nitrate filter	*Botryococcus braunii*	5.49	Cheng et al. (2013)
Perfused system with horizontal support				
Porous substrate photobioreactor (PSBR)	Electrostatic flocking cloth (EFC)	*Spirulina platensis*	6–7 (Indoor) 46–70 (Outdoor)	Zhang et al. (2015)
Attached cultivation system	Cellulose acetate/ nitrate filter	*Acutodesmus obliquus*	9.16	Ji et al. (2014)
Porous substrate bioreactor	Glass fiber filter	*Anabaena variabilis*	2.8	Murphy and Berberoglu (2014)
Filter in test tube	Chromatography filter	*Trentepohlia aurea*	1.0	Abe et al. (2003)
Perfused system with inclined support				
Algal biofilm reactor (ABR)	Nonwoven spun bond fabric	Mixed culture (*Chlorella* and *Phormidium*)	3.1–4.0	Choudhary et al. (2016b)
Constantly submerged system with vertical support				
Attached cultivation systems	Glass fiber-reinforced plastic	*Nannochloropsis oculata*	3.87	Shen et al. (2014b)
Biofilm bioreactor	Foam polyvinylchloride	Wastewater consortium	3.1	Posadas et al. (2013)
Biofilm flow cell	Polyvinyl chloride	Wastewater consortium	7.7	Boelee et al. (2012)

(continued)

Table 1 (continued)

Biofilm cultivation system with types	Attachment material	Species	Productivity (g m^{-2} day^{-1})	Reference
Flow-lane incubator	Polycarbonate	Cyanobacteria isolates	3.33	Bruno et al. (2012)
Constantly submerged systems with horizontal support				
Biofilm system	Polyester mesh discs	Cyanobacterial strains (dominated *by Phormidium or Oscillatoria)*	0.26 mg chl *a* mg^{-1} P 0.12 mg chl *a* mg^{-1} N	Rai et al. (2016)
Continuous flow-lane incubator	Polycarbonate slides	*Anabaena augstumalis* VRUC163, *Calothrix* sp. VRUC166 *Nostoc* sp. VRUC167	1.29 1.19 1.19	Di Pippo et al. (2013), Gismondi et al. (2016)
Intermittently submerged systems with horizontal support				
Algal Turf Scrubbers (horizontal/inclined)	Polyethylene	Wastewater consortium	25	Mulbry et al. (2008a, b)
Algal Turf Scrubber	Polyethylene	Wastewater consortium	9.4	Kebede-Westhead et al. (2006)
Intermittently submerged systems with rocking support				
Rocking cultivation chamber	Glass fiber-reinforced plastic	*Chlorococcum* sp.	4.26	Shen et al. (2014a)
Rocking attached cultivation system	Polystyrene	*Chlorella* sp.	2.57	Johnson and Wen (2010)
Intermittently submerged systems with rotating support				
Rotating algal biofilm reactor (RABR)	Cotton cord	Consortium from pond	0.96	Shayan et al. (2016)
Drum biofilm reactor (DBR)	Canvas	*Chlorella vulgaris*	54.46	Shen et al. (2016)
Revolving Algal Biofilm (RAB) growth system	Cotton duct canvas	*Chlorella vulgaris*	29.58 (trough based) 15.2 (raceway-based)	Gross et al. (2015b)
Algadisk (rotating biological contractor)	Stainless steel mesh	*Chlorella sorokiniana*	20.1	Blanken et al. (2014)
Pilot-scale rotating algal biofilm system	Cotton duct	*Chlorella vulgaris*	4.29	Gross et al. (2013)
Photo-rotating biological contractor	Polyvinyl chloride	Acid mine drainage consortium	0.74	Orandi et al. (2012)

(continued)

Table 1 (continued)

Biofilm cultivation system with types	Attachment material	Species	Productivity ($g\ m^{-2}\ day^{-1}$)	Reference
Bench-scale rotating algal biofilm reactor Pilot-scale rotating algal biofilm reactor RABR-enhanced raceway	Cotton rope	Wastewater consortium	1.9 14 6.57	Christenson and Sims (2012)

4.1 Attachment Material/Biofilm Growth Support

Extensive research has been done to study the effects of various supports on algal biomass production (Kesaano and Sims 2014). The surface characteristics and material composition of support determine surface area for biofilm growth and protection against hydraulic shear forces (Hoh et al. 2016). Johnson and Wen (2010) screened six different materials (nylon sponge, cardboard, polystyrene foam, polyethylene, and landscape fabric) for biofilm growth support and found polystyrene foam as the best performer in terms of firm attachment and high biomass yield. Lee and Ahn (2014) compared the algal biomass productivity of biofilm harvested from polycarbonate and polyethylene plates and nylon and stainless steel mesh. The mesh made of nylon and stainless steel facilitated easier harvesting and exhibited the highest biomass productivity as compared to plates. Christenson and Sims (2011) used eight different substrata for construction of cord (acrylic nylon, cotton, polypropylene, and jute) and sheet (low thread cotton, polyester, and high thread cotton) to be used as attachment material. Among all the materials, cotton cord was found as an effective substratum. Authors concluded that the cellulosic nature of cotton resulted in high surface energy and hence achieved greater attachment than synthetic polymers which are generally characterized by low surface energy.

Researchers have proposed various mechanisms like hydrophobic interactions or acid-base interactions to describe the attachment and colonization of cells (Ozkan and Berberoglu 2013; Palmer et al. 2007). In order to study the influence of surface attachment, surface roughness, pH of the medium, culture age, culture density, and presence of organic and bacterial films on the adhesion of *Nitzschia amphibia*, Sekar et al. (2004) tested titanium, stainless steel, and glass surface as attachment materials. The study showed higher attachment of the algae onto the hydrophobic materials (titanium, perspex, and stainless steel) as compared to hydrophilic surfaces (glass). Irving and Allen (2011) also tested a range of materials like polyethylene, borosilicate glass, and polyurethane and poly(methyl methacrylate) to assess the effect of hydrophobicity (water-material contact angle) on cell adhesion and concluded that while hydrophobicity is an important factor in algal adhesion, its

exact effects can vary from species to species. However, understanding of the exact surface properties responsible for algal attachment needs detailed research. Apart from physiochemical properties, texture (surface roughness) also plays an important role in algal attachment. Cao et al. (2009) proposed that on increasing the surface texture (roughness), zones are created where velocity is slow enough to allow algal cells to settle on the surface. The appropriate texture also minimizes the shear forces and reduces cell sloughing (Cui et al. 2013).

In addition to attachment efficiency, durability of the attachment materials is an equally important factor for longevity of a biofilm system; otherwise, it reduces the productivity and increases operation downtime and operating costs (Gross et al. 2015b). The attachment material should be unaffected by the force applied during mechanical harvest and resist extremely moist and high salinity conditions of wastewaters. In a study by Gross et al. (2013), cotton-based duct canvas and ropes although showed superior attachment efficiency but deteriorated within 2–3 months.

In summary, a large number of materials have been tested for roughness, porosity, hydrophobic property, and biological affinity to identify the best attachment material in terms of high biomass productivity, longevity, cost, and ease of harvesting. The present understanding can be used to identify specific attachment materials for particular microalgae strains and applications.

4.2 Biofilm Thickness and Harvesting Frequency

As biofilm develops on the surface of attachment material, it grows to form layers of cells, and eventually biofilm of appropriate thickness is formed (Hoh et al. 2016). With expansion of biofilm, new layers of cells are stacked on top of existing layers, which may increase the shading of the underlying cells (Ozkan and Berberoglu 2013). Due to shading, nutrient and mass transfer limitations could prevail in the bottom layer. Hence, it is necessary to maintain the appropriate thickness to avoid the loss of productivity due to mass transfer limitations (Katarzyna et al. 2015). Additionally, the nutrient-limited underlying cells which act as inoculum for next growth cycle will cause extended lag phase in growth of new biofilm. In summary, the harvest time is critical parameter for maintaining appropriate thickness dominated by actively growing cells.

Boelee et al. (2014b) tested different harvesting frequencies (2, 4, and 7 days) and found maximum biomass production rate of 7 g m^{-2} d^{-1} on the seventh day. Authors reported that longer harvesting period of more than 1 week decreases the biomass productivity due to loss of cells through detachment. With increasing biofilm thickness, the collisions between cells increase that start removing less dense newly formed biomass on outer layers of biofilm and cause detachment (Kwok et al. 1998).

5 Biofuel Potential of Algal Biofilm

In spite of extensive research on biofilm systems, most of the studies are focused on wastewater treatment rather than biofuel production. Few authors have evaluated the biofilm composition for only biodiesel potential and neglected other biofuel routes (biogas and bioethanol). The detailed biomass characterization studies revealed the presence of extracellular polymeric substance (EPS) consisting of polysaccharides, proteins, nucleic acids, and phospholipids in addition to intracellular lipids and proteins of algae (Mata et al. 2010; Williams and Laurens 2010). The rich composition of algal biofilm indicated high potential of their conversion into biodiesel, bioethanol, biogas or bio-oil (Pulz and Gross 2004). Most of the studies on biofilm production are focused on lipid production and their further application in biodiesel production.

Johnson and Wen (2010) used rocker cultivation system for cultivation of *Chlorella* sp. as biodiesel feedstock, with dairy manure wastewater as growth medium. Results showed higher total fatty acid (TFA) content (9% w/w) in attached culture as compared to suspended culture (8.9% w/w). In a similar study, Christenson and Sims (2011) used rotating biological contactors (RBC) for generation of biomass for biodiesel applications and reported FAME productivity of 2.2–2.5 g m^{-2} day^{-1}. Schnurr et al. (2013) used a horizontal biofilm reactor to assess neutral lipid productivities of *Scenedesmus obliquus* and *Nitzschia palea* and found increase in the neutral lipid concentrations under nutrient starvation. *Nitzschia palea* having higher lipid concentrations (15 % w/w) than that of *S. obliquus* (8% w/w) showed significantly higher lipid productivities (0.45 g m^{-2} d^{-1}). Similar study was done by Bernstein et al. (2014), who characterized the biofilm biomass and extracted neutral lipid fractions to assess the biodiesel potential. Results showed modest increases of extractable precursor concentrations in the nitrate deplete biofilms, as compared to the nitrate replete conditions. The accumulation of 2.9% and 5.1% (w/w) precursor molecules and FAMEs, respectively, was not significantly higher. Hence, it requires further optimization and experimentation of field-scale system for control of community composition for high lipid accumulation.

Among biofilm-based lipid extraction studies, cyanobacteria have received less attention than microalgae despite their successful commercial cultivation and distinct properties, making them a promising candidate for biofuel feedstock. In this context, Bruno et al. (2012) characterized biofilm-forming cyanobacteria for potential use in biodiesel production. The maximum lipid concentration was found to be 18% (w/w), and palmitic acid has been identified as the main fatty acid (FA). Authors concluded that high proportion of saturated fatty acids observed in cyanobacterial isolates along with the occurrence of monounsaturated FAs was optimal from a fuel quality standpoint.

From the life cycle analysis of algal biodiesel process by Brentner et al. (2011), the energy requirements were estimated to be 616 kWh for cultivation and 2500 kWh for harvesting of biomass (centrifugation) for a functional unit of 1 GJ

of biodiesel. The biofilm cultivation would save a total of 3116 kWh of energy used in cultivation and harvesting, and energy would only be invested in extraction (Heimann 2016).

In spite of having significant biofuel potential and feasibility of algal biofilm systems, no reports are available on the utilization of biofilms for other biofuel routes like bioethanol and biogas. Due to preferential utilization of wet biomass as biogas feedstock, anaerobic digestion of algal biofilms grown on wastewater seems most suitable as a conversion system (Choudhary et al. 2015). Studies have reported better biogas production potential of algae (23–31 m^{-3} day^{-1}) as compared to conventional feedstock like cow dung (13 m^{-3} day^{-1}) (Prajapati et al. 2014). The integration of biofilm production with wastewater treatment is already well established by many authors (Boelee et al. 2014a, b; Gismondi et al. 2016; Gross et al. 2015b; Shayan et al. 2016; Mulbry et al. 2008a; Pizarro et al. 2002; Rai et al. 2016) and successfully implemented at pilot scales. Further coupling of these systems with algal biogas production could make the systems energy efficient and economically feasible.

6 Commercial Applications

Unfortunately, all biofilm-based systems typically focused on nutrient removal and less on growth and biomass accumulation for biofuel production. A number of organizations (BioProcess Algae, Hydromentia, OneWater Inc., GreenShift Corp.) have successfully implemented the use of wastewater as a growth medium to generate algal biomass commercialized in the form of products such as compost and cattle feed, whereas trials on biofuel production are still in infant stage (Kesaano and Sims 2014). BioProcess Algae LLC, based in Omaha, Nebraska, is using Grower Harvester™ bioreactors to convert biomass into biofuels as well as animal feed, fish feed, and nutraceuticals (URL 2016a). Their currently running demonstration plant at the Green Plains Inc. ethanol plant in Shenandoah, Iowa, is directly coupled with the plant's CO_2 exhaust gas which facilitates the reuse of CO_2 emissions for high-value algae production. After successful implementation of ATS technology for point source and non-point source pollution control in water bodies, Hydromentia (URL 2016b) is targeting the utilization of harvested biomass for production of bioethanol and other biofuels. Apart from ATS technology, rotating algal biofilm reactors (RABR) have also been implemented successfully at pilot scales for biomass and biofuel production by researchers of Utah State University and Iowa State University (Christenson and Sims 2012; Gross et al. 2013).

7 Research Needs and Recommendations

In spite of availability of novel and innovative biofilm designs having high productivity potential and easy harvesting facility, there is a large gap due to heterogeneity and lack of standardization among reported studies. Most of the reported systems are tested within a narrow set of environmental parameters and species and hence limit the scope of application. This also limits the understanding and identification of the basic drivers of algal biomass production and its subsequent conversion into biofuels. In addition, the extensive research on wastewater remediation using biofilms lacks variability in growth conditions and outdoor applications.

Successful integration of algal biofilms into wastewater treatment processes requires engineering versatile systems that should be tested under more realistic conditions. Studies on correlating wastewater characteristics from different sources with biofilm growth, species composition, nutrient removal trends, and biochemical composition are required for utilization of biomass for diversified applications. Instead of studying effect of parameters like pH, CO_2, light, temperature, and nutrient availability which cannot be controlled under outdoor conditions, research should be directed toward selection of attachment materials and choice of wastewater as nutrient medium. The research should be focused on understanding the fundamental algal biofilm processes such as mass transport mechanisms as determined by its structure, heterotrophic-autotrophic interactions, and community characterization for designing a multifaceted biofilm reactor. Finally, standard operating procedures (SOPs) should be developed, validated, and tested for reporting nutrient removal and biomass production (Kesaano et al. 2015). In order to provide a broader foundation for comparisons between cultivation systems and with physiology of algal biofilms, uniformity is recommended to report composition and quantity in an application context. More research on application of biofilms for diverse biofuel routes is required to make algal biofuel technology a reality.

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Algal Technologies for Wastewater Treatment and Biofuels Production: An Integrated Approach for Environmental Management

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Contents

1 Introduction

The algae play very important role in mobilizing elements in the aquatic environments and received more attention in recent years as they have ability to absorb and detoxify heavy metals (Ye et al. 2012). Synthesis of phytochelatins and metallothioneins by algae helps in forming complexes with heavy metals and their detoxification (Suresh and Ravishankar 2004). Heavy metal uptake and

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accumulation in algae from wastewater depend on adsorption process and uptake metabolism (Lomax et al. 2011). For removing organic and inorganic contaminants, nutrients, and metals from wastewater different microalgae have been used (Hirooka et al. 2005; Fierro et al. 2008; Jácome-Pilco et al. 2009). Wastewater treatment by algae is more feasible due to their low cost and high efficiency as they remove contaminants by uptake and accumulation. Algae are capable of biotransforming and mineralizing nutrients and metals growth and development (Semple et al. 1999). Therefore, an integrated algal system can be utilized for wastewater treatment to remove organic matter, nutrients (N&P) from industrial effluents, sewage, and other wastes (Sivakumara et al. 2012).

Despite algal wastewater treatment being promoted as long ago as the 1950s (Oswald and Gotaas 1957), it has yet to be adopted as a conventional approach (Pittman et al. 2011).

Wastewater treatment by algae may be a sustainable option over conventional wastewater treatment which are costly and require energy for operation. There are some limitations in wastewater treatment by algae like problem in separating the algae from the treated water due to their small size (de la Noüe et al. 1992). Algal biomass is being utilized as feedstock for generation of biogas. Recently, several algae have been reported for biogas production. Biogas production potential of *Chlorella spp*. and *Chroococcus spp*. has been explored in earlier studies (Prajapati et al. 2013, Prajapati et al. 2014). Similarly, digestion studies of *Scenedesmus obliquus* and *Phaeodactylum tricornutum* under mesophilic and thermophilic temperature conditions have been conducted (Zamalloa et al. 2012). Green algae and cyanobacteria are distinct photosynthetic organisms able to rapidly convert solar energy to carbon based compounds. They are attractive raw materials for biofuel production because they are more capable to capture carbon dioxide and have less impact compared to other biofuel crops cultivation on agriculture and land availability. However, for commercialization of algal based biofuels including biomethane, high cost of nutrients is required in growing algae which may be overcome by utilizing wastewaters to provide nutrients. Recently, algae have been grown in the laboratory under volume reactor anaerobic digestion of wastewater (Kinnunen et al. 2014). The advantage of wastewater treatment by algae is the production of algal biomass which can be further used for biofuel production (Prajapati et al. 2013).

Algal biomethane process is also affected by lower activity of anaerobic microflora due to imbalanced carbon and nitrogen (C/N ratio) which may be overcome by codigestion with carbon rich substrate (Zhao and Ruan 2013). For example, a significant increase in biogas production has been observed by co-digesting Taihu blue algae with corn straw at C/N ratio of 20/1 (Zhong et al. 2012) and optimized C/N ratio to be 15/1 for co-digestion of Taihu algae and kitchen wastes (Zhao and Ruan 2013). Therefore, anaerobic digestion of algal biomass may be increased by codigestion with carbon rich substrate. Importantly, the economic value, energy, and resource efficiency of photosynthetic biofuels can be considerably improved when employing waste streams as feedstocks (Pittman et al. 2011, Samorì 2013). Nutrient resource reuse is particularly important in the face of diminishing

phosphorus reserves and threats to global food security (Cordell et al. 2009). From the above discussion, it is clear that integrated approach of wastewater treatment and codigestion of resulting algal biomass for biofuel production may be a sustainable way for environmental management.

2 Wastewater Treatment by Algae

2.1 Wastewater Type and Composition

Generally water after consumption by different source is termed wastewater or water enriched with different types of compounds is known as wastewater. In wastewater different type of pollutants are present which directly or indirectly affects the health of people around the world. About more than 50% diseases are water borne. The main source of wastewater is effluent emitted from household, urban, municipal, agricultural, chemical, and industrial resource. Point and non-point sources of wastewater from different anthropogenic activities have detrimental effects on water quality when directly discharge into the river without any treatment (Rai et al. 2011). The wastewater discharge can be characterized into two types, i.e., organic and inorganic waste. Organic waste constitutes the carbon containing biodegradable substances while inorganic waste constitutes nitrate, phosphate, heavy metal, etc. As for organic waste is concern, it was degraded by the activity of microbes and oxidation processes. However, inorganic pollutant particularly heavy metals cannot be treated effectively through simple measure and persist in the environment for long time. Untreated sewage may contain different heavy metals like cadmium, zinc, nickel, lead, chromium, cobalt and copper release into the waterbodies, and sediments leads to their bioaccumulation and bio-magnification into the aquatic flora and fauna (Gochfeld 2003).

2.2 Algae Based Wastewater Treatment Technologies

Technologies have been put forth for the treatment of wastewater still could not mitigate the pollution at satisfactory level. Moreover, technologies used are high cost and are not eco-friendly. Therefore, there is a noteworthy requirement for some cost-effective green technology which can treat wastewater in a sustainable manner. In this regard plant based management of wastewater could be a boon over water pollution.

The algae based treatment systems have been reported for efficient treatment of sewage, agricultural waste, and industrial effluent (Kaplan et al. 1988; Ma et al. 1990). More frequent algal based treatment systems used are the Algal Turf Scrubber (ATS) and High Rate Algal Ponds (HRAP) (Craggs et al. 1996; Oswald

1988) for growing algal biomass of green algae (*Scenedesmus* sp., *Chlorella* sp., and *Cladophora* sp.) and cyanobacteria (*Spirulina* sp., *Oscillatoria* sp., and *Anabaena* sp.). Currently algae and cyanobacteria draw more attention towards sustainable wastewater treatment. Algae and cyanobacteria have potential to treat different types of wastewater from surrounding. Algae are small in size can grow autotrophically like green algae or heterotrophically as cyanobacteria with high tolerance to heavy metals and other water contaminants. Synthesis of phytochelatin and potential for genetic manipulation in algal cell makes them more efficient for removal of pollutants from wastewater (Cai et al. 1995). The interesting idea of wastewater treatment by algae has been launched by Oswald and Gotaas (1957) in the USA. Different algae such as *Chlorella, Ankistrodesmus, Scenedesmus, Euglena, Chlamydomonas, Oscillatoria, Micractinium, and Golenkinia* have been reported from waste stabilization ponds and may be utilized for water treatment (Palmer 1974).

2.3 Nitrogen and Phosphorus Removal Efficiency of Algae

Nitrogen and phosphorous are very essential for algal growth; however, they have detrimental effects at higher concentration in wastewater. Algae can thrive in wastewaters containing high concentration of nitrogen and phosphorus (Pittman et al. 2011) and which can be applied not only to remove, but also to mobilize these nutrients as fertilizer in the terrestrial environment. Algal bioremediation to remove metals and nutrients from wastewater is widely accepted as an efficient and cost-effective method (Barrington et al. 2009; Neori et al. 2004) (Table 1).

2.4 Heavy Metals Removal Potential of Algae

As for heavy metal is concern, ability of algae to accumulate heavy metals has been recognized for many years (Megharajet al. 2003; Wang et al. 1995; Bursali et al. 2009; Al-Homaidan et al. 2011). Algae particularly microalgae remove metals from

Table 1 Different potential algae used in wastewater treatment

Algae	Type of wastewater	References
Spirulina	Anaerobic effluents of pig waste	Lincoln et al. (1996)
Phormidium bohneri	Sewage	Talbot and de la Noue (1993)
Chlorella sp.	Municipal wastewater	Li et al. (2011)
Euglena	Domestic wastewater	Mahapatra et al. (2013)
Desmodesmus sp. TAI-1 and *Chlamydomonas*	Industrial wastewater	Wu et al. (2012)
Scenedesmus quadricauda	Campus sewage	Han et al. (2015)

Table 2 Potential algae used for heavy metal accumulation/removal from wastewater

Algae	Algae group	Heavy metal accumulation/ removal	References
Nitella pseudoflabellata	Red algae	Cr, Cd	Gomes and Asaeda (2013)
Cystoseira indica, *Nizmuddinia zanardini*, *Sargassum glaucescens*, and *Padina australis*	Brown algae	Ni	Pahlavanzadeh et al. (2010)
Chlamydomonas reinhardtii	Green algae	Cu, Pb	Flouty and Estephane (2012)
Chlorella vulgaris, Chlamydomonas sp.	Green algae	Pb	Golab and (Smith 1992)
Chlamydomonas reinhardtii and *Scenedesmus obliquus*	Green algae	As	Wang et al. (2013a, b)
Cladophora sp.	Green algae	Pb	Cao et al. 2015
Microcystis aeruginosa	Green algae	As	Wang et al. 2014
Laminaria hyperborea, Bifurcaria bifurcata, Sargassum muticum, and *Fucus spiralis*	Brown algae	Cd, Zn, Pb	Freitas et al. (2008)

wastewater either by metabolism dependent uptake at low concentrations or by absorption process (Matagi et al. 1998). In recent years, green algae such as *Enteromorpha* and *Cladophora* have been used to estimate metal concentrations (Al-Homaidan et al. 2011). The potential of algae for bioaccumulation and biotransformation of metals has led to their widespread utilization in ecosystem's biomonitoring studies(Mehta and Gaur 2005). The Cyanobacteria *Phormidium* has been used successfully for bioaccumulation cadmium, zinc, lead, nickel, and copper (Wang et al. 1995). The algae *Caulerpa racemosa* has been utilized for boron removal from wastewater (Bursali et al. 2009). Therefore, removal of metals and nutrients by algae may provide a cost-effective and environmental friendly method for wastewater treatment (Table 2).

The functional groups, hydroxyl (–OH), phosphoryl (–PO), amino (–NH), carboxyl (–COOH), and sulfydryl (–SH), present on algal cell which bound metals (cations) from wastewater (Xue et al. 1988; Romero-González et al. 2001). Different algae have been manipulated for overexpression of metal binding protein (metallothionein) and removal of metal from wastewater. Genetic engineering was first done with an Hg^{++} transport system for overexpressing metal binding protein (metallothionein) (Chen and Wilson 1997; Li et al. 2011).

3 Algal Biomass for Biofuel Production

Algae are fast growing and most abundant photosynthetic plants in the world and organism of bulk production of different types of products like chemicals, biofuel, lipid, EFA, and secondary metabolites (Wijffels et al. 2013). Generally algae grow

in two basic system: open systems include turf scrubber and tanksetc and closed systems consisting bioreactor, biocoil, bags, etc (Borowitzka 1999). Closed system of growing algae can improve yields by protecting algal species from different contaminations and provide temperature control (Darzin and Pienkos 2010).

3.1 Biomass Production

Basically two concepts, i.e., open pond reactor and closed photobioreactor of algae production are used nowadays. Prior to biofuel preparation some common steps, i.e., cultivation, collection, harvest, and a processing steps, are being used regardless of the biomass feedstock. In the production of biodiesel lipid content is one of the important components which should be high in algae with high lipid production efficiency (Xu and Hu 2013). The algae such as *Chlorella*, *Scenedesmus*, and *Botryococcus braunii* have been reported for biodiesel production (Wang et al. 2013a, b; Nascimento et al. 2013). The lipid obtained from algal biomass contains triacyglyceride and different sterol (Pruvost et al. 2009; Pruvost et al. 2011) which may or may not affect the biodiesel production efficiency. Therefore proper fatty acid profile of the algae need to be done before the start of production process of transesterification (Xu and Hu 2013; Bogen et al. 2013). Lipids having high content of mono unsaturated fatty acids (MUFA) with low content of poly unsaturated fatty acids (PUFA) are preferred for biodiesel as they can be easily transesterified during the processes (Mandotra et al. 2014).

3.2 Harvesting

Different strategies viz. centrifugation, flocculation, and filtration have been applied for the effective removal of algae from water. In centrifugation processes algae are removed through gravitational force by rotating water sample to high speed in centrifuge and algal mass collected at the bottom of tubes. Different types of centrifuge are in used for obtaining biomass form algae which are slightly varies in mechanism to separate materials (Shuler 2002) like centrifugal force, flow rate, biomass settling rate and settling distance of centrifugation (Williams and Laurens 2010). In flocculation process chemicals like polyacrylamides are added to clump algae together and easily separated. In the closed system limiting the supply of CO_2 causes the cells to clump. Often, in the flocculation generally salt of aluminum and iron are used (Grima et al. 2003). Filtration is also the best process and algae can be filtered by applying water pressure of water to pass through a membrane. The easiest form of filtration is dead-end filtration (Fig. 1).

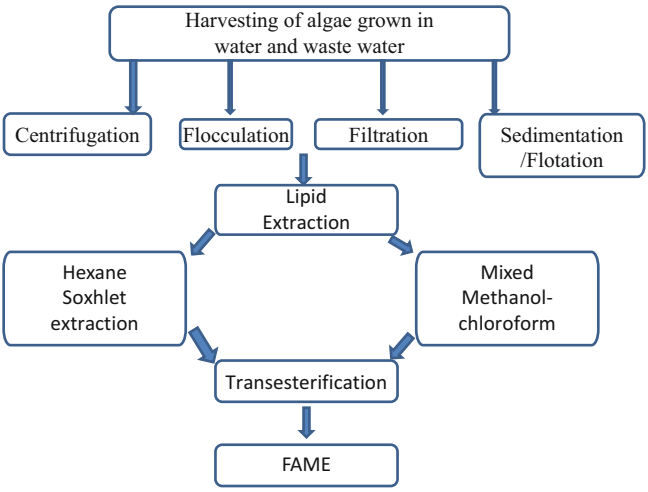

Fig. 1 Schematic representation of processes involved in biofuel production from algae

3.3 Processing

Lipid extraction is a key practice prior to transesterification. Lipid is extracted from the biomass of algae which is usually performed by the hexane Soxhlet extraction and mixed methanol–chloroform (2:1 v/v) (Bligh–Dyer method) (Bligh and Dyer 1959) process. In transesterification lipid compounds (triglycerides) react with alcohol in the presence of catalyst (Van Gerpen et al. 2004; Francisco et al. 2010). In the transesterification process propanol, butanol, methanol, ethanol, and amyl alcohol are used (Naik et al. 2006). Methanol and ethanol are utilized most frequently for transesterification process. The transesterified product is known as Fatty Acid Methyl Esters (FAME) which is known as biodiesel fuel. The main purpose of transesterification is to lower the viscosity of oil (Indhumathi et al. 2014).

4 Factor Affecting on Wastewater Treatment and Energy Production Efficiency of Algae

Algal biomass cultivation is difficult due to low survival under harsh conditions, heterotrophic species, climatic, and effluent water (Varshney et al. 2015). However, closed system improves yields by protecting algal species from water contaminants. Quality of wastewater and climate related impacts viz. rainfall, evaporation, and diurnal and seasonal temperature fluctuations affects growth, type, and abundance of algae. In case of land based aquaculture operations, wastewater treatment is often limited by strict environmental regulations around water quality of point-source discharges (Abreu et al. 2011 and de Paula Silva et al. 2008). Algae producing

lipids with high content of mono unsaturated fatty acids are more suitable and efficient for biodiesel production.

5 Conclusion and Future Prospect

It may be concluded from the literature that efficient algae can be cultivated for algal biomass production utilizing wastewater containing nutrients (nitrogen and phosphorus) and metals to remove them. Additionally, the resultant algal biomass from water treatment could be utilized for biofuel production, it may be a more viable, cost-effective and eco-friendly integrated method for wastewater treatment and biofuel generation. Moreover, treated water may be utilized for irrigation in agricultural field for crop production which further strengthen the feasibility of the algae based treatment of wastewater on sustainable manner. Further, algae based biotransformation and detoxification of water contaminants may contribute to reduce the toxicity of their bioaccumulation in crops for sustainable agriculture and environmental monitoring. Therefore, an integrated approach of wastewater treatment and biofuel production by algae could be a viable method for environmental cleanup, biofuel production, and environmental management in the coming future.

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Exploring Microalgae Consortia for Biomass Production: A Synthetic Ecological Engineering Approach Towards Sustainable Production of Biofuel Feedstock

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1 Introduction

Due to the day by day increased use of fossil fuels, a drastic shift in the climate has been reported which alternatively has disturbed the normal weather. Therefore, careful monitoring and the use of renewable fuels are very necessary to get the relaxation from these serious issues. Due to the increasing crisis of fuels, environmental concerns, and higher demands of renewable energy, microalgae are being perceived as affordable biofuel feedstock option to reach the industrial scale reality (Scaife et al. 2015; Shao et al. 2000; Sayre 2010). Consequently, people are looking towards other photosynthetic systems as an alternate source (Jegathese and Farid 2014; Medipally et al. 2015). Published literature showed that microalgae are one group of prominent organisms that can generate enormous amounts of high quality biomass which might be useful in generating biofuels such as biodiesel, bioethanol, or biogas.

Different physical, chemical, and biological methods are being implemented on the algal biomass to produce the biodiesel or bioethanol (Dexter and Fu 2009; Ducat et al. 2011; Georgianna and Mayfield 2012). Large scale ethanol is being produced from the algae through consolidated bio-processing, or through genetic engineering approaches. However, because of their rapid growth and higher lipid contents, they are main focus to produce the biodiesel (Griffiths and Harrison 2009). Biodiesel is mono-alkyl esters of the long chain fatty acids that can be produced via chemical or biological trans-esterification (Hempel et al. 2012). Algal biomass represents a potential source of lipids and carbohydrates that make them an emerging source of potential feedstock for the production of sustainable biodiesel and bioethanol, respectively. They are group of photosynthetic organisms, chiefly involving the green algae and cyanobacteria, belonging to the phylum Chlorophyta and Cyanophyta, respectively. Green algae are eukaryotic organisms while the cyanobacteria are prokaryotic in nature. Green algae and cyanobacteria are greenish to bluish green in appearance with photo-autotrophic habit to make their own food by utilizing the photons from sunlight, CO_2 from the environment, and a very little amount of nutrients from the environment (Desikachary 1959). As a biofuel feedstock, microalgae have several advantages such as no to minimal use of arable land and potential to improve the air quality by sequestering the plenty of CO_2. Therefore, rational utilization of microalgae could pull together these advantages, and consequently make an important contribution not only in catering the major energy demands, but also in providing the environmental reimbursements (Brennan and Owende 2009).

In general, most of the marine macroalgae grow in a diversity of water reservoirs (Eidens et al. 2014), with characteristic biomass yields and photosynthetic efficiency that exceeds over terrestrial plants. As aquatic in nature; algae do not need any land, therefore, avoiding the issues related to the food vs. fuel, large scale monoculture, and land changes thereof. Establishing the macroalgal farms in nutrient rich aquatic areas like original upwelling in the ocean or those that receive nutrients from the industries has been wondered (Eidens et al. 2014; Lanari and

Coutinho 2014; Hessing–Lewis et al. 2015). The excess of nutrients can be stripped from the waste streams by cultivation of microalgae; therefore, eutrophication and related ecological events can be avoided. However, still macroalgae cultivation is an appeal in the present scenario that alternatively limits their extensive utilization. These photosynthetic aquatic organisms create a link between CO_2 mitigation, carbohydrate and lipid assimilation, and biomass feedstock synthesis that is several folds higher than the market dominating first and second generation biomass feedstocks (Scaife et al. 2015).

Cyanobacteria and algae as a third generation feedstock can be a good tool to address the short falls in the first and second generation biofuel feedstocks. It offers increased yields and opportunity to produce the lipid enriched biomass by sequestering the nutrients from the waste streams that could be expanded to the biodiesel and other biofuels (Packer 2009). Cultivation of cyanobacteria and microalgae limits competition for the potable water and arable land. Heterotrophic microalgae and cyanobacteria have the inherent traits to fully utilize the nitrates and phosphates from the wastewater streams, therefore, being used to reduce the problem of eutrophication. However, third generation biomass feedstocks are relatively new, generally lack complement of suitable traits in a single species (Griffiths and Harrison 2009; Hempel et al. 2012). Instead of having several limitations in photosynthesis, many microalgae and cyanobacteria candidate species are able to grow under constant light, the logistics of such a feat for biofuel production makes it unrealistic. Therefore, efforts are ongoing to increase the photosynthetic efficiency through applying the principles of synthetic ecology; however, in this regard, a little success has been achieved (Cardinale et al. 2006; Cardinale 2011). It is the need of hour to develop the consortia of these organisms through synthetic ecology to ensure higher biomass yield that could be a possible way to limit the "food vs fuel" crises.

Fourth generation biodiesel feedstock includes the genetically manipulated microalgae strains. It largely involves the synthetic biology for generating the genetically manipulated green algae or cyanobacteria mutants that could be a source of high quality lipid profile to produce the good quality biodiesel (Chen et al. 2014).

1.1 Emergence of the Need of Algal Consortia

The imposing morphological types of cyanobacteria with a broad range of inherent features providing them unicellular to filamentous forms, and making them a good target to be used as cell factories for the sustainable and renewable biofuels (Madigan et al. 2011). They are endowed with a number of important traits which provide them faster growth rates to produce higher biomass. They have the novel photosynthetic systems to capture a wide range of irradiation to produce carbohydrates and lipids (Borowitzka and Hallegraeff 2007). They are being used as cell factories for a list of important value-added products such as carotenoids and

phycocyanin. In general, lipid content of 5–30% has been reported in the biomass of different cyanobacteria (Borowitzka 1995), and being targeted for the production of biodiesel through the process of trans-esterification, and in petrol or gasoline through cracking and distillation. Efforts are underway at global scale to enhance the biomass and lipid production by fine-tuning their growth needs, and by adding some growth promoters or elicitors.

In spite of their inherent traits as good biofuel agents, a number of hurdles are blocking their importance to make them commercially sustainable and viable fuel technology (Hannon et al. 2010). Therefore, regular search for robust species with higher growth potential, lipid and carbohydrate enriched biomass, improved photosynthetic ability, high quality lipid profiles, and resistance against the bacterial contaminants is ongoing. It is the dire need of society to identify an ideal cyanobacteria candidate species that can grow competently in a varying environmental condition, and can tolerate a wide range of variations in irradiation, pathogenic loads, salinity, and temperature to make sure their exploration in industrial sectors.

Screening of the suitable microalgae crop strains and to optimize their growth requirements (Mollers et al. 2014; Smith and McBride 2015; Bartley et al. 2015) or to manipulate their genome for higher growth rates and biomass yields with desired compounds production (Dexter and Fu 2009; Wang et al. 2012; Ducat et al. 2011) are the major ongoing thrust areas of algal biotechnology. Search for robust cyanobacteria species from diverse habitats with better fatty acid profiles to get the high quality biodiesel is underway (Griffiths and Harrison 2009; Nascimento et al. 2013; Hempel et al. 2012). However, microalgal biotechnology presents unique challenges to search the suitable single candidate species for a variety of biofuel producing traits. It has invigorated the use of vigorous and highly productive species microalgae to establish their synthetic consortia to get higher biomass, carbohydrates, and high quality lipid profiles by applying the principles of synthetic ecology (Georgianna and Mayfield 2012; Smith and Crews 2014; Kazamia et al. 2014).

2 Algae in its Natural Habitat

The freshwater and marine water are common habitats of different microalgae. Freshwater habitats include lakes, ponds, river, or a running water stream while marine habitat usually includes the saltwater, salt lakes, or the seas. In comparison to the freshwater forms, marine algae have unusual features, such as the secretion of secondary compounds: toxins, antibiotics, etc. Continuous exposures of these microalgae to the intense sun irradiation provoke the expression of the scytonemin and mycosporin like sun-protecting amino acids that alternatively affect the overall structure and services of the original community. These compounds are being used as sun-protecting agents in several pharma skin ointments. Presence of the mutagenic or carcinogenic agents in the habitat of these organisms is responsible for the development of new species. For instance, mutation in the genes of the prokaryotic

operon mreBCD that provides the rod shape to cyanobacteria, in general, completely absent in the spherical cells. A similar observation was made in the case of *Anabaena* sp. PCC 7120 that usually has rod-shaped cells (Hu et al. 2007). However, a mutation in mreBCD operon generated the truncated circular morphotypes of *Anabaena* sp. Furthermore, these algae and cyanobacteria have good interactions with the surrounding living and non–living things, which determine that what type of animal or plant genus would dominate in that particular habitat. The water ferns are well known for their interaction with different cyanobacteria. For instance, *Azolla* and *Anabaena* are well known for their mutualistic interaction in paddy fields.

Due to their inherent features, these organisms in natural habitat produce a variety of primary and secondary compounds that drive how to control the effects of biotic and abiotic factors to modulate the entire community structure accordingly. In general, pond sustain a fairly diverse ecological community that include several species of green algae, cyanobacteria, floating and rooted aquatic plants, grazing snails, clams, crustaceans, insects, fishes, reptiles, and amphibians. The insects and a number of small fishes feed on these algae and cyanobacteria as a predator. Therefore, the predator–prey relationship or predation depends on the type of cyanobacteria or microalgae genera as present in a particular habitat, and alternatively it determines that what predator species could survive, and become a part of that community. These photosynthetic organisms are the chief members of phytoplankton groups. Phytoplankton becomes a rich source of nitrogen and carbon that alternatively supports the growth of aquatic plants and animals.

Besides, they have also been reported in a number of wetland, estuary, and ocean ecosystems, and significantly contribute in defining the overall community structure. Since, they have strong competition for nutrients such as nitrogen and phosphorus, therefore, cyanobacteria that have the potential to fix the atmospheric nitrogen, or to solubilize the phosphates, play a serious role in fine-tuning overall growth of the community, re-constructing its structure, and modulating its services in a favorable direction. Consequently, such features allow them to dominate in the community at the end of succession. For instance, pathogens especially bacteria or fungi sequester the nutrients from the living organisms in the community, or engaged as saprophytes which alternatively increases the competition, and fine-tune the entire community structure as well as services.

To illustrate the relationship among the different algal species of a habitat, Cardinale (2011) synthesized an artificial algal community and described that with increasing the species biodiversity, the biomass productivity of algal community increases due to the coexistence of a variety of genus in a common habitat. However, he also elucidated that instead of being the part of a common habitat, each alga occupied distinct microhabitats commonly known as "niche" or "microhabitat." He found that filamentous algae such as *Melosira and Stigeoclonium* susceptible to shear were abundant in low water velocity niches whilst single-celled diatoms like *Achnathidium and Synedra* that can grow prostrate to a surface grew with higher densities in the high velocity habitats. Therefore, this work drew an important conclusion that instead of being present in a common habitat, different

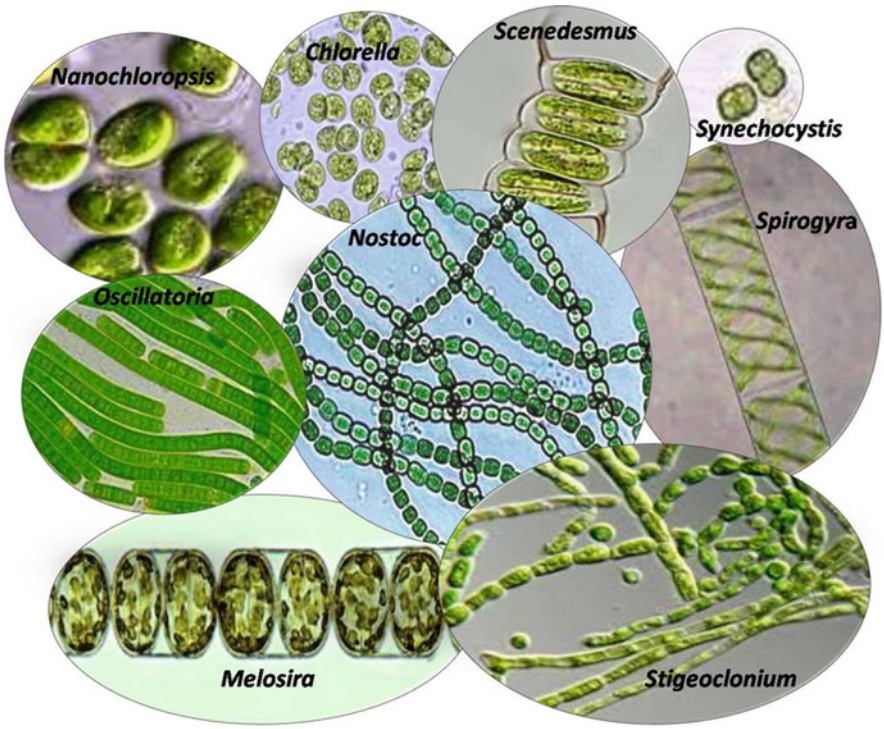

Fig. 1 Synthetic consortia and niche positioning: Hypothetical diagram was originally produced to show that how the individual microalgae can confined to a particular niche position in a habitat due to their inherent features. In general, filamentous forms occupy the low velocity zone of the habitat whilst single-celled forms inhabit the high velocity zone

algae have distinct inherent features that determine their growth requirements and niche partitioning. A hypothetical diagram has been shown (Fig. 1) that defines how the different algae and cyanobacteria interact with each other in a synthetic consortium or in a natural community.

3 Cultivation of Algae

Cultivation of these green algae and cyanobacteria requires the supplementation of little amounts of micro and macronutrients. In general, all the algae have common growth requirements except for some genus. In the laboratory, most of the microalgae are cultivated in the $BG11^{+}$ media, under irradiation of 30–100 μmol m^{-2} s^{-1} photons in a 14:10 light–dark regime, in a temperature range between 25 ± 5°C, and in a pH range between 7.2 and 8.0. However, most of the microalgae grow optimally at the pH 7.8 (Rippka et al. 1979; Stanier et al. 1971; Desikachary 1959). Algal cultures could grow optimally at the temperature 25 ± 1°C with supplementation of artificial

daylight by using the fluorescent lamps between the irradiance of 30–100 μmole photons m^{-2} s^{-1} in a regime of 14:10 light–dark periods in controlled ponds or in photo-bioreactors. The cell density of each microalga can be determined by measuring the optical density at 730 nm through spectro-photometric methods and simultaneous cell counting through the microscopic measurements and microbiological methods. Furthermore, relationship between the cell density and biomass productivity can also be established to gather the information for screening of the species that could be helpful in higher biomass production. The sigmoidal, logistic regression model can be used to generate the relationship between the corresponding numbers of cells (cell density) and the optical density at 730 nm (OD_{730}), or the optical density and biomass productivity for different microalgae species. However, it requires initial standardizations of relationship between the optical density and biomass productivity, or cell density. However, the growth of some genus requires special environments, such as the cultivation of *Spirogyra*, some psychrophilic strains that grow below 15°C temperature, or salt loving freshwater forms such as *Chlamydomonas nivalis* require supplementation of very minute amounts of the sodium chloride in $BG11^{+}$ media.

Cultivating these novel tiny plants to generate huge amounts of biomass either in open ponds or in closed bio-systems requires regular high throughput, monitoring with special regards to the structural changes in the community. Yet, cultivation in open ponds may be economically viable in comparison to the closed bioreactors, but generating the biomass with desired lipid and carbohydrate profile, or biogas profile requires the inputs and maintenance of selected species in the consortia that could be possible only in the closed systems. Since, different algal species would be inoculated in the open ponds, and as they start multiplying, there are chances for the high degree contamination by pathogens, which might modulates the original structure of community that is a strong reason for the loss of the original community structure. In contrary, in consortia these monocultures may be protected from the pathogens or high light intensity due to the increased diversity. Therefore, careful planning could be a positive way to grow these organisms in the open ponds, under varying light intensities that may limit the excessive cost, and high throughput online monitoring. It alternatively opens the door for the sustainable production of algal biomass through eco-engineering approaches. It could be an economically viable methodology to reach the commercial scale reality of synthetic consortia for the biomass production (Kazamia et al. 2014).

Furthermore, with varying light conditions, the growth rates of these microalgae are altered, even the morphology is affected to decrease or to increase the surface to volume ratios (Foy 1980). It directly influences the biomass productivity and its biochemical features. Therefore, to establish a sustainable community for the biomass feedstock, microalgae species should be characterized under the same culture environments to get the real information about their inherent traits.

Considering the industrial scale cultivation either in open pond or in closed bioreactors, these organisms can be efficiently grown in pond water with regular supplementations of phosphates and nitrates. The superphosphates and urea are being used as phosphate and nitrate sources, respectively (Geldenhuys et al. 1985).

Although, they can be grown efficiently in the freshwater open ponds, their productivity is also dependent on the seasonal variations (Kloser et al. 1993). For instance, in winters, low temperature tolerating species dominates in the ponds and clumps are formed to cope up with adverse environmental conditions. Therefore, considering the appropriate species with better biomass yields and high quality lipid profiles should be fruitful effort to cultivate these novel organisms in open ponds. Therefore, to avoid the temperature related issues, cultivation ponds should be equipped with spargers, and online robotic monitoring of physical and chemical features of the pond water is necessary. If needed, some heaters should also be equipped to maintain the temperature and to avoid the clumping.

In photo-bioreactors, computer simulations can be made easily, and the growth of consortia or monocultures can be modulated accordingly through online robotic monitoring programs. Continuous photo-bioreactors can be good tools to get the higher yields of primary products from these organisms. Moreover, the light intensity, temperature, and nutrients' concentrations can be modulated to get the higher yields. However, it is less time consuming and highly productive in comparison to the open ponds, but it is expensive, and requires high throughput screening and skilled technicians which alternatively added up to total production cost.

4 Relevance of Monocultures for Synthetic Consortia

Contrary to the scenario in natural setup, most of the cultivation attempts try to maintain a monoculture of selected species with advantageous traits. Different monoculture species of cyanobacteria and green algae have the different biofuel producing attributes such as growth rates, carbohydrates, photosynthesis, lipid profiles, and total biomass production. Therefore, to select a suitable microalgae candidate to include in the consortia formulation should be carried out with high degree of precautions. The agonistic microalgae species either with higher biomass yields and good lipid profiles or species having higher carbohydrate and biomass yields or with the higher biomass, carbohydrate, and good lipid profiles could be mixed separately to get the functionally distinct communities. However, before proceeding for the synthesis of a particular type of microalgae consortia, the screening of various biofuel producing traits of each constituent monoculture is necessary. Each monoculture has distinct inherent traits that discriminate it from others in various ways. For instance, if willing to have a functional community for higher biomass and good lipid profiles to get better quality biodiesel, careful monitoring of monocultures is necessary (Fig. 2). Therefore, it needed a good microbiology and biochemistry hands to screen the suitable species with desirable features to establish the successful consortia.

Concerning the issues related to the stability of consortia, the initial inoculums size of different constituent species, duration of log to stationary phases of these monocultures, carrying capacities, and toxin, non-antibiotic producing features of

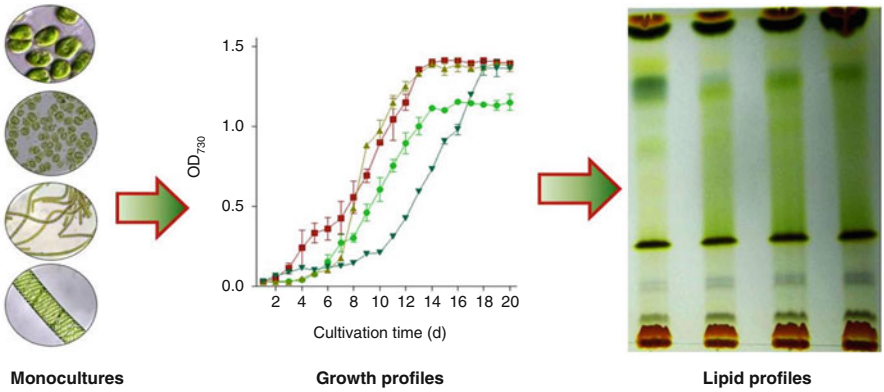

Fig. 2 Steps in the screening of different micro-algae species for the growth, biomass, and lipid profiles

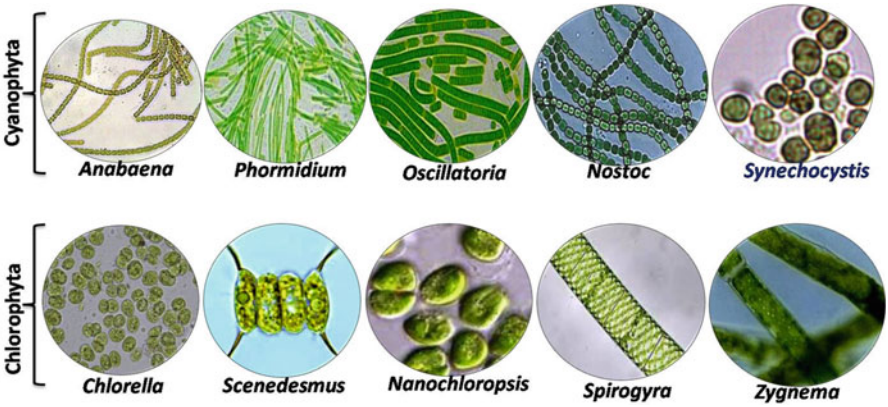

Fig. 3 Monocultures of green algae and cyanobacteria (each micrograph was originally recorded below 63x objective of compound microscope)

these species against each other, and maintenance of their original features in the consortia might be considered to get a highly productive and stable community. The consortia should have a variety of organisms with a number of distinct features such as nitrogen fixation, heavy metal detoxifying nature, high CO_2 sequestration, and utilization of additional phosphates. Once such a group of organisms would be identified they can be mixed to get highly productive consortia. Below micrographs are provided for monocultures of some cyanobacteria and green algae (Fig.3). The nitrogen fixing species may fulfill the limitation of nitrogen source. In contrast, phosphates from anthropogenic sources may be utilized by them to enhance their growth, and heavy metal detoxifying strains such as *Chlorella* can reimburse the environmental conditions. More diverse community has more biomass production

(Cardinale 2011). Therefore, involving both cyanobacteria and green algae in consortia may provide the successful utilization of these organisms in production of biomass pellet.

4.1 Synthetic Consortia and Increased Productivity

Yet, different microalgae have some inherent biochemical features when grown under axenic culture conditions; it may alter during the cultivation with other microalgae species. However, it largely depends on the co-cultivating species and its inherent features such as the secretion of growth promoting compounds and nitrogen fixation by any species within community highly affects the growth and biomass production of whole community (Brauer et al. 2015). Such species can be called as keystone species, and it may rely on the growth of other organisms within this synthetic ecosystem. In contrary, a species with negative attributes may diminish the overall productivity of the ecosystem by secreting some antibiotic secondary compounds. The presence of such organism within synthetic or natural ecosystem might be responsible for diminished growth rates and biomass production. Therefore, considering these important facts, species with positive attributes that could be helpful in whole community growth and biomass yields should be selected that alternatively can promote the growth and biomass yields of whole consortia. By doing so, a fast growing community can be developed to get the higher biomass yields. In contrast, the community for desired features such as lipid or carbohydrate enriched microalgae may be grown together to get the high quality biomass and overall higher yields than monocultures.

5 Ecological Community of Algae and Synthetic Consortia

Following the footsteps of nature, a number of microalgae species with common desired traits can be grown in a single cultivation tank for productivity and stability. With this approach, particular type of biomolecules enriched biomass production in valuable amounts is possible with sustained yield. However, growing different microalgae species simultaneously in a common cultivation tank requires the preliminary screening for the agonistic growth of these photosynthetic organisms. Since, some of these microalgae produce and secrete the secondary metabolites and bioactive compounds against the other members of same or another group (Morais et al. 2015). Therefore, screening for such compounds should be included in the mandates of synthetic ecology (Smith et al. 2010). It may create a good assemblage of cyanobacteria and green algae to get the biomass enriched in lipid or carbohydrate in less culture duration. In natural ecological communities, such discrimination

cannot be made; therefore, it results in slower growth rates and lower yields. However, in synthetic consortia such drawbacks can be easily removed through eliminating such species.

Furthermore, within microalgae community different individuals perform separate functions. For instance, nitrogen fixing cyanobacteria such as *Nostoc muscorum, Anabaena cylindrica, Lyngbya, Phormidium*, and *Calothrix* can aid the nitrogen to the cultivation media which can be utilized by other non-nitrogen fixing members of the synthetic community. The individuals of community have the different functional roles; therefore, each of them would occupy a distinct niche in their synthetic habitat. In general, filamentous forms usually occur in floating conditions, and unicellular forms are homogenous scattered in upper portion of the cultivation tanks.

The lipid and carbohydrate enriched high CO_2 sequestering and/or bio-hydrogen producing species such as *Nannochloropsis, Scenedesmus dimorphus,* and *Chlamydomonas reinhardtii*, and *Synechocystis* PCC 6803 etc can be grown together to get the higher CO_2 sequestration, bio-hydrogen production, lipid and carbohydrate enriched biomass production, or improved biogas production and cost-effective hydrothermal–thermochemical processing (Pandey et al. 2014). Furthermore, these microbes should be mixed together in such an amount that it can be developed in the successful synthetic consortia to reach the industrial scale reality. Since, including microbes with antagonistic features such as anti-algal/cyanobacterial compound producing strain(s) may lead to the suppression of growth of other organisms; therefore, such organisms should be avoided during the consortia formulation.

6 Functional Traits and Services of Synthetic Microalgae Consortia

Although, monocultures with varying attributes can be mixed together to get the desired production of primary and secondary metabolites, functional traits of these organisms get altered within synthetic consortia. For instance, the growth rates and photosynthesis are highly affected. In general, the total yields of a particular biomolecule get increased in the community which illustrates that the cumulative functional traits of community are superior in comparison to the monocultures. The microalgae with higher growth rates, lipid and carbohydrate enriched biomass yields can be considered to develop the synthetic consortia to get the sustainable biofuel feedstock.

6.1 Altered Metabolic Traits of Consortia

Some of the microalgae produce secondary metabolites with antibiotic features against another algae or cyanobacteria. Therefore, despite having rapid growth and higher biomass yields of such organisms, their presence in the consortia highly affects the growth and productivity of other microalgae. Sometimes, these microalgae produce growth promoting compounds that could be helpful in growth regulation of other organisms in consortia (Safonova and Reisser 2006). Sometimes, addition of some growth promoting phytohormones or similar chemicals in culture media enhances the growth of cyanobacteria and algae. Therefore, chemical priming can also be targeted as an alternate tool to enhance the growth of synthetic microalgal consortia (Patel et al. 2014, Modiri et al. 2015, Yu et al. 2015, Li et al. 2015). Some people are also generating the chemical mutants of cyanobacteria and algae to get the higher biomass with improved lipid and carbohydrate (Patel et al. 2016). These mutants can be grown synergistically to get the improved biofuel production. Considering these serious facts, before establishing the consortia, each microalga should be characterized for its inherent features. By doing so, a highly productive consortia with desired features can be established.

Some efforts have been made to study the role of algal consortia to water quality reimbursement by alleviating the levels of chromium and other toxic heavy metals through employing the algal consortia (Bose et al. 2011). Green algae such as *Chlorella* have been found to play an important role in the chromium contaminated water purification (Singh et al. 2012). Efforts have been made to study the effects of microalgae consortia in the heavy metal removal.

7 Exploiting Synthetic Microalgae Consortia for Summed up Positive Traits

Different micro-organisms can be mixed in a desired way to get the production of industrial compounds. Minty et al. (2013) developed the synthetic consortia of bacteria and fungus and directly produced iso-butanol from the cellulosic biomass through applying the principles of synthetic microbial ecology. In the similar way, the microalgae consortia with distinct traits can be processed to get the industrial scale reality of a desired compound. The lipid enriched algae such as *Nannochloropsis* and *Scenedesmus* can be mixed together with nitrogen fixing cyanobacteria to get the lipid enriched biomass which can be alternatively processed to the biodiesel through extraction and trans-esterification of lipids from the biomass. Similarly, the microalgae with inherent traits for carbohydrate enriched biomass production may be grown together in a synthetic consortium to get the more carbohydrates which subsequently may be converted into the ethanol through consolidated bio-processing with cellulolytic, xylanolytic, and ethanologenic micro-organisms.

8 Synthetic Ecology: A Fair Choice Over Synthetic Biology

Synthetic ecology is an emerging area of biology. It has several advantages over the other approach called synthetic biology which includes the genetic manipulation of micro-organisms for the desired product formation (Fig. 4). Synthetic biology involves the expression of target genes in a suitable chassis organism to get the improved and higher production of a desired compound in a pre-determined way. Therefore, a single cell may be used as the cellular factory for the increased production of novel biomolecules. However, to generate such novel strains is a very difficult task; since, it requires a high throughput screening at each and every stage. In addition, these methods are comparatively costly and environmentally not safe due to the use of antibiotic resistance genes as screening agents in the chassis organism.

On the other hand, synthetic ecology is the way through which the goal of targeted metabolic engineering can be achieved. It does not involve the production of undesired compounds. Yet, through applying the principles of synthetic ecology, desired compound(s) can be produced. However, beside that compound(s); in addition, a number of undesirable compounds are also produced that may inhibit the overall production of the community. In contrast, synthetic ecology also has

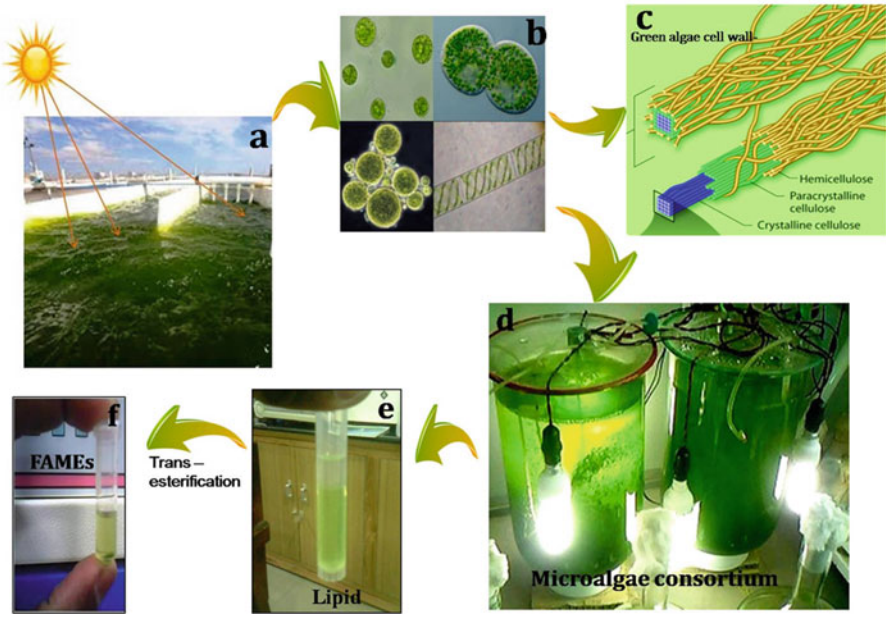

Fig. 4 Hypothetical diagram showing the basic approaches of synthetic ecology to establish the microalgae consortia with desired features. (**a**) Microalgae in natural habitat, (**b**) isolation, purification, and characterization of microalgae strains, (**c**) composition of microalgae cell wall, (**d**) synthetic microalgae consortia, (**e**) lipid extraction and trans-esterification, and (**f**) crude biodiesel or fatty acid methyl esters (FAMEs)

several advantages such as it is almost similar to the natural growth and does not involve any foreign chemicals that may cause mutation and subsequently evolution, the total production cost is very low when compared to the synthetic biology methods. It could be only a possible way to reach the industrial scale production of affordable and cost-effective algal biomass for various aspects of biofuels. Therefore, instead of manipulating the genome of a chassis organism for a desired compound through genetic engineering tools, utilization of algal consortia with desirable functional traits may lead to the production of desirable compounds in an eco-friendly way that could be more acceptable as a better technology than synthetic biology.

9 Insights into the Altered Traits of the Synthetic Consortia: Role of Molecular Ecology

The altered traits of microalgae could be easily identified through using the various molecular methods. For instance, the over or underexpression of a particular gene can be identified by Denaturing Gradient Gel Electrophoresis (DGGE), next generation gene sequencing (NGS), metagenomics and 16S and 18S pyrosequencing, metabolome, and proteome analysis (Minty et al. 2013, Faust and Raes 2012, Ma et al. 2011, Du et al. 2012, Phelan et al. 2012). The altered functional traits related to the CO_2 sequestration can be easily identified by identifying the changes in expression profiles of carbonic anhydrase enzyme in monocultures and in consortia (Wilbur and Anderson 1948, Miura et al. 2004, Bharti et al. 2014). Since photosynthesis is the signature for the growth profiles of algae, therefore, study of various aspects of photosynthesis and chlorophyll fluorescence may clarify the possible changes in community due to the presence or absence of a particular monoculture (Govind 2005). UV visible scanning of intact cells may provide the information about the production of a wide range of compounds such as scytonemin and mycosporin like amino acids or other bioactive compounds (Pichelt and Castenholz 1993, Rastogi and Incharoensakdi 2014). Photochemical and non-photochemical quenching may directly elucidate that among the incident photons, how many are being utilize in photosynthesis and how many are involved in chlorophyll fluorescence. Since, the values of Fv/Fm are directly related to the photosynthesis and growth profiles; therefore, the growth of consortia in comparison to monocultures can be elucidated through that spectro-photometric tool. The photosynthesis and chlorophyll fluorescence can also be used for the characterization of their altered summed up traits. Furthermore, the species richness or evenness may also be identified within the synthetic consortia through the DGGE analysis of common conserved gene sequences in constituting organisms. Consequently, the molecular markers could be developed for the regulating enzymes of high lipid or carbohydrate, or any other compound of pharmaceutical importance for important microalgal species which alternatively may be used to screen the similar species from the natural habitat from the diverse habitat.

10 Conclusion

Synthetic ecology is an emerging area of biology that could lead to the sustainable production of biomass to reach the industrial scale reality of a desired compound production in a cost effective and environmentally safe way. It can be utilized to generate huge quantity of biomass enriched with desired commodity chemicals that might be helpful in generating various biofuels. The growth and biomass productivity of selective synthetic consortia can be promoted by adding the phytohormones or related chemicals which can be a good effort to defeat the fuel scarcity and increasing prices. Furthermore, combined use of synthetic ecology and bio-process fermentation technology may lead it to industrial scale, and therefore, can replace the fossil fuel. It could generate good job opportunity in the rural setup by generating the microalgal biomass and selling it to pharma industries. Several nongovernment organizations (NGOs) or government institutions may also work in this regard. This technology is environmentally safe and cost effective. Therefore, it could be applied on different algal groups.

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Modeling the Effects of Operational Parameters on Algae Growth

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1 Algae Growth Versus Substrate Concentration

Generally, algal growth can be described by five phases (Fig. 1) as follows (Vaccari et al. 2006; Richmond 2003):

Phase 1, "lag phase," where an initial delay in growth occurs due to the physiological adjustments to change in culture and/or new environmental conditions
Phase 2, "exponential phase," where cells exponentially grow and reproduce as a function of time, as long as substrates/nutrients and light intensity are saturated
Phase 3, "linear growth phase," where growth rate is linear as a function of time
Phase 4, "stationary growth phase," where the growth rate remains approximately steady, along with luxury storage/uptake/consumption of nutrients
Phase 5, "decline or death phase," where microorganisms' death occurs due to the decrease in the concentration of substrate/nutrients and/or accumulation of inhibitory substances

2 Nutrient Addition

Algal medium should be enriched with nutrients to develop cultivation. According to Richmond (2003), the nutrients include total salt content; ionic components such as K^+, Na^+, Mg^{2+}, Ca^{2+}, $SO_4{}^{-2}$, and Cl^-; nitrogen species in terms of ammonia, nitrate, and urea; carbon source either CO_2 or $HCO_3{}^-$; phosphorus; trace elements such as ethylene diamine tetra acetic acid (EDTA); and vitamins.

3 Light Distribution Modeling

Light is an essential parameter for the growth of microalgae, since it is used to undertake the photosynthetic process. Photosynthetic is the process of light energy utilization by green plant, where carbon dioxide and water are converted to organic

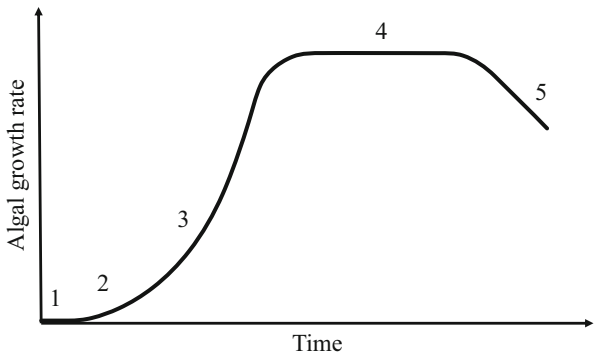

Fig. 1 Typical algal growth in a batch culture, where 1 is lag phase, 2 is exponential phase, 3 is linear growth phase, 4 is stationary growth phase, and 5 is decline or death phase (Richmond 2003)

compound (mainly glucose) with the release of oxygen gas. The basic chemical equation of the photosynthetic process can be expressed by Eq. (1) (Carvalho et al. 2011).

$$6H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1)$$

Microalgae can't absorb all the photons, and thus not all the applied light energy can be stored by microalgae. Moreover, too much light can cause light inhibition for the surface layer of microalgae. The chlorophylls, phycobilins, and carotenoids in microalgae can be absorbed in the visible light range as listed in Table 1.

The correlation between light intensity and photosynthesis for individual cells is noted as "*P–I* relationship"; where "*P*" and "*I*" refer to photosynthetic rate and light intensity, respectively. This relationship has three distinct light regimes, as shown in Fig. 2:

Phase 1: At low "*I*," the "*P*" is usually proportional to "I_2," and photosynthesis is limited by the rate of capture of photons.

Phase 2: When "*I*" reaches a saturation threshold, noted as "I_k," algae become light saturated (Crill 1977). Under this environment, the "*P*" reaches its maximum value and becomes independent of "*I*."

Phase 3: Further increase in "*I*" over an inhibitory threshold "I_{inhib}," the "*P*" starts to decline. This trend could be due to the deactivation of key proteins in the photosynthetic units (Rubio et al. 2003).

Saturation constant of light is defined as the intensity of light at which the specific biomass growth rate is half its pick value, i.e., light intensity at $\mu = ½\,\mu_{max}$. After increasing the light intensity over a certain value, the algal specific growth rate starts to decline, referring to the photoinhibition phenomenon (Fig. 3). This phenomenon occurs when the applied light intensity becomes higher than the light intensity at which the specific growth rate peaks. An excessive light intensity can damage cells, leading to oxidative stress and photoinhibition. To achieve

Table 1 Photonic features of major pigments in microalgae (Carvalho et al. 2011)

Pigment group	Color	Ranges of absorption bands (nm)	Hydrophobic and hydrophilic surfaces	Pigments
Chlorophylls	Green	450–475 630–675	Hydrophobic	Chlorophyll *a* Chlorophyll *b* Chlorophyll c_1, c_2, *d*
Phycobilins	Blue, red	500–650	Hydrophilic	Phycocyanin Phycoerythrin Allophycocyanin
Carotenoids	Yellow, orange	400–550	Hydrophobic	*β*-Carotene *α*-Carotene Lutein Violaxanthin Fucoxanthin

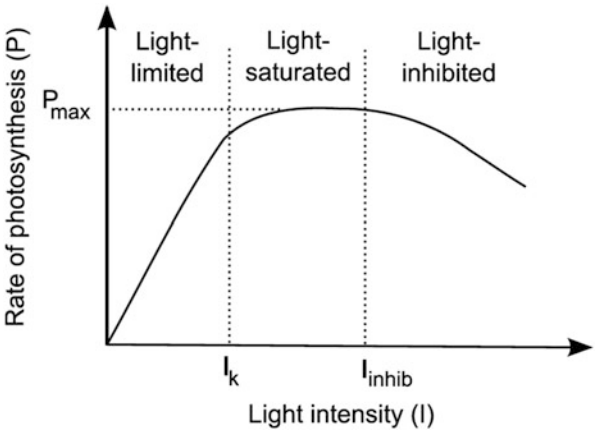

Fig. 2 Typical "*P–I* relationship" of the three phases for microalgae light response: Phase 1, light limited ($I < I_k$); phase 2, light saturated ($I_k < I < I_{\text{inhib}}$); and phase 3, light inhibited ($I > I_{\text{inhib}}$)

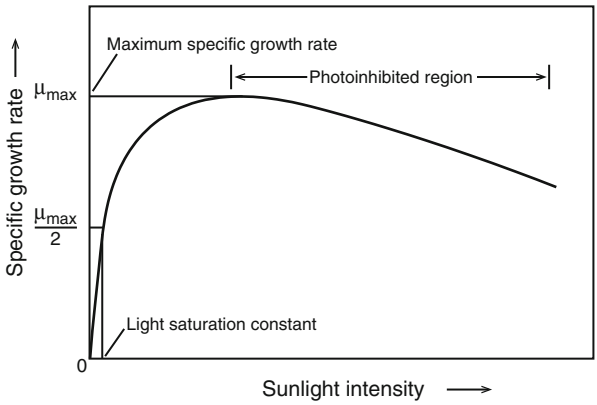

Fig. 3 Effect of light intensity on specific growth rate of microalgae (Chisti 2007)

satisfactory algal productivity, photoinhibition phenomenon should be avoided, and light utilization needs to be optimized.

Light distribution modeling assumes that the culture is subjected to an average light intensity, estimated according to light intensity at reactor surface (Richmond 2003). A beam of incident light that falls on a material can be scattered, absorbed, or transmitted. Transmitted light happens when light propagates in the same direction as the incident light, whereas scattered light occurs when light emerges in a different direction from the incident light. Absorbed light is the energy from light that is absorbed in the volume of the material.

4 Beer-Lambert Law

Beer-Lambert Law (or Beer's law) is a linear relationship between absorbance and concentration of an absorbing species. Consider light incident on a material with area A and thickness dx and concentration of molecules C (i.e., number cm^{-3}).

- Number of molecules illuminated by light of incident intensity I_x is $CAdx$.
- Total effective area that the molecules present is $\sigma CAdx$.

Probability of light being absorbed or scattered out of the beam in thickness dx is expressed in Eq. (2):

$$-\frac{dI_x}{I_x} = \frac{\sigma CA}{A}dx \tag{2}$$

where dI_x is the change in intensity across dx.
By integrating both sides, Eq. (3) can be derived:

$$\int_{I_0}^{I} \frac{dI_x}{I_x} = -\int_0^x \sigma C dx$$
$$\ln\left(\frac{I}{I_0}\right) = -\sigma Cx \tag{3}$$

Therefore, the intensity of light is exponentially reduced according to the Lambert-Beer law according to Eq. (4) (Richmond 2003):

$$I = I_0 \cdot e^{-\alpha XL} \tag{4}$$

where I is light intensity at distance L (μmol m^{-2} s^{-1}), I_0 is light intensity incident on reactor wall (μmol m^{-2} s^{-1}), α is absorption coefficient (m^2 g^{-1}), L is distance from reactor wall into culture (m), and X is biomass concentration in reactor (g m^{-3}).

5 Growth Rate Modeling

Growth of the algal cell can be estimated considering an energy balance from photons to carbon stored in the biomass (Quinn et al. 2011). The energy balance takes into account the losses by respiration, uptake of nutrients, and the consumption of photosynthetically fixed carbon to synthesize the cellular macromolecules carbohydrates, proteins, and lipids (Eq. 5). Photosynthesis comprises chains of reactions starting with light absorption followed by synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) as intermediate energy-conserving compounds. ATP is the energy source, while NADPH is

the reducing agent that adds high-energy electrons to form sugar. These reactions lead to carbon fixation in the Calvin cycle (Williams et al. 2002).

$$\mu = P_c - rR_c - \xi \cdot rN \tag{5}$$

where P_c is photosynthetic rate (carbon) (h^{-1}), rR_c is maintenance respiration rate for carbon (h^{-1}), rN is specific uptake rate of nitrogen (h^{-1}), and ζ is biosynthetic efficiency (g g^{-1}).

Moreover, Platt and Jassby (1976) expressed the growth rate of algae by fitting the data to the hyperbolic tangent function (Eq. 6):

$$\mu = \mu_m \tanh\left(\alpha \frac{I}{\mu_m}\right) \tag{6}$$

where μ is specific growth rate at light intensity (h^{-1}), μ_m is maximum growth rate at optimum light intensity, α is initial slope of light-saturation curve (W^{-1} m^2 h^{-1}), I is x-axis intercept or compensation irradiance (W m^{-2}), and tanh is hyperbolic tangent function.

6 Monod Model

The Monod model is a general kinetic model used for describing the correlation between the microorganism growth and concentration of the limiting substrate (or nutrient). The model is defined by two parameters: a nutrient-saturated growth rate and a half-saturation constant (Eq. 7) (Sommer 2011).

$$\mu = \mu_{\max}\left(\frac{S}{K_S + S}\right) \tag{7}$$

where μ is specific growth rate (h^{-1}), $\mu_{\max}$ is the maximum specific growth rate (h^{-1}), S is substrate concentration (mg L^{-1}), and K_S is the half-saturation constant (mg L^{-1}).

7 Droop Model

Luxury uptake (or luxury consumption) of nutrients and further storage for growth can lead to a temporal uncoupling between reproductive rates and dissolved nutrient concentrations (Droop 1973). The Droop quota model is used to present a correlation between the growth rate of microorganisms and the internal substrate (or nutrient) content of a cell rather than the substrate (nutrient) concentration around the medium. According to the Droop model, nutrient limitation can be

modeled by multiplying maximum photosynthesis rate with an efficiency factor for nutrient limitation (Lemesle and Mailleret 2008). The Droop model can be expressed as Eq. (8).

$$\mu = \mu_{\max}\left(1 - \frac{q_0}{q}\right) \tag{8}$$

where μ is specific growth rate (h^{-1}), $\mu_{\max}$ is maximum specific growth rate (h^{-1}), q_0 is limiting cell quota for the limiting substrate or cell quota at which the growth rate approaches zero (g g^{-1}), and q is cell quota for the limiting substrate (g g^{-1}).

8 Kinetic Models Related to Inorganic Carbon Concentration

Monod's parameters and the optimal carbon concentration for algae growth in carbon limiting cultures can be calculated from Eq. (9) (Goldman et al. 1974; Hsueh et al. 2009):

$$\mu = \mu_{\max}\left(\frac{S_C}{K_{S,C} + S_C}\right) \tag{9}$$

where, μ is specific growth rate (h^{-1}); $\mu_{\max}$ is maximum specific growth rate (h^{-1}); $K_{S,C}$ is half-saturation constant of the inorganic carbon (mg L^{-1}) (the inorganic carbon concentration at which the specific growth rate is half of the maximum); S_c is inorganic carbon concentration (mg L^{-1}).

9 Kinetic Models Related to Nitrogen Concentration

The Monod model in Eqs. (10) and (11) can also be used to describe the relationship between the algae-specific growth rate and the nitrogen concentration in a nitrogen-limited culture (Aslan and Kapdan 2006):

$$\mu = \mu_{\max}\left(\frac{S_N}{K_{S,N} + S_N}\right) \tag{10}$$

$$R = R_{\max}\left(\frac{S_N}{K_{S,N} + S_N}\right) \tag{11}$$

where μ is specific growth rate (h^{-1}); μ_{max} is maximum specific growth rate (h^{-1}); $K_{S,N}$ is half-saturation constant of the nitrogen (mg L^{-1}); S_N is concentration of nitrogen; R is the nitrogen uptake rate (h^{-1}); R_{max} is the maximum nitrogen uptake rate (h^{-1}).

10 Kinetic Models Related to Phosphorus Concentration

The Monod model (Eq. 12) is used to illustrate the correlation between specific growth rate and phosphorus concentration (Grover 1991). Moreover, Flynn (2002) modified the Droop model to derive functions that link the growth rate of algae to the phosphorus quota:

$$\mu = \mu_{max}\left(\frac{S_P}{K_{S,P} + S_P}\right) \tag{12}$$

where μ is specific growth rate (h^{-1}), μ_{max} is maximum specific growth rate (h^{-1}), $K_{S,P}$ is half-saturation constant of the phosphorus concentration (mg L^{-1}), and S_P is phosphorus concentration (mg L^{-1}).

For studying the removal of phosphorus from synthetic wastewater by algae, a study by Aslan and Kapdan (2006) used Eq. 13:

$$(\text{Chl}a)_f - (\text{Chl}a)_i = Y_P\left[(PO_4 - P)_0 - (PO_4 - P)_f\right] \tag{13}$$

where Y_P is the yield coefficient for phosphorus removal; $(\text{chl}a)_f$ and $(\text{chl}a)_i$ are the final and initial chl*a* concentrations, respectively (mg L^{-1}); and $(PO_4 - P)_0$ and $(PO_4 - P)_f$ are the initial and the final $PO_4 - P$ concentrations, respectively (mg L^{-1}).

Yao et al. (2011) used the Langmuir equation to describe the adsorption/desorption process of phosphorus by algae. The adsorption and desorption processes can be described as Eqs. (14) and (15):

$$R_a = K_a W_P\left(1 - \frac{S_P}{S_{P,max}}\right) \tag{14}$$

$$R_d = K_d A_P \tag{15}$$

where R_a and R_d are the adsorption and desorption rates, respectively (μmol m^{-3} h^{-1}); A_P is the surface-adsorbed phosphate concentration (μmol m^{-3}) and can be calculated from $A_P = S_p \times N$; S_p is the amount of surface-adsorbed phosphate per algal cell (10^{-8} μmol $cell^{-1}$); S_{pmax} is the maximum of S_p (10^{-8} μmol $cell^{-1}$); N is the algal cell density (10^8 cells m^{-3}); K_a and K_d are the adsorption and desorption constants, respectively (h^{-1}); and W_P is the phosphate concentration in the substrate (μmol m^{-3}).

11 Kinetic Models Related to Light Intensity

The relationship between specific growth rate and light intensity can be described by either the Monod model (Eq. 16) (Sasi et al. 2011) or the exponential model (Eq. 17) (Martinez et al. 1997):

$$\mu = \mu_m \left(\frac{I}{K_{S,I} + I} \right) \tag{16}$$

$$\mu = \mu_m \left(1 - e^{-I/K_{S,I}} \right) \tag{17}$$

where μ is specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), $K_{S,I}$ is saturation light intensity (μmol m^{-2} s^{-1}), and I is light intensity (μmol m^{-2} s^{-1}).

The empirical correlation between growth rate and irradiance was determined by Steele (1965) as follows (Eq. 18):

$$\mu = \frac{\mu_{\max T} I}{I_{\text{opt}T} \exp\left(I - I_{\text{opt}T}\right)} \tag{18}$$

where μ is specific growth rate at light intensity I, $\mu_{\max T}$ is the estimated maximal growth rate at temperature T, and $I_{\text{opt}T}$ is the optimal light intensity at temperature T.

Peeters and Eilers (1978) described a photoinhibition model for describing photosynthesis in phytoplankton. The equation is as follows (Eq. 19):

$$\mu_{(T,15/9)} = \mu_m \times 2 \times (1 + \beta) \times I' / \left(I'^2 + 2 \times I' \times \beta + 1 \right) \tag{19}$$

where μ is specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), β is the attenuation coefficient, I is irradiance, and I_{opt} is optimum irradiance, with $I' = I/\mathrm{I}_{\text{opt}}$.

12 Kinetic Model Considering Inhibition

Due to the presence of toxic/inhibitory substances in the medium, algal growth can be less than the maximum value. The most common model used for describing the substrate inhibition is a modification of the Monod expression, known as Andrews model (Eq. 20) (Vaccari et al. 2006).

$$\mu = \mu_{\max} \frac{S}{S + K_S + {}^{S}/_{K_I}} \tag{20}$$

where μ is specific growth rate (h^{-1}), $\mu_{\max}$ is maximum specific growth rate (h^{-1}), K_S is half-saturation constant (mg L^{-1}), S is substrate concentration (mg L^{-1}), and K_I is the inhibition coefficient (mg L^{-1}).

13 Kinetic Model Related to Temperature

A linear relationship between algal growth rate and water temperature was reported in a study by Sterner and Grover (1998). This correlation is based on the Monod model and can be expressed as Eq. (21):

$$\mu = T\mu_T \left(\frac{[R]}{K + [R]} \right) \tag{21}$$

where μ is specific growth rate (day^{-1}), $[R]$ is the concentration of dissolved nutrient (mg-N L^{-1} or mg-P L^{-1} according to the culture), T is the temperature, μ_T is the coefficient of temperature dependence for growth, and K is the half-saturation constant for nutrient-limited growth.

In another study, Lehman et al. (1975) set a model of temperature-dependent maximum growth rate as Eqs. (22) and (23):

$$\mu_{\max T} = \mu_{\max} \exp\left(-2.3 \left(T - T_{\text{opt}} \right)^2 / B^2 \right) \tag{22}$$

$$I_{\text{opt}T} = I_{\text{opt}} \exp\left(-2.3 \left(T - T_{\text{opt}} \right)^2 / B^2 \right) \tag{23}$$

with $B = T_{\text{sup}} - T_{\text{opt}}$ if $T > T_{\text{opt}}$ and $B = T_{\text{inf}} - T_{\text{opt}}$ if $T < T_{\text{opt}}$, where $\mu_{\max T}$ is maximum growth rate at temperature T (h^{-1}), $\mu_{\max}$ is maximum growth rate (h^{-1}), $I_{\text{opt}T}$ is optimal light intensity at temperature T, I_{opt} is optimal light intensity, T_{opt} is the optimum temperature, and T_{inf} and T_{sup} are the lowest and highest temperatures, respectively, with $\mu_{\max T} = 0.1 \times \mu_{\max}$ and $I_{\text{opt}T} = 0.1 \times I_{\text{opt}}$.

Bordel et al. (2009) modeled the algal growth rate in consideration of temperature and light intensity, as expressed in Eq. (24).

$$\mu = \mu_{m,o} \frac{I_{av}}{K + I_{av}} \exp\left(-\frac{E_a}{kT} \right) \tag{24}$$

where μ is specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), I_{av} is average light intensity in the culture (μmol m^{-2} s^{-1}), K is light constant

(μmol m^{-2} s^{-1}), E_a is activation energy for photosynthesis (J), k is Boltzmann constant (J K^{-1}), and T is temperature (K).

Roels (1983) modeled the rate of deactivation of enzymes as a function of temperature as Eq. (25):

$$\mu = \mu_m \frac{\exp\left(-\frac{E_a}{KT}\right)}{1 + \exp\left(-\frac{E_a'}{KT}\right)} \tag{25}$$

where μ is specific growth rate (h^{-1}); μ_m is maximum specific growth rate (h^{-1}); k is Boltzmann constant (J K^{-1}); T is temperature (K); E_a and E_a' are activation energy for photosynthesis and enzyme denaturation, respectively (J); and K is dimensionless constant.

Bernard and Rémond (2012) expressed the maximum specific growth rate as a function of temperature as follows (Eq. 26):

$$\mu = \mu_m \frac{(T - T_{\max})(T - T_{\min})^2}{(T_{\text{opt}} - T_{\min})\left[(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)\right]} \tag{26}$$

where μ is specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), and $T_{\min}$, $T_{\max}$, and T_{opt} are the minimum, maximum, and optimum temperatures for photosynthesis, respectively.

14 Respiration Rate Modeling

During daytime, short-term respiration can utilize up to 25% of the chemical energy (in the form of ATP and NADPH) generated during photosynthesis (Falkowski and Owens 1978). The rate of daytime respiration can be expressed as Eq. 27, assuming that the consumption is directly proportional to the rate of photosynthesis (Geider et al. 1997).

$$R_D = -\xi \mu X \tag{27}$$

where R_D is rate of daytime respiration (g m^{-3} h^{-1}), ζ is dimensionless constant, μ is specific growth rate (h^{-1}), and X is cell concentration (g m^{-3}).

However, during nighttime, long-term respiration can cause significant biomass losses. The rate of nighttime maintenance is usually modeled using first-order kinetics with regard to cell concentration as Eq. (28) (Torzillo et al. 1991).

$$R_N = -\lambda X \tag{28}$$

where R_N is the rate of nighttime maintenance (g m^{-3} h^{-1}), λ is a constant (h^{-1}), and X is the cell concentration (g m^{-3}).

Dark respiration rates are found to be linearly related to growth rates of microalgae (Geider and Osborne 1989). This correlation can be expressed by Eq. (29):

$$r_d = r_0 + b\mu \tag{29}$$

where r_d is dark respiration rate (h^{-1}), r_0 is minimum dark respiration rate observed at $\mu = 0$ (h^{-1}), μ is specific growth rate (h^{-1}), and b is dimensionless constant.

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Recent Advances in Improving Ecophysiology of Microalgae for Biofuels

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1 Introduction

The increase in greenhouse gas emission and fossil fuel depletion has raised the demand to focus on alternative biomass derived fuels (Chen et al. 2009; Chisti 2007; Mutanda et al. 2011). Microalgae have long been recognized as a promising source of biomass for biofuel production and nutritional supplements like omega-3 fatty acids or carotenoids (Chen et al. 2009; Laurens and Wolfrum 2011). Microalgae are diverse group of organism with capacity to survive in diversified ecosystems starting from freshwater to harsh environments like estuaries and marine water. Up till now over 20,000 algal species are reported with ability to generate more than 50,000 kg/acre/year of energy-rich biomass without necessary requirement of arable land for cultivation (Gimpel et al. 2013; Radmer 1996). This diversified population of microalgae can synthesize large amounts of neutral lipids (up to 60% of cell dry weight) called triacylglycerol (TAG), which is an important feedstock for biofuel particularly biodiesel and renewable jet fuel (Bio-Synthetic Paraffinic Kerosene) (Chisti 2008; Hu et al. 2008). Some species of microalgae can also produce large amount of carbohydrates, particularly starch, which could be fermented to produce bioethanol (Brányiková et al. 2011). This capacity of microalgae to produce large amount of TAG or starch rich biomass makes algae as most promising feedstock for biofuel generation.

Biofuel from microalgae is most feasible energy-rich alternative, which could also decrease our dependence on fossil fuels. Biofuel is non-toxic, biodegradable, and extremely low CO_2 emitting fuels (Lam and Lee 2012). However, the complete commercialization of biofuel from microalgae is linked with several challenges such as low productivity with respect to biomass; complex harvesting methods and extraction techniques; finding efficient strain, and maintaining right culture conditions (Brennan and Owende 2010; Pragya et al. 2013). In general we can say that high growth rate with high lipid or starch content is the main challenge in front of scientific community, before algal biofuel can become a commercial reality. Several biochemical, genetic engineering including transcription factor engineering techniques are tried to enhance the lipid and carbohydrate accumulation in microalgae with limited success.

2 Factors Affecting Microalgae Growth and Biofuel Production

To harness algae potential as microbial cell factories requires better understanding of its genetic and metabolic processes. Advances in functional genomics like transcriptomic, proteomic, and metabolomic are becoming helpful in understanding some of the dynamics of genes, proteins, and metabolite abundance in algae. These techniques can provide details about metabolic changes under different environmental/growth conditions and can be useful in optimization of algal cell factories.

The process of lipid or carbohydrate accumulation in microalgae is strongly influenced by availability of nutrients and light conditions (Xin et al. 2010). For instance, cells grown in certain conditions might not be optimum for growth or cellular processes but might divert the carbon flux towards synthesis of energy-rich compounds like lipid and carbohydrates. Therefore, accumulation of these energy-rich compounds can be optimized by controlling physical parameters like temperature, pH, light intensity, and chemical parameters such as nutrient deprivation (De Bhowmick et al. 2015). Some of the strategies to enhance lipid and starch accumulations like nutrient variation and genetic manipulation for metabolites synthesis are discussed further.

2.1 Nitrogen Depletion

Nitrogen (N) is one of the abundant elements of intracellular components, it plays vital role in regulating protein and nucleic acids synthesis as well as cell division of the algae (Schnurr et al. 2013). Microalgae are capable to utilizing N in the form of nitrite, nitrate, ammonia as well as urea. However, the form of N taken up is algae specific, but commonly ammonia is the most preferred form (Rashid et al. 2014). It has been well documented that, during N deplete conditions, the production of microalgal lipids increases with decrease in biomass, leading to reduction in total lipid.

Increase in lipid accumulation during N stress condition is mainly employed by lipid synthesis pathway. Earlier studies have shown that increase in lipid content leads to decrease in protein content of microalgae (Ho et al. 2012; Sun et al. 2014). Table 1 shows the change in the lipid content under nitrogen starve condition in various microalgal strains. Under N depletion in growth medium, most of the carbon flux is diverted towards the synthesis of energy-rich compounds like lipids and carbohydrates (Ho et al. 2014b). Synthesis of these energy-rich compounds is mainly algae specific. The microalga such as *Nannochloropsis oculata* accumulated 48% of lipid content in N deplete condition, similarly, *Nannochloropsis* sp. F&M-M24 accumulated significant amount of lipid during N starve condition, whereas, *Scenedesmus obliquus* and *Chlorella vulgaris* showed increase in carbohydrate content upto 49 and 51%, respectively, under N starve condition (Ho et al. 2013a, b; Siaut et al. 2011). Reduction of chlorophyll content was also reported in N starved microalgal cells. Msanne et al. (2012) demonstrated huge reduction in the enzyme Rubisco, β subunit of tryptophan synthase, and cytosolic ribosomes, leading to intensive loss of amino acid biosynthesis, cytosolic protein translation as well as photosynthetic machinery of N starve cells.

Starch is another important energy-rich compound, which reported to get accumulated within 2 days of N deprivation (Msanne et al. 2012). However, on prolonged N starvation the amount of lipid within the cells increases and major part of lipid synthesis is synthesized at the expense of already fixed carbon in the form of starch. Whereas, some small portion of lipid is synthesized by de novo

Table 1 Effect of phosphate and nitrogen starvation on lipid accumulation in algae

Algae	Nutrient	Effect	References
Chlorella sp. 227	Nitrogen	Lipid content was increased from 63.0 to 143.3 mg with the decrease in nitrogen source from 2 to 0.2 mM	Cho et al. (2011)
Isochrysiszhangjiangensis (Haptophyta)	Nitrogen	Lipid content was increased from 15.0 to 22.6% during nitrogen starve condition	Wang et al. (2015)
Chlorella sp. FC2 IITG	Phosphorus	Neutral lipid content increased from 1.00 to 28.60% in phosphate starve condition	Muthuraj et al. (2014)
Scenedesmus sp. LX1	Phosphorus	Increase in lipid content from 23 to 28% at the phosphate concentration of 0.2–2.0 mg L^{-1} to 53% at 0.1 mg L^{-1} phosphate concentration	Xin et al. (2010)
Isochrysis galbana U4	Phosphorus	Algal cells accumulated up to 50% lipid during phosphate starve and limit condition	Roopnarain et al. (2014)
Chlorella zofingiensis	Nitrogen	Lipid content was increased from 6.2 to 24.5% during nitrogen starve condition	Zhu et al. (2014)
Neochloris oleoabundans UTEX 1185	Nitrogen	Lipid productivity was increased from 62 to 202 mg L^{-1} day^{-1} during nitrogen deficient condition	Breuer et al. (2012)
Chlorella ellipsoidea	Phosphorus	Increase in lipid content from 15.2% (standard phosphate concentration) to 41.8% (phosphate starve condition)	Satpati et al. (2016)
Chlorococcum infusionum	Phosphorus	Lipid content was increased from 12.1 to 31.3% in phosphate starve condition	Satpati et al. (2016)
Scenedesmus obliquus UTEX 393	Nitrogen	Lipid productivity was increased from 68 to 360 mg L^{-1} day^{-1} during nitrogen deficient condition	Breuer et al. (2012)

pathway using newly fixed carbon and remaining portion is synthesized by consumption of carbon stored in cellular components due to ribosomal degradation (Li et al. 2011).

2.2 Phosphorus Depletion

Apart from nitrogen, phosphate (P) is another essential macronutrient that plays important role in plants and algae. Many metabolic processes such as nucleic acid metabolism, cell signal transduction, phospholipid metabolism, and ATP synthesis require P as essential component (Bajhaiya et al. 2015; Mandotra et al. 2016). Similarly like N starvation, P starvation also known to cause increases in lipid

content of microalgae; therefore, P starvation coupled with N starvation strategies is widely employed to enhance the overall lipid content. Apart from microalgae, P starvation is also known to increase lipid content in *Saccharomyces cerevisiae* (yeast) (James and Nachiappan 2014). Table 1 shows the increase in lipid content in various microalgae during P starve conditions.

It is reported by several researchers that, during P limiting conditions, the photosynthetic rate is less commonly affected as compare to cell division. The carbon flux from breakdown of intracellular molecules like carbohydrate, protein, and pigments directed towards accumulation of lipids, resulting into increase of cells size and volume (Liang et al. 2013; Muthuraj et al. 2013; Spijkerman and Wacker 2011). The P starved cells also have reduced amount of chlorophyll content, due to impaired chlorophyll synthesis mechanism. However, continuation of cell division still occurs with the breakdown of already fixed chlorophyll (Roopnarain et al. 2014). Study conducted by Khozin-Goldberg and Cohen (2006) on *Monodus subterraneous* reveals that phospholipids can also act as a source of phosphate during P starvation. They observed continuous increase in triacylglycerol with simultaneous reduction in phospholipids content of microalgae cells. This phenomena of lipid accumulation during phosphate or nitrogen starvation is explained by the fact that the oxidation of lipids molecules generate more energy upon oxidation and can serve as best energy reserve in unfavorable conditions (Muthuraj et al. 2013).

Study performed by James and Nachiappan (2014) demonstrated enhanced expression of lipid synthesizing genes such as acyl-CoA sterol acyltransferase-1 and acyl-CoA sterol acyltransferase-2 in P starved condition. Another study in P free growth medium showed reduced synthesis of ATP and NADPH (required for lipid synthesis), resulting into highly reduced lipid synthesis. This reduction in ATP and NADPH is attributed to decreased concentration of chlorophyll, which hampers capturing of solar energy and compromising the photosynthetic activity of the cell. Therefore, harvesting of algal culture is recommended at a particular time period where there's a considerable amount of phosphate concentration and possibility of getting maximum lipid content in the cells (Roopnarain et al. 2014).

2.3 *Effect of pH*

The pH of the growth medium is considered to be an important factor, as it can influence growth and various metabolic processes of microalgae. Unlike nitrogen and phosphate concentration, the optimum pH range for growth of different algae is species dependent. However, in most of the cases, the higher growth has been seen towards the alkaline pH of growth medium (Ho et al. 2014c). Study performed with *Scenedesmus abundans* revealed that, with the increase of pH form 5 to 8, the total growth of microalgae increases; however, increase in lipid content was reported at pH 6 (Mandotra et al. 2016). Another study, conducted by Muthuraj et al. (2014), on *Chlorella* sp. FC2, optimal growth was recorded between pH 6 and 8. Bartley et al.

Table 2 Effect of pH, temperature, and light intensity on lipid accumulation in algae

Microalgae	Stress factor	Effect	References
Scenedesmus abundans	pH	Higher biomass content (769.0 mg L^{-1}) at pH 8 and higher lipid concentration (179.47 mg L^{-1}) was at pH 6	Mandotra et al. (2016)
Botryococcus braunii	Temperature	With the increase in temperature saturated fatty acid content increased	Sushchik et al. (2003)
Tetraselmis suecica	pH	Biomass yield of 900 mg^{-1} with lipid productivity of 92 mg L^{-1} day^{-1} was achieved at pH 7–5	Moheimani (2013)
Scenedesmus obliquus	Light intensity	Sufficient light intensity (180 μmol m^{-2} s^{-1}) induced the accumulation of saturated and monounsaturated fatty acids	Ho et al. (2012)
Neochloris oleoabundans	Light intensity	Higher light intensity (300 μmol m^{-2} s^{-1}) accumulated saturated and monounsaturated fatty acids	Ho et al. (2012)
Euglena gracilis	Temperature	Higher growth rate at temperature range of 27–31°C	Kitaya et al. (2005)
*Chlorell*a sp.	pH	Biomass yield of 1600 mg^{-1} with lipid productivity of 99 mg L^{-1} day^{-1} at pH 7	Moheimani (2013)
Nannochloropsis oculata	Temperature	Lipid percentage increased with the increase in temperature from 20 to 25°C	Converti et al. (2009)
Phaeodactylum tricornutum	Temperature	Poly unsaturated fatty acid content increased with the decrease in temperature from 25 to 10°C	Jiang and Gao (2004)
Nannochloropsis salina	pH	Maximum cell growth was observed at pH 8 and 9, highest lipid accumulation at pH 8	Bartley et al. (2014)
Chlamydomonas sp.	pH	Long chain saturated fatty acid was higher at pH 6	Tatsuzawa et al. (1996)
Neochloris oleoabundans HK-129	Light intensity	Light intensity of 200 μmol m^{-2} s^{-1} induced synthesis of higher percentage of C16/C18 fatty acids than in 100 μmol m^{-2} s^{-1}	Sun et al. (2014)
Botryococcus spp.	Light intensity	Lipid content increased with increase in light intensity from 33 to 49.5 μmol m^{-2} s^{-1}, decreased when light intensity increased to 82.5 μmol m^{-2} s^{-1}	Yeesang and Cheirsilp (2011)
Chlorella vulgaris	Temperature	With the increase in temperature saturated fatty acid content increased	Sushchik et al. (2003)

(2014) have shown higher growth rates at pH 8 in microalga *Nannochloropsis salina*. During unfavorable conditions, such as higher alkaline conditions, the higher pH hinders the cell division process by inhibiting release of autospores. The autospores are known to utilize lipids during cell division cycle, thereby, stopping autospore can enhance lipid content of cells with some decrease in membrane lipids (glycolipids and polar lipids) (Guckert and Cooksey 1990).

Besides increasing lipid content, the pH of the culture medium is one of the factors that determine the fatty acid profile of microalgae. Table 2 shows the effect

of pH levels on biomass and lipid content of the algae. Study conducted by Santos et al. (2013) on *Neochloris oleoabundans* revealed that at higher pH levels under nitrogen deplete condition, the MUFA content (C18:1) increases up to twofold with sharp decrease in the PUFA (C18:3) content. Another study on *Chlamydomonas* sp., at pH 1, has shown higher proportion of TAG accumulation than culture grown in higher pH (Tatsuzawa et al. 1996).

2.4 *Effect of Light Intensity*

Apart from availability of nutrients and favorable pH for algal growth, availability of light plays most important role in controlling photosynthesis, cell growth, and CO_2 fixation in microalgae. During photosynthesis CO_2 is converted into energy-rich compounds (triacylglycerol and starch) via primary precursor glyceraldehydes-3-phosphate (G3P) (Williams and Laurens 2010). Appropriate light intensity has been reported to change the concentration of NADPH, Mg^{2+}, and pH levels in the stroma that could regulate G3P, a key metabolite for lipid accumulation (Ho et al. 2014a). Apart from lipid accumulation, different light intensities also known to regulate the level of phosphoglucomutase (PGM), a key enzyme involved in starch synthesis (Neuhaus and Stitt 1990).

Three different phases of light intensities such as light limitation, light saturation, and light inhibition can influence the growth of microalgae. Ho et al. (2012) reported that the growth rate of microalga *Scenedesmus obliquus* CNW-N increased with increasing light intensity during light limiting phase. The highest growth rate (1.8 d^{-1}) was recorded during the light saturation phase of 180 μmol m^{-2} s^{-1} followed by light inhibition phase, as they keep on increasing light intensity to 540 μmol m^{-2} s^{-1} resulted in significant decrease in biomass and CO_2 fixation rate.

Acetyl-CoA carboxylase (ACCase) is rate limiting enzyme for the synthesis of fatty acid that can convert acetyl-CoA into malonyl-CoA (Ohlrogge and Browse 1995). Optimum alkaline pH and higher concentration of NADPH and Mg^{2+} ions augment in vivo activity of ACCase. The absorption of photons during photosynthesis leads to movement of H^+ ions from stroma to thylakoid membrane with the accumulation of Mg^{2+} ions inside the stroma. As a result of this, stromal pH increases, which could also increase the activity of ACCase and further leads to increase in lipid accumulation (Lv et al. 2010).

In most of the microalgae, intensity of light decides the fate in the sense that which particular energy molecule (lipid and carbohydrate) will be accumulated in the cell. The higher light intensities can reduce the synthesis of membrane polar lipids in microalgae with considerable increase in accumulation of neutral lipids (Ho et al. 2014c). On the other hand, low-light intensity induces the synthesis of membrane polar lipids, especially sulfoquinovosyldiacylglycerols, phosphatidylglycerol, and phosphatidylcholine (Sharma et al. 2012). Table 2 shows the effect of different light intensities on microalgae. Study conducted by Sun et al. (2014) demonstrated that, at

sufficiently higher light intensity (300 μmol m^{-2} s^{-1}), *Neochloris oleoabundans* HK-129 synthesized higher percentage of saturated and monounsaturated (C16:0, C18:0, and C18:1) fatty acids.

2.5 Effect of Temperature

Like other abiotic factors, temperature is also known to modulate the growth and lipid metabolism in algae. Favorable temperature for higher growth rate and lipid production, however, differs for different algal species (Vitova et al. 2015). Many microalgal species tend to alter their morphology during different temperature regime. For example, increase in cell size of microalga *Acutodesmus dimorphus* was observed at 35 and 38°C (Chokshi et al. 2015). Various microalgae have been studied for the effect of different temperature on the overall lipid percentage of the cell for biofuel production. Three different species of *Chlorella* viz. *Chlorella*-Des, *Chlorella*-Arc, and *Chlorella*-Nat have achieved highest lipid percentage at 3, 10, and 21°C, respectively (Yang et al. 2016). The lipid percentage of *Monoraphidium* sp. decreased as the temperature was increased from 25 to 35°C (Wu et al. 2013), whereas maximum lipid productivity of *Desmodesmus* sp. was achieved at 35°C and decreased when cultivated at 25°C (Ho et al. 2014b). Therefore, it can be concluded that the effect of temperature on microalgae is also species dependent. Table 2 shows the effect of cultivation temperature on different microalgae.

Effect of temperature can also be seen on the fatty acid profile of microalgae, with the decrease in temperature, significant increase in unsaturation of fatty acids has been reported. On the other hand, higher temperature favors the synthesis of saturated fatty acids. Membrane physical properties are directly influenced by the temperature of the growth medium, during unsaturation; the carbon–carbon double bonds are loosely packed as compared to saturated fatty acids, therefore during lower temperature membrane fluidity is maintained by the synthesis of unsaturated fatty acids that provides an adaptation for the cell to perform its normal function (Bell 1989; Sharma et al. 2012). Study conducted with *Chlorella* spp. grown at cold temperature has shown significant increase in the ratio of saturated fatty acid to unsaturated fatty acid with more increase in poly unsaturated fatty acid content of cells (Yang et al. 2016).

3 Strategies for Manipulating Microalgae

Apart from trying different growth conditions, many metabolic engineering strategies were also tried to enhance lipid or carbohydrate accumulation in microalgae for biofuel production. The nutrient stress conditions cause reduction in growth rate, biomass, and overall lipid content or quality (Scott et al. 2010). Thus, metabolic engineering strategies, which can enhance lipid or carbohydrate content and

quality, without reducing biomass and yield, can be effective method for biofuel industries. Up till now total 20 algal genomes have been fully sequenced, starting with green algae—*Chlamydomonas reinhardtii* (Merchant et al. 2007), *Ostreococcus lucimarinus* CCE9901 (Palenik et al. 2007), *Coccomyxa* sp. C-169, *Micromonas* CCMP 1545 (Worden et al. 2009), *Ostreococcus tauri* (Derelle et al. 2006), and *Volvox carteri* (Prochnik et al. 2010); diatoms: *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bowler et al. 2008); red alga: *Cyanidioschyzon merolae* Strain:10D (Matsuzaki et al. 2004); brown alga: *Ectocarpus siliculosus* (Cock et al. 2010). These fully sequenced organisms can helpful in identification of different genes and enzymes involved in the lipid and carbohydrate biosynthesis. This genetic information can be strategically used for manipulating algal strains to enhance lipid and carbohydrate synthesis.

With the advances in genetic engineering and system biology various algal genome manipulation tools have came in existence. Microalgae can be transformed with foreign genes using methods such as glass beads (Kindle 1990), microprojectile particle bombardment (Apt et al. 1996), electroporation (Tang et al. 1995), agrobacterium mediated transformation (Kumar et al. 2004), and agitation in the presence of silicon carbide whiskers (Dunahay 1993). Manipulation of metabolic pathways in microalgae can be performed by various strategies such as by down-regulating competing pathways; site-directed mutagenesis to improve the efficiency of key enzymes/proteins; over-expressing of transcription factors, or rate limiting enzymes controlling synthesis of desired product (De Bhowmick et al. 2015).

Depending on the desired metabolites, genes of different metabolic processes can be targeted and transformed in microalgae. Some of the strategies such as optimizing light utilization; altering carbon flow pathways; modifying enzymes of lipid, carbohydrate synthesis, and transcriptional engineering has been tried for increasing efficiency of algae for biofuel production (Gimpel et al. 2013; Work et al. 2012).

3.1 Improving Photosynthetic Efficiency and Light Utilization

Earlier studies have suggested that algal photosynthesis can convert approximately 5–7% of incident light energy into biomass in controlled culture conditions (Blankenship et al. 2011; Peers 2014). The photosynthetic efficiencies can be push further by genetic tools, which might be helpful to reduce the land area utilization and associated infrastructure needed for biofuels production (Simionato et al. 2013).

Naturally microalgae have large light harvesting complex (LHCs), which is useful to maximize light absorption in low-light environment. In artificial culture conditions, algal cells try to dissipate out extra light energy through heat and

fluorescence quenching by LHCs (Gimpel et al. 2013). The excess energy, which cannot be dissipated, can lead to photodamage or photoinhibition due to production of excess reactive oxygen species (Ort et al. 2011). The larger size of LHCs is also a problem as it can reduce light penetration in artificial culture conditions leading to reduced growth and biomass for biofuel production. To overcome these limitations RNAi silencing studies are performed by Mussgnug et al. (2007) on all 20 LHC protein isoform of *C. reinhardtii*. All RNAi transformed lines showed reduced LHC mRNA (0.1–26% lower relative to control) and protein accumulation with 68% less chlorophyll resulting about 290% higher light transmittance in artificial culture conditions (Mussgnug et al. 2007). Further reduced fluorescence quenching was observed, leading to substantial increase in photosynthetic quantum yield. Although in high-light conditions, transformed cells grew faster with less photoinhibition but increase in cell density or biomass was not observed. Mussgnug et al. (2007) also down-regulated LHC expression, using the redox-dependent translational repressor of LHC protein family called NAB1. In this case effect of down-regulation was less dramatic with only 20% reduction chlorophyll per cell compared to 68% in RNAi lines (Beckmann et al. 2009).

One of the important targets of photodamage is photosystem II (PS II), which is a multiprotein complex and performs light-driven oxidation of water. Under excess light, degradation of D1 subunit of PSII can significantly increase (Keren and Krieger-Liszkay 2011). Rea et al. (2011) amplify algal D1 coding sequences of PSII using error-prone PCR and selected mutant under ionizing radiation. Unfortunately selected lines performed worse in artificial light conditions (10% midday sunlight), suggesting that targeting some of these PSII proteins might not be a good strategy to increase biomass for biofuel production (Rea et al. 2011).

Similar study was performed by Gimpel and Mayfield (2013), they express heterologous D1 protein (psbA) in *C. reinhardtii* chloroplasts and concluded that heterologous gene expression is not the best approach for enhancing photosynthesis. However, D1 proteins of cyanobacteria, Synechoccocus (species. PCC 7942), were expressed in *C. reinhardtii* and gave high-light and low-light phenotypes related to D1 isoforms. Interestingly, low-light phenotype showed 11% higher yielded in dry biomass compared to high-light phenotype and endogenous D1 expressing strain, making low-light phenotype strain as desirable trait for biofuel industries (Gimpel et al. 2015).

3.2 Modification of Carbon Assimilation

Increasing carbon flux and its utilization within the microalgal cell can be an important component for biofuel industries. In general algae are known to be good in carbon sequestration but its efficiency can be enhanced further by genetic engineering of key enzymes. The amount of CO_2 fixation plays major role as it can significantly affect cell metabolic process including lipids, carbohydrate, and biomass synthesis (Wang et al. 2008). It is report that under phototropic conditions the

fixation of environmental carbon dioxide into ribulose-1,5-biphosphate (RuBP) to form 3-phosphoglycerate is catalyzed by enzyme called RuBP carboxylase/oxygenase (Rubisco). Several other enzymes with ATP and NADPH are required during Calvin cycle to regenerate RuBP (Raines 2011). Light-driven activity of photosystems I and II supplies required amount of ATP and NADPH for Calvin cycle.

The activity of Rubisco is major bottleneck for carbon flux through Calvin cycle when there is not enough amount of CO_2 available in media or in high-light and temperature condition, which can easily happen in commercial large scale ponds used for algal biomass production (Ducat and Silver 2012; Whitney et al. 2011). Several studies have also suggested that RuBP carboxylase/oxygenase is slow and confused enzyme (Gimpel et al. 2013). For sustainable carboxylation rate, very large amount of Rubisco is needed and its affinity for oxygen can increase counter-productive reactions. Rubisco deficient strains of *C. reinhardtii* were generated by researchers and these strains can grow heterotrophically, unlike plants (Whitney et al. 2011).

To further evaluate the role of Rubisco, only smaller subunit of Rubisco (rbcS) from Sunflower and arabidopsis was transformed into rbcS deficient *C. reinhardtii*. In transformed lines about 11% increase in CO_2/O_2 specificity factor (V) was reported with no change in V_{max} of carboxylation (Genkov et al. 2010). PCR-based gene shuffling was also tried with larger subunit of Rubisco, after three rounds of gene shuffling and strain selection about 20% increase in V and 56% increase in V_c was reported. In spite of increase in CO_2 affinity in both the mutants, no significant increase in growth rate of cells or biomass was observed (Zhu et al. 2010). Another strategy to increase efficiency of algae to utilization of carbon, nitrogen, and light energy was tried by tuning abundance of Rubisco in culture/environmental conditions. Rubisco was engineered by altering expression of rbcL mRNA maturation factor MRL1 in *c. reinhardtii*, different mutant lines with difference in expression of MRL1 were generated. Expression of Rubisco was lowered upto 15% compared to wild type, while maintaining phototrophic growth (Johnson 2011).

It has been reported that some algae are strict heterotrophs and are very selective for their source of organic carbon. The heterotrophic growth has few advantages such as controlled culture conditions in closed reactors, higher cell densities and lipid production per volume per day, and ability to utilize more nutrients from culture media or wastewater (Chen et al. 2011). *C. reinhardtii*, *Phaeodactylum tricornutum*, *Volvox carteri*, and *Cylindrotheca fusiformis* were transformed with hexose transporter gene (HUP1) resulting utilization of glucose as carbon source (Doebbe et al. 2007; Fischer et al. 1999; Hallmann and Sumper 1996; Zaslavskaia et al. 2001). However, adding hexoses to culture media is not suitable as it will increase risk of contamination and cost of biofuel production.

3.3 Genetic Modification of Lipid Metabolism in Microalgae

In spite of whole genome sequence of different microalgae the knowledge about lipid metabolism is very limited. Several attempts were made to engineer microalgal fatty acid and TAG biosynthesis pathway to enhance the lipid content of microalgal cells. The synthesis of any particular metabolite is dependent on the activity of responsible enzymes in that pathway. Therefore, attempts to enhance the activity of enzymes by overexpression of individual genes responsible for lipid biosynthesis were made. Below are some of the examples of overexpression strategies.

Some of the acyltransferases of Kennedy pathway such as acyl-CoA: glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA: diacylglycerol acyltransferase (DGAT), and the acyl-CoA: lysophosphatidic acyltransferase (LPAAT) are important enzymes involved in synthesis of fatty acid patterns of TAGs (Khozin-Goldberg and Cohen 2011). In diatom *Thalassiosira pseudonana* and chlorophyte *Ostreococcus tauri*, two of the GPAT and one DGAT have been cloned and their function was characterized based on available genome information (Wagner et al. 2010). Similarly overexpression of two isoforms of LPAAT in *Brassica napus* and GPAT in yeast gat1 mutant resulted in increase of phosphoinositol and TAG content (Khozin-Goldberg and Cohen 2011; Tonon et al. 2002). The starchless mutants of *C. reinhardtii* also showed increase in transcript abundance of DGAT2 gene as compared to wild type, suggesting the importance of DGAT2 for hyper-accumulation of TAG in microalgae (Khozin-Goldberg and Cohen 2011). The fivefold increase in TAG content in amyloplast of potato tubers was also observed when ACCase from *Arabidopsis thaliana* was over-expressed.

As it's mentioned above that many microalgae can accumulate lipid on nutrient stress but they need to compromise with biomass yield, which is not ideal for biofuel synthesis. Therefore it will be advantageous if microalgae-specific inducible promoters can be used for inducing overexpression of lipid biosynthesis gene. Inducible promoter system can be used to induce expression of specific lipid or carbohydrate synthesis gene, when sufficiently high amount of cell density is reached. An example of this approach is overexpression of DGAT in *C. reinhardtii*, using in P starvation inducible promoter, sulphoquinovosyldiacylglycerol 2 (SQD2). The DGAT engineered strain showed 2.5-fold increase in TAG accumulation as compared to wild type (Iwai et al. 2014). Similar approach was also used for overexpression of copper-responsive elements (CuREs) in *C. reinhardtii* (Quinn and Merchant 1995).

Overexpression study of endogenous thioesterase on diatom *P. tricornutum* was also performed with 72% increase in total fatty acid content without change in relative chain length compositions (Gong et al. 2011). Further to produce biodiesel stocks with shorter-chain fatty acids to enhance cold flow properties of biodiesel, two thioesterases from different terrestrial plants were transformed into *P. tricornutum*. These transformed thioesterases increased C12 and C14 ratio of fatty acids in *P. tricornutum* and most of these fatty acids were incorporated into TAG, which is important feedstock for biofuel production (Radakovits et al. 2011).

3.4 Genetic Modification of Carbohydrate Metabolism in Microalgae

Apart from lipid, algae can also produce good amount of carbohydrate, as primary store from photosynthesis (e.g., cellulose) and transient energy storage such as starch, glycogen, or chrysolaminarin (Santelia and Zeeman 2011). Starch is extensively used as feedstock for biofuels, through conversion to alcohols. Starch is an important energy-rich reservoir in some class of algae such as Chlorophyta (green algae), Glaucophyta, Rhodophyta (red algae), and Dinophyta (dino-flagellates). In few other algal classes, like Bacillariophyceae (diatoms) and Phaeophyceae (brown algae), glucans stores in laminarin and chrysolaminarin (Work et al. 2012). Algal carbohydrates can be hydrolyzed and fermented by yeast to make ethanol or they can be used as carbon source for producing biofuels from microorganisms (Harun et al. 2010). Making algal cells efficient for synthesis of carbohydrate can be very advantageous for biofuel industries. Therefore several genetic engineering strategies like overexpression of starch biosynthesis enzymes (e.g., ADP-glucose pyrophosphorylase (AGPase) or isoamylase), knockout studies of key starch degrading enzymes (e.g., glucan-water dikinases and amylases), and alter secretion to export soluble carbohydrates were proposed and tried by several researchers (Work et al. 2012).

During starch synthesis in microalgae or plants, key reaction of glucose-1-phosphate with ATP to form ADP-glucose and is catalyzed by AGPase. Several studies were performed to alter catalytic and allosteric properties of AGPase in higher plants to enhance starch synthesis. Starch-synthesizing enzymes were also expressed in cytosol of microalgae to enhance starch accumulation for biofuel production (Deschamps et al. 2008; Smith 2008).

Similarly complementation studies of isoamylase in mutant of *C. reinhardtii* were performed and resulted into "starch excess" strains, which can produce about three to fourfold higher starch compare to wild type. However, this increase in starch excess' strains causes reduction in cell division and protein synthesis (Work et al. 2010). Recently, overexpression studies of phosphate starvation response (PSR1) transcription factor in *C. reinhardtii* have reported significant increase in starch accumulation. Several starch synthesis genes showed PSR1 mediated regulation in both P sufficient and deficient conditions with no significant change in biomass (Bajhaiya et al. 2015). This study suggested that PSR1 can be a potential regulator for starch biosynthesis and expression of PSR1 in higher starch producing strain can be useful to develop superior starch producing strain.

3.5 Advances in Transcriptional Engineering

As algae are diverse with non-uniform class of lipids and carbohydrates; therefore, single gene target approach has been extensively used to study individual variations

in different algal strains. This traditional method of identifying and manipulating single gene is an effective approach but considering the interest in final product, lipid or carbohydrate, this method does not seem to develop into economically viable system (De Bhowmick et al. 2015). With further advances in molecular engineering, researcher has made some attempts to engineer transcription factors (TFs), as TFs can control larger number of lipid and starch biosynthesis genes and can be useful to develop a metabolic switch to control specific metabolic process.

TFs are proteins, which interact with *cis*-elements in promoter regions of genes and can regulate the expression of downstream genes involved in various metabolic processes. They can directly interact with DNA polymerase to activate it to enhance the transcription of specific group of genes or can act as repressors to control certain metabolite synthesis (Latchman 1997). TFs can be engineered to act as triggers for diversion of metabolites leading to stress-free production of high value product and biofuels. Several strategies for transcription factor engineering (TFE) are possible, which can be used to control desired pathways for synthesis of specific metabolites. However, this alternative approach of modifying metabolism by TFE is still in its premature stage in microalgae. Several attempts of TFE on higher plants, animals, and microorganisms have been performed and shown success in modifying composition with overproduction of valuable metabolites like lipid and starch (Cernac and Benning 2004; Fu and Xue 2010).

In microalgae, up till now only five transcription factors, PSR1, CHT7, ROC40, NRR1, and soybean transcription factor GmDof4 are engineered with successful alteration of metabolites. The transcription factor PSR1, member of MYB-CC (MYB coiled-coil domain) transcription factor family, is reported to express in phosphate starvation condition and control carbon storage metabolism by controlling specific lipid and starch bio-synthesis genes (Moseley et al. 2006). PSR1 comes out to be regulator of starch as well as lipid biosynthesis genes under both nitrogen and phosphorus starvation (Bajhaiya et al. 2015; Ngan et al. 2015). Some of earlier studies have also suggested that PSR1 plays important role in integration of signaling pathways between sulfur and phosphorus starvation responses (Moseley et al. 2009). Another transcription factor called compromised hydrolysis of triacylglycerols 7 (CHT7) was known to express in N-starvation conditions. Study of CHT7 mutants demonstrated that CHT7 can act as a repressor of cellular quiescence and control TAG degradation after re-supply of N (Tsai et al. 2014). Simultaneous overexpression of PSR1 and CHT7 can be a good target for the engineering microalgae for higher lipid and starch accumulation.

Another MYB-related transcription factor ROC40 was reported to express in N-deprived conditions. ROC40 mutant showed reduction in TAG accumulation suggesting ROC40 regulation of TAG genes; however, overexpression studies were not performed to confirm its role (Goncalves et al. 2016). Similarly a potential N response transcription factor, NRR1, was also identified with enhanced expression under N deprivation. The Chlamydomonas mutant of nrr1 under N deprivation showed about 50% reduction in TAG accumulation compared to parental strain. NRR1 is reported to controls expression of DGTT1, and AMT1D, which suggest

that NRR1 can work as regulatory TF in controlling TAG accumulation as well as, N assimilation (Boyle et al. 2012).

Apart from endogenous TFs of algae, heterogeneous-expression of TF such as GmDof4 from soybean was also expressed in *C. ellipsoidea*. GmDof4 comes from soybean Dof-type (DNA binding with one finger) TF family and know to regulate lipid content in soybean seeds. Hetero-expression of GmDof4 in *C. ellipsoidea* alters expression of 22 lipid biosynthesis genes and the increase in accumulation of lipid without affecting growth rate in mixotrophic culture conditions was also observed (Zhang et al. 2014). TF engineering is capable of wider regulation of metabolic genes with higher chances of success to increase accumulation of desired metabolite. Up till now, most of the studied TFs on algae were aimed to increase either lipid or starch biosynthesis for biofuel production. Some of recent transcriptomic and proteomic studies have suggested several new algal TFs, which can be engineered to alter metabolite synthesis for production of high value compounds as well as biofuels.

To study *Nannochloropsis*, an oleaginous microalga have reported at least 11 TFs with regulatory role in lipid metabolism, few of these TFs were found to be orthologs of TFs which are reported to be involved in lipid metabolism in higher plants (Hu et al. 2014). In 2008, total 147 TFs and 87 transcription regulators were reported in *C. reinhardtii*; however, the biological relevance of most of these TFs is still not determined (Courchesne et al. 2009). It is important to find out the specific TFs regulating lipid and carbohydrate biosynthesis as it can help to develop strains with enhanced accumulation of metabolites without affecting photosynthesis and total biomass. TFE in microalgae is still at very early stage compared to single gene engineering; however, it has great potential for multigene targeting and ability to improve desired metabolite synthesis for biofuel production.

4 Conclusion

The increase in energy crisis and environmental concern has brought new challenges for scientific community. Most of the challenges revolve around development of economic, environmental sustainable, and alternative renewable energy sources. Biofuel from biomass is a potential solution but there are several technological hurdles. Microalgae biomass is the most promising source of biomass, with faster growth rates, higher lipid contents with limited requirement of arable land and freshwater. However, the amount of neutral lipid in microalgae under natural conditions is not enough but can be enhanced by altering growth conditions or by making suitable genetic manipulations. Several nutrient stress conditions starting from macronutrients to micronutrients are tried and demonstrated limited success.

Genetic engineering coupled with transcriptomic and proteomic has brought new hopes. New genetic targets suggested by several transcriptomic and proteomic studied could bring breakthrough for algal industries. Initially genetic engineering was focusing on targeting single gene or pathway, which results in some success

with increase in lipid or carbohydrate. However, more innovative approaches like silico analyses, metabolic control, analyses of microalgal strains with engineering of regulatory elements like transcription factors could bring solution for enhancing lipid or carbohydrate content. These approaches coupled with biochemical and bioprocess studies could help to find a genetically stable and environmentally robust microalgal cell lines for biofuel production.

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Phycoremediation of Heavy Metals Coupled with Generation of Bioenergy

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Contents

1 Introduction

Algae are a large group of versatile plant species with excellent photosynthetic efficiency which can adopt and grow in any environment (Latiffi et al. 2015; Rawat et al. 2011). They require mainly sunlight, CO_2, nitrogen and phosphorus and microelements for photosynthesis and growth. Algae are the largest group of primary producers and contribute approximately 32% in the overall global photosynthesis (Priyadarshani et al. 2011). The term phycoremediation is used to

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denote the remediation (removal, degradation, assimilation, etc.) from various types of algae and cyanobacteria (Olguín and Sánchez-Galván 2012).

Among diverse environmental problems faced by the modern world, heavy metal pollution is of major issue of concern (Chabukdhara and Nema 2012a, b). Heavy metals are defined as elements with metallic properties and atomic number greater than 20 (Jing et al. 2007; Srivastava 2007), and atomic density is greater than 20 g/cm^3 or five or more times greater than water (Duruibe et al. 2007; Herrera-Estrella and Guevara-Garcia 2009). Wang and Chen (2009) grouped various heavy metals into three different categories, i.e. Ni, As, Cd, Cr, Zn, Hg, Pb, etc., and are termed as toxic metals; Ag, Pt, Pb and Au are considered as precious metals, whereas U, Ra and Th are termed as radionuclides. Heavy metal pollution is of serious concern due to its long biological half-lives, non-biodegradable nature, bioaccumulation and high toxicity (Kabata-Pendias and Pendias 1992; Radha et al. 1997; Li et al. 2004). Heavy metal contamination of aquatic ecosystems may threaten aquatic organisms and associated ecosystem. Heavy metals can cause severe effects to humans, once entered into the human food chain via consumable aquatic plants (Khan et al. 2000; Chabukdhara and Nema 2012a), vegetables (Chabukdhara et al. 2016) and aquaculture products (Yılmaz et al. 2010; Leung et al. 2014; Gupta et al. 2015a). It can cause malfunction of various vital organs such as cardiovascular, kidney, bone, etc., and previous studies have shown most of the heavy metals are carcinogenic, teratogenic and/or mutagenic for human beings (WHO 1992; Steenland and Boffetta 2000; Jarup 2003; Oskarsson et al. 2004).

Rapid urbanization, industrialization, agricultural practices and other anthropogenic activities are responsible for generation of heavy metals that contaminate water bodies when discharged untreated. As far as environmental contamination is concerned, the major anthropogenic sources of heavy metals are mining, metal processing, electroplating, textiles, tanneries, batteries, refineries, paints and pigments, pesticides, paper industries, etc. Heavy metals such as Pb, As, Hg, Ag, Cd, Cr, Ni, Mn, etc. may originate from natural sources. Sediment and soil are the natural sink and source of metals and other pollutants (Chabukdhara and Nema 2012b, 2013).

Currently, high population density lives in urban areas throughout the world, which generate a huge volume of wastewater. In the present scenario, recycle and reuse of the wastewater is considered as a potential option to cope up with the increasing water crisis. The focus is now shifted from pollution control to recycle and reuse of wastewater due to technical advancement and to meet the economic and societal needs and sustainable development.

The treatment of heavy metals containing industrial wastewater before discharge to the aquatic ecosystems is crucial so that the risk of heavy metal contamination of the ecosystem and its associated food chain can be avoided. Various physicochemical techniques such as ion exchange, chemical precipitation, membrane filtration, adsorption, oxidation with ozone/hydrogen peroxide, photocatalytic degradation, coagulation flocculation, electrochemical and floatation methods, etc. are used, if the metal concentration is below 100 mg/L (Fu and Wang 2011). However, most of such technologies are energy intensive and, hence, expensive. Moreover, huge

amount of toxic sludge is being generated in such treatment which poses disposal problems. Metal sorptions using biotechnological approach have been found comparatively more effective for the treatment of heavy metal-contaminated water and wastewater (Schiewer and Volesky 1995; Vieira and Volesky 2000).

Phycoremediation has evolved as a cost-effective eco-friendly alternative from the pioneering work carried out by Oswald (1963), which can be used for the remediation of soil and wastewaters contaminated by heavy metals (Olguín 2003). Phycoremediation can also be used for several other bioremediation applications such as treatment of wastewater and industrial effluents containing high concentration of organics, nutrient removal from wastewaters, removal of pathogenic microbes, etc. Live or dead algal biomass can also be used as biosorbents for removal of xenobiotic contaminants from water and wastewaters. Along with it, the algal systems can also be used for treatment of acidic and metal wastewaters, CO_2 sequestration, transforming and degradation of xenobiotics and detection of toxic compounds by algae-based biosensors (Olguín 2003; Kwarciak-Kozłowska et al. 2014; Dominic et al. 2009; Gupta et al. 2016). The algae can grow on various types of wastewaters so they do not require arable land for cultivation. Moreover, most of the freshwater and marine algal species can be used for the biomass production alongside due to its inherent high productivity. Therefore, such properties make algae as a special candidate for the phycoremediation and bioremediation in general (Gupta et al. 2015b). Algae-based technologies are capable of treating various types of wastewaters efficiently and economically in one go, whereas conventional wastewater treatment technologies do the same but in multistep energy-intensive processes and are, therefore, very expensive.

In socioeconomic development, along with clean water, the energy also plays a significant role in overall growth of a country; in fact they are the engines of growth for global economy. Rapid urbanization and industrialization are the root causes of global crisis for fresh water and energy. Limited availability of fossil fuel is now revived the worldwide focus towards numerous alternative options and the development of sustainable technologies for alternative fuel production (Jang et al. 2012). Now, it has been established that for the economic as well as the environmental sustainability, the use and production of carbon neutral renewable biofuel are the only options. Biofuels have been recognized as promising alternative renewable sources of energy. Nevertheless, first-generation biofuels were questioned due to the utilization of food crops as raw materials for bioenergy production. Biofuel production processes using microalgae biomass coupled with wastewater treatment seem to be a promising alternative (Khan et al. 2009). The algal biofuels produced from the wastewater can help in developing a sustainable economy and can also help in reducing fresh water demands for biofuels. Thus, the need of day is that the wastewater treatment by phycoremediation must be ensured for both remediation as well as biomass production which can be utilized for the value-added products and biofuels (Malla et al. 2015).

This chapter is an attempt to give an overview of algal technologies used for the heavy metal removal from wastewater and its potential for use in energy production as well. The information may help the scientific societies and stakeholders to

understand the current status of research and advancements made in this field. Such concurrent information help in taking suitable initiative and measures towards improving available techniques and its applications.

2 Application of Algal Technologies for the Remediation of Heavy Metals from Wastewater

2.1 Mechanisms of Heavy Metal Removal from Wastewater by Algae

Several species of algae (both freshwater and marine) are known to remove heavy metals from wastewater and possess excellent potential to accumulate heavy metals as well as various other inorganics within their cells. Several studies have established that use of algae is an economic method for heavy metal sequestration from various types of wastewater, and such treated wastewater can be reused without additional treatment (Kiran et al. 2007; Nasreen et al. 2008; Bhat et al. 2008; Pandi et al. 2009; Afkar et al. 2010; Kumar and Gaur 2011; Chen et al. 2012; Gupta et al. 2016). The presence of heavy metals in the industrial wastewaters has been depicted elsewhere (Singh et al. 2016).

It has been established now that microalgae possess excellent potential of metal sequestration, and in the past few decades, live algae or immobilized algal biomass have been used for metal biosorption studies using various types of bioreactors such as batch, continuous flow, packed bed column, flow-through twin-layer, tubular photo, continuous-contact mode operations, etc. due to its multiple advantages over inert conventional materials. Presently, live or dead microalgal biomass is trending due to its ability of rapid metal uptake, saving of energy and time, user- and eco-friendliness, ease of handling and its occurrence throughout the year, low cost, recyclability as well as reusability, large surface-to-volume ratio and comparably faster growth rate. Moreover, algae also possess the ability to bind metal ions up to 10% of their biomass, with selectivity that enhances their performance without the requirement of synthesis. Phycoremediation of metals is also free of toxic waste generation; therefore, it is applicable in both continuous and batch modes. Due to such properties, the phycoremediation is capable to treat wastewater either of high or of relatively low contaminant levels (Monteiro et al. 2012). Algae can effectively adsorb and metabolize trace metals due to their large surface/volume ratio, high affinity towards metals, presence of metal-binding groups on their cell surfaces and efficient metal uptake and storage systems (Rajamani et al. 2007). Molecular mechanisms of microalgae allow them to discriminate non-essential heavy metals from essential ones, for their growth (Perales-Vela et al. 2006). Monteiro et al. (2012) explained the merit of using living and nonliving biomass of microalgae for removing heavy metals even at low concentration. Monteiro et al. (2012) demonstrated phycoremediation wastewaters contaminated

with Cr(VI) using charophyte, *Nitella pseudoflabellata*, and the findings suggested optimum remediation through passive (short-term) and active (long-term) treatments (Gomes and Asaeda 2009). A combination of high and low Ca and Cr (VI) was used for the active and passive treatments.

Kumar et al. (2007) investigated the different living colour forms (pale yellow, green and brown) of a marine algae (seaweeds) named *Kappaphycus alvarezii* to remove heavy metals from wastewater. Results demonstrated that brown colour form could efficiently adsorb good amount of Cd 3.064 mg/100 g f.wt. and Co 3.365 mg/100 g f.wt. in laboratory conditions. Brinza et al. (2007) reviewed the biosorption properties of different micro- and macro marine algal species for the removal of metals. A host of literatures on heavy metal uptake by both living and dead algal has been reviewed. Brown algae are found to be more efficient in absorbing heavy metals than red and green algae. Saunders et al. (2012) studied bioremediation of Cd and Zn present in the wastewater of coal-fired power generation units using three algal species, *Rhizoclonium* sp., *Oedogonium* sp. and H*ydrodictyon* sp. Similarly, Kwarciak-Kozłowska et al. (2014) investigated the potential of microalgae in removal or biotransformation of integrated nutrients (N and P) and heavy metals (iron, manganese and zinc) from municipal wastewater using two microalgae species, i.e. *Chlorella vulgaris* and *Scenedesmus armatus*. Results showed that *Chlorella vulgaris* performed better for removal of total nitrogen and bioaccumulation of heavy metals from effluent wastewater, whereas *Scenedesmus armatus* was highly efficient in removal of heavy metals from influent wastewater. Maria et al. (2016) studied four different species of brown macro-algae (*Pelvetia canaliculata*, *Fucus spiralis*, *Ascophyllum nodosum* and *Laminaria hyperborea*) as natural cation exchangers for the remediation of petrochemical wastewater contaminated with selected transition metals. The results revealed that *L. hyperborea* was found to be superior to the rest of the algae species. El-Sheekh et al. (2015) studied toxic pollutant removal efficiencies of two different types of algae, i.e. freshwater and marine *Chlorella* species for various types and strength of sewage, sea, and well water samples, and the results suggested excellent phytoremediation potential of both algae species with specific reference to the reduction in biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), ammonia and nitrate, sulphate and phosphate, sodium, potassium, magnesium and calcium, as well as selected heavy metals (Mn, Ni, Zn, Co, Cu, Cr and Fe) and the coliform bacteria within a short period of treatment (10 days). In this study, it was recorded that *C. vulgaris* was comparably more efficient than *C. salina* in treating the water samples. Kumar et al. (2012) explored the possibility of using dry algal biomass of *Spirogyra hyalina* as biosorbent for the removal of varying concentrations of different heavy metals (As, Hg, Pb, Co and Cd) at varying contact time. Results suggested that dried biomass of *S. hyalina* was efficient in treating wastewater by adsorbing the heavy metals. Due to the immense capability of adsorbing metals, algae are considered potential candidates for treating wastewater. Heavy metals first bind to cell surface of algae and then get internalized. Biosorption capacities of four different species of red seaweeds for heavy metals from aqueous solutions were evaluated by observing the effects of

biomass dosage, contact time and pH. The biomass of red seaweed *G. oblongata* was found most efficient (84% biosorption efficiency) in removal of heavy metal. Therefore, for removing of the heavy metals, the use of seaweeds as a biodegradable sorbent biomaterial is promising, highly efficient and economic as well (Ibrahim 2011). The heavy metal removal efficiencies of various microalgae species are presented in Table 1.

The mechanism of phycoremediation of heavy metals could be either bioaccumulation, biotransformation and bioaugmentations by living cells or biosorption by nonliving microalgal biomass or biomass products. Elements such as Zn, Mg, Mn, Cu, Ni, Bo, Mo, Co, Fe and Ca are important for the growth of microalgae up to a certain limit (Patidar et al. 2015). Waste detoxification processes in living cells occur by bioaccumulation, while dead biomass can also rapidly accumulate HM ions by selective adsorption (Aksu 1998). Heavy metal bioaccumulation by both living and nonliving microalgae cells occurs typically by an initial non-metabolic, rapid and essentially reversible removal process, occurring at the cell surface (adsorption, coordination, ion exchange, chemisorption, complexation, micro-precipitation, chelation, entrapment and diffusion) followed by a much slower metabolic irreversible process by mechanism such as diffusion into the cell interior, precipitation, covalent bonding, crystallization, precipitation and redox reactions (Al-Qunaibit 2004), occurring only within living cells (Monteiro et al. 2012).

Among different heavy metal removal mechanism in living algal cells, though ion exchange dominates, complexation and micro-precipitation are also most efficient algal resistance mechanisms (Mehta and Gaur 2005). Metal ion binding at the cell surfaces, insoluble metal complex precipitation, masking of metal toxicity by complexation with excreted metabolites, efflux mechanism to maintain low element levels in cell interior, enzymatic conversion and methylation of toxic form to lesser by change of the oxidation state and prevention to react with ASH groups inside the cell, respectively, metal conversion to volatile chemical species and binding to proteins or polysaccharides to constrain metal toxicity are different stages of algal resistance mechanism (Monteiro et al. 2012). The cell wall in microalgae act as first barrier followed by counter-ion interactions of proteins, carbohydrates and lipids with metallic species inside the cell (Crist et al. 1981; Dönmez et al. 1999; Monteiro et al. 2012). This is due to the interaction between functional groups such as OH, amino, COOH, sulfhydryl, −SH, etc. with metal ions. During heavy metal exposure, the plasma membrane transporters consisting of group A and B transporters represent the first line of defence by decreasing the excess metal concentration in the cytoplasm by exocytosis. Group A transporters include Fe transporter, Cu transporter, etc. and group B transporters are vacuolar iron transporter, cation diffusion facilitator, P1B-type ATPases, Ferroportin, etc. Up to 90% metal uptake by microalgal cells take place via ion exchange process and rest by adsorption to cell surface (Chojnacka et al. 2005; Tiantian et al. 2011; Monteiro et al. 2012; Maznah et al. 2012). In addition to adsorption, coordination bonding takes place between metal ions and carboxyl groups and amino groups of the polysaccharides of the cell wall which lead the biosorption of Cu in *C. vulgaris*

Table 1 Heavy metal removal efficiency of selected algae (Updated from Kumar et al. 2015)

Algae	Type of biomass	pH range	Metal uptake (mg/g)	Reference	Country
AER Chlorella	Nonliving	3–7	Cd, 7.74	Sandau et al. (1996)	Germany
AER Porphyridium	Nonliving	3–7	Cd, 7.55	Sandau et al. (1996)	Germany
AER Spirulina	Nonliving	3–7	Cd, 7.28	Sandau et al. (1996)	Germany
Chlorella vulgaris	Nonliving	6	Cd, 12.45; Cu, 10.90; Zn, 6.42	Sandau et al. (1996)	Germany
Chlorella vulgaris	Nonliving	3–7	Cd, 8.41	Sandau et al. (1996)	Germany
Porphyridium cruentum	Nonliving	3–7	Cd, 8.84	Sandau et al. (1996)	Germany
Spirulina platensis	Nonliving	6	Cd, 12.08; Cu, 10.33	Sandau et al. (1996)	Germany
Spirulina platensis	Nonliving	3–7	Cd, 8.06	Sandau et al. (1996)	Germany
Microcystis spp.	Nonliving	9.2	Cu, 0.003; Fe, 0.03	Singh et al. (1998)	India
Aulosira fertilissima	Nonliving	5	Cd, 14.57; Cu, 21.77; Ni, 4.16; Pb, 31.12; Zn, 19.15	Singh et al. (2007)	India
Hydrodictyon reticulatum	Nonliving	5	Cd, 7.2; Cu, 08.72; Ni, 13.86; Pb, 24; Zn, 3.7	Singh et al. (2007)	India
Pithophora oedogonia	Nonliving	5	Cd, 13.07; Cu,23.08; Ni,11.81; Pb, 71.13; Zn, 8.98	Singh et al. (2007)	India
Spirogyra neglecta	Nonliving	5	Cd, 27.95; Cu, 40.83; Ni, 26.3; Pb, 90.19; Zn, 31.51	Singh et al. (2007)	India
Calothrix parietina TISTR	Nonliving	7	Cd, 79; Hg, 19	Inthorn et al. (2002)	Thailand
Chlorella vulgaris BCC 15	Nonliving	7	Cd, 76; Hg, 18; Pb, 127	Inthorn et al. (2002)	Thailand
Chlorella vulgaris CCAP211/ 11B	Nonliving	7	Cd, 62; Hg, 16; Pb, 39	Inthorn et al. (2002)	Thailand
Scenedesmus acutus IFRPD 1020	Nonliving	7	Cd, 110; Hg, 20; Pb, 90	Inthorn et al. (2002)	Thailand
Tolypothrix tenuis TISRT 8063	Nonliving	7	Cd, 90; Hg, 27; Pb, 31	Inthorn et al. (2002)	Thailand
Calothrix parietina TISTR 8093	Nonliving	7	Pb, 45	Inthorn et al. (2002)	Thailand

(continued)

Table 1 (continued)

Algae	Type of biomass	pH range	Metal uptake (mg/g)	Reference	Country
Chlamydomonas reinhardtii	Cell wall	5.5	Cd, 5.75; Co, 0.89; Cu, 6.42	Macfie and Welbourn (2000)	Canada
Chlamydomonas reinhardtii	Without cell wall	5.5	Cd, 3; Co, 11.3; Cu, 7.54	Macfie and Welbourn (2000)	Canada
Chlamydomonas reinhardtii	Ca alginate	6	Cd, 28.9; Hg,35.9; Pb, 230.5	Bayramoğlu et al. (2006)	Turkey
Chlamydomonas reinhardtii	Immobilized	6	Cd, 79.7; Hg, 106.6; Pb, 380.7	Bayramoğlu et al. (2006)	Turkey
Chlamydomonas reinhardtii	Nonliving	6	Cd, 42.6; Hg, 72.2	Tüzün et al. (2005)	Turkey
Chlamydomonas reinhardtii	Nonliving	5	Pb, 96.3	Tüzün et al. (2005)	Turkey
Chlamydomonas reinhardtii		2.3	Cd, 2.3	Munoz and Guieysse (2006)	Sweden
Chlorella homosphaera	Nonliving	8.4	Cd, 8.4; Zn, 15.6	Munoz and Guieysse (2006)	Sweden
Chlorella pyrenoidosa		6.8–7.0	Cd, 2.8	Munoz and Guieysse (2006)	Sweden
Chlorella vulgaris		6.8–7.0	Cd, 2.6	Munoz and Guieysse (2006)	Sweden
Euglena gracilis	Nonliving		Zn, 7.5	Munoz and Guieysse (2006)	Sweden
Chlorella sorokiniana	Nonliving	5	Cd, 33.5	Akhtar et al. (2003)	Pakistan
Chlorella sorokiniana	Immobilized	5	Cd, 192		
Chlorella vulgaris	Nonliving	4	Cd, 62.3	Aksu (2001)	Portugal
Chlorella sp. HA-1	–	–	Cd, 21.6	Chen et al. (2012)	Taiwan
Scenedesmus abundans			Cd, 0.64	Chen et al. (2012)	Taiwan
Scenedesmus obliquus CNW-N	Nonliving	6	Cd, 24.4–108.5	Chen et al. (2012)	Taiwan
Spirulina spp.			Cd, 0.463	Chen et al. (2012)	Taiwan
Chlorella vulgaris		4; 4.5	Cd, 86.6; Ni, 58.4	Aksu and Dönmez (2006)	Turkey

Chlorella vulgaris	Nonliving	4; 4.7; 4	Cd, 33.72; Ni, 24.06; Pb, 97.38; Zn, 24.19	Klimmek et al. (2001)	Germany
Cyclotella cryptica	Nonliving	6	Cd, 22.24; Cu, 26.28; Hg, 11.92; Pb, 36.68; Zn, 242.9	Schmitt et al. (2001)	Germany
Phaeodactylum tricornutum	Nonliving	6	Cd, 1.24; Cu, 1.67; Hg, 0.51; Pb, 1.49; Zn, 14.52	Schmitt et al. (2001)	Germany
Porphyridium purpureum	Nonliving	6	Cd, 0.42; Cu, 0.27; Hg, 0.51; Pb, 0.32; Zn, 2.02	Schmitt et al. (2001)	Germany
Scenedesmus subspicatus	Nonliving	6	Cd, 7.29 Cu, 13.28; Hg, 9.2; Pb, 38.71;	Schmitt et al. (2001)	Germany
Scenedesmus subspicatus	Live	6	Zn, 72.06	Schmitt et al. (2001)	Germany
Desmodesmus pleiomorphus	Nonliving	-	Cd, 58.6	Monteiro et al. (2011)	Portugal
Scenedesmus obliquus	Nonliving	11.4	Cd, 60.8; Zn, 22.3	Monteiro et al. (2011)	Portugal
Phormidium spp.	Nonliving	5	Cd, 9.6; Cu, 10.1; Ni-5.7; Pb-13.6; Zn-9.4	Wang et al. (1998)	USA
Chlorella vulgaris	Nonliving	2	Cu, 16.14; Fe, 24.52	Romera et al. (2006)	Spain
Chlorella vulgaris	Nonliving	4	Cu, 34.89	Romera et al. (2006)	Spain
Chlorella vulgaris	Nonliving	4.5	Cu, 48.17	Romera et al. (2006)	Spain
Spirogyra insignis	Nonliving	4	Cu, 19.3	Romera et al. (2006)	Spain
Spirogyra insignis	Nonliving	6	Cd, 22.9; Ni, 17.5; Pb, 51.5; Zn, 21.1	Romera et al. (2007)	Spain
Spirulina platensis	Nonliving	7–8	Cd, 357	Solisio et al. (2008)	Italy
Spirogyra hyalina	Nonliving		Cd, 18.18; Co, 12.82;Hg, 35.71; Pb, 31.25	Kumar and Oommen (2012)	India
Spirulina platensis TISTR 8217	Nonliving	7	Cd, 98.04	Rangsayatorn et al. (2002)	Thailand
Spirulina platensis TISTR 8217	Silica immobilized	4–7	Cd, 36.63	Rangsayatorn et al., 2004	Thailand
Tetraselmis chui	Nonliving		Cd, 210.54	da Costa and deFranca (1998)	Brazil
Tetraselmis chui	Live		Cd, 292.6	da Costa and deFranca (1998)	Brazil

(continued)

Table 1 (continued)

Algae	Type of biomass	pH range	Metal uptake (mg/g)	Reference	Country
Spirulina platensis	Immobilized		Cd, 47.89	Murugesan et al. (2008)	India
Spirulina platensis	Live		Cd, 44.56	Murugesan et al. (2008)	India
Spirulina spp.	Nonliving	7.5	Cd, 0.46; Co, 0.01; Hg, 1.34; Ni, 0.19; Zn, 0.17	Chojnacka et al. (2004)	Poland
Spirulina spp.			Cu, 0.271	Chojnacka et al. (2004)	Poland
Chaetoceros calcitrans	Live	8	Cd, 1055.27	Sjahrul (2012)	Indonesia
Tetraselmis chui	Live	8	Cd, 13.46	Sjahrul (2012)	Indonesia
Desmodesmus pleiomorphus	Nonliving	5	Zn, 360.2	Monteiro et al. (2009)	Portugal
Desmodesmus pleiomorphus (ACOI561)	Live	4	Cd, 85.3	Monteiro et al. (2010)	Portugal
Desmodesmus pleiomorphus (L)	Live	4	Cd, 61.2	Monteiro et al. (2010)	Portugal
Scenedesmus abundans	Live	7.8–8	Cd, 574	Monteiro et al. (2010)	Portugal
Isochrysis galbana	Live	–	Cd, 0.02; Cu, 0.11; Zn, 0.3	Sbihi et al. (2012)	Morocco
Planothidium lanceolatum	Live	7	Cd, 275.51;Cu, 134.32; Zn, 118.66	Sbihi et al. (2012)	Morocco
Pseudochlorococcum typicum	Live	7	Cd, 5.48; Hg, 15.13; Pb, 4.49	Shanab et al. (2012)	Egypt
Synechocystis sp.	Live		Cd, 199.83; Pb, 155.63	Tiantian et al. (2011)	China
Oscillatoria angustissima	Nonliving	4	Co, 15.32	Mehta and Gaur (2005)	India
Chlorella vulgaris	Free	4.5	Ni, 59.29; Cu, 76.71	Mehta and Gaur (2001)	India
Chlorella vulgaris	Immobilized	4.5	Ni, 111.41; Cu, 63.08	Mehta and Gaur (2001)	India
Chlorella miniata	Nonliving	3	Cr, 14.17	Han et al. (2006)	Hong Kong
Chlorella miniata	Nonliving	4	Cr, 28.72	Han et al. (2006)	Hong Kong
Chlorella miniata	Nonliving	4.5	Cr, 41.12	Han et al. (2006)	Hong Kong
Spirulina spp.	Nonliving		Cr, 143; Ni, 515	Doshi et al. (2007)	India
Chlorella spp.	Nonliving		Cr, 98	Doshi et al. (2008)	India
Spirulina spp.	Nonliving		Cr, 16; Cu, 100	Doshi et al. (2007)	India

Chlorella spp.	Nonliving		Cr, 104; Cu, 108	Doshi et al. (2008)	India
Spirulina	Live		Cr, 304	Doshi et al. (2007)	India
Spirulina	Live		Cr, 333; Ni, 1378	Doshi et al. (2007)	India
Chlorella spp.	Live		Ni, 183	Doshi et al. (2008)	India
Spirulina sp. (HD-104)	Live		Cr, 306; Cr, 226; Ni, 1108; Cu, 576	Doshi et al. (2008)	India
Chlorella spp.	Live		Cu, 220	Doshi et al. (2006)	India
Spirulina	Live		Cu, 389	Doshi et al. (2006)	India
Chlamydomonas reinhardtii	Nonliving native	2	Cr, 18.2	Arıc et al. (2005)	Turkey
Chlamydomonas reinhardtii	Heat treated	2	Cr, 25.6	Arıc et al. (2005)	Turkey
Chlamydomonas reinhardtii	Acid treated	2	Cr, 21.2	Arıc et al. (2005)	Turkey
Chlorella vulgaris	Nonliving	2, 4, 5, 6, 5	Cr, 23; Cu, 40; Cu, 1.8; Cu, 7.5; Ni, 42.3	Dönmez et al. (1999)	Turkey
Synechocystis spp.	Nonliving	2	Cr, 19.2	Dönmez et al. (1999)	Turkey
Scenedesmus obliquus	Nonliving	2; 4.5; 5	Cr, 15.6; Cu, 20; Ni, 18.7	Dönmez et al. (1999)	Turkey
Scenedesmus quadricauda	Nonliving	4	Cu, 2.8	Dönmez et al. (1999)	Turkey
Spirulina platensis	Nonliving	6	Cu, 10	Dönmez et al. (1999)	Turkey
Synechocystis sp.	Nonliving	4.5; 5	Cu, 23.4; Ni, 15.8	Dönmez et al. (1999)	Turkey
Chlorella fusca	Live	6	Cu, 3.2	Dönmez et al. (1999)	Turkey
Chlorella vulgaris	4	Nonliving	Cr, 23.6; Cu, 37.6; Pb, 90; Zn, 24.5	Aksu and Kutsal, 1990	Turkey
Dunaliella sp.*1*	Nonliving	2	Cr, 58.3	Dönmez and Aksu (2002)	Turkey
Dunaliella sp.*2*	Nonliving	2	Cr, 45.5	Dönmez and Aksu (2002)	Turkey
Nostoc muscorum	Nonliving	3	Cr, 22.92	Gupta and Rastogi (2008)	India
Spirogyra spp.	Nonliving	5	Pb, 140.84	Gupta and Rastogi (2008)	India
Oscillatoria nigra	Nonliving	8.2	Cr, 1.86	Dwivedi et al. (2010)	India
Oscillatoria tenuis	Nonliving	8.2	Cr, 7.35	Dwivedi et al. (2010)	India
Phormidium bohneri	Nonliving	8.2	Cr, 8.55	Dwivedi et al. (2010)	India
Ulothrix tenuissima	Nonliving	8.2	Cr, 4.56	Dwivedi et al. (2010)	India
Chlamydomonas angulosa	Nonliving	8.2	Cr, 5.32	Dwivedi et al. (2010)	India

(continued)

Table 1 (continued)

Algae	Type of biomass	pH range	Metal uptake (mg/g)	Reference	Country
Asterionella formosa	Nonliving	4.0–5.0	Cu, 0.53	Tien et al. (2005)	Taiwan
Aulacoseira varians	Nonliving	4.0–5.0	Cu, 3.03	Tien et al. (2005)	Taiwan
Ceratium hirundinella	Nonliving	4.0–5.0	Cu, 5.75	Tien et al. (2005)	Taiwan
Chlorella vulgaris	Nonliving	4.0–5.0	Cu, 4.26	Tien et al. (2005)	Taiwan
Eudorina elegans	Nonliving	4.0–5.0	Cu, 2.13	Tien et al. (2005)	Taiwan
Microcystis aeruginosa	Nonliving	4.0–5.0	Cu, 12.62	Tien et al. (2005)	Taiwan
Anabaena cylindrical	Live	4.0–5.0	Cu, 8.73	Tien et al. (2005)	Taiwan
Anabaena spiroides	Live	4.0–5.0	Cu, 1.1	Tien et al. (2005)	Taiwan
Asterionella formosa	Live	4.0–5.0	Cu, 2.29	Tien et al. (2005)	Taiwan
Aulacoseira varians	Live	4.0–5.0	Cu, 2.3	Tien et al. (2005)	Taiwan
Ceratium hirundinella	Live	4.0–5.0	Cu, 3.63	Tien et al. (2005)	Taiwan
Chlorella vulgaris	Live	4.0–5.0	Cu, 3.96	Tien et al. (2005)	Taiwan
Eudorina elegans	Live	4.0–5.0	Cu, 8.21	Tien et al. (2005)	Taiwan
Microcystis aeruginosa	Live	4.0–5.0	Cu, 2.47	Tien et al. (2005)	Taiwan
Aphanothece halophytica	Living	6.5–7	133	Incharoensakdi and Kitjaharn (2002)	Thailand
Chlorella miniata	Nonliving	6	Cu, 23.26; Ni, 20.37	Lau et al. (1999)	Hong Kong
Chlorella vulgaris	Nonliving	6	Cu, 18.72; Ni, 12.06	Lau et al. (1999)	Hong Kong
Chlorella miniata	Live	7.4	Ni, 1.37	Wong et al. (2000)	Hong Kong
Chlorella vulgaris	Live	7.4	Ni, 0.64	Wong et al. (2000)	Hong Kong
Chlorella vulgaris		4.5	Ni, 58.4	Aksu and Dönmez (2006)	Turkey
Chlorella vulgaris	Live	5	Ni, 15.4	Al-Rub et al. (2004)	UAE
Chlorella vulgaris	Nonliving	5	Ni, 15.6	Al-Rub et al. (2004)	UAE
Chlorella vulgaris	Immobilized	5	Ni, 28.6	Al-Rub et al. (2004)	UAE
Anabaena flosaquae			Pb, 70	Arunakumara et al. (2008)	China

Spirulina (Arthrospira) platensis	Live		Pb, 188	Arunakumara et al. (2008)	China
Arthrospira (Spirulina) platensis	Nonliving	5.0–5.5	Pb, 102.56; Zn, 33.21	Ferreira et al. (2011)	Brazil
Chlorella vulgaris	Nonliving	5.0–5.5	Pb, 131.36; Zn, 43.41	Ferreira et al. (2011)	Brazil
Chlorella pyrenoidosa	Live	7	Pb, 2.4	Yan and Pan (2002)	China
Closterium lunula	Live	7	Pb, 0.5	Yan and Pan (2002)	China
Scenedesmus obliquus	Live	7	Pb, 1.8	Yan and Pan (2002)	China
Chlorella spp.	Immobilized	7	Pb, 33.4	Maznah et al. (2012)	Malaysia
Chlorella spp.	Nonliving	4	Zn, 28.5	Maznah et al. (2012)	Malaysia
Spirulina platensis	Live	9	Cu, 0.85	Nalimova et al. (2005)	Russia
Sargassum sp.	Nonliving	5	Cu, 38	Volesky et al. 2003	Canada
Sargassum fluitans	Nonliving		Cu, 51	Kratochvil et al. (1997)	Canada
Microcystis novacekii	Nonliving	8	Pb, 80	Ribeiro et al. (2010)	Brazil
Oscillatoria laetevirens	Live	5	21.6	Miranda et al. (2012)	India
Spirulina maxima	Intact biomass	5.5	Pb, ~32	Gong et al. (2005)	China
Spirulina maxima	Pretreated biomass	5.5	Pb, ~42	Gong et al. (2005)	China
Stigeoclonium tenue	Nonliving	6.8	Pb, 0.86; Zn, 0.88	Pawlik-Skowrońska (2003)	Poland
Stigeoclonium tenue	Nonliving	8.2	Pb, 0.38; Zn, 0.77	Pawlik-Skowrońska (2003)	Poland
Scenedesmus obliquus	Nonliving		6.67	Omar (2002)	Egypt

(Aksu et al. 1992). Similar biosorption also takes place due to ionic and covalent bonds (Gadd 1990). Decrease in solubility of metal ions is associated with precipitation of metal in the cell (Perpetuo et al. 2011; Ahalya et al. 2003).

Heavy metal sequestration also occurs by metal-binding peptides called phytochelatins (PCs), and presence of several metals and metalloids assists in vivo and in vitro activation of phytochelatin synthase (PCS), in responsible for PCs synthesis (Grill et al. 1987; Chen et al. 1997; Pawlik-Skowrońska 2001; Bačkor et al. 2007; Torres et al. 2008). Algae-metal interactions are also influenced by metallothioneins (MTs) which are structurally diverse, cysteine-rich and low-molecular-weight polypeptides present in the cells (Perales-Vela et al. 2006). MTs play a significant role in detoxification of metal ions via chelation and reduce the concentration of cytotoxic, free metal ions in the cells. Some are also involved in zinc and copper homeostasis (Robinson 1989). Based on microscopical and X-ray analyses, Shanab et al. (2012) demonstrated the transport of metals-MtIII complex into the vacuole of algae and explained the heavy metal tolerance mechanism.

In addition to metal compartmentalization in the vacuole, storage in an inert form leads to lower toxicity as compared to cation association with polyphosphate bodies and corresponding trafficking into vacuoles causing high metal toxicity (Wang and Dei 2006). Polyphosphate bodies could provide a "storage pool" for certain metals and enable a "detoxification mechanism" (Dwivedi 2012). Metals ions get sequestered in chloroplast and mitochondria of microalgae (Nagel et al. 1996; Avilés et al. 2003; Mendoza-Cózatl et al. 2004; Soldo et al. 2005; Perales-Vela et al. 2006). Production of proline in response to heavy metal stress is also a mechanism involved in metal remediation (Perales-Vela et al. 2006).

Metal sequestration by nonliving algal biomass depends on several factors such as species type, taxonomy cell structure and several physicochemical factors like metal ion and its binding patterns, metal solution and chemical composition that influence its binding to the algal biomass, thus the removal (Aksu 1998). Ion exchange process causes metal ions biosorption by algae through competition with protons for the binding sites on the cell wall which are basically negatively charged (Gardea-Torresdey et al. 1990; Michalak and Chojnacka 2010). Some advantages associated with nonliving microalgae for metal removal through biosorption include rapid treatment of large volumes, cleansing of mixed wastes and removal of multi-metals, high selectivity and specificity for HMs, non-requirement of growth media and nutrients and, more importantly, feasibility of significant metal recovery from the biomass (Kumar et al. 2015). That is why, the bioremediation of heavy metal-contaminated water and wastewater by micro- or macro-algae has gained much popularity, and the added advantages are the ease of handling. Figure 1 (adopted from Kumar et al. 2015) shows schematic mechanisms witnessed in HM-exposed microalgae. Despite well-adapted resistance mechanism in algal cells, acute exposure of high concentrations of metal ions lead to the formation of reactive oxygen species (ROS) that damage the algal cells whereas, in chronic exposure to lower concentrations of HMs, lead to accumulations heavy metals (Pinto et al. 2003; Singh et al. 2016).

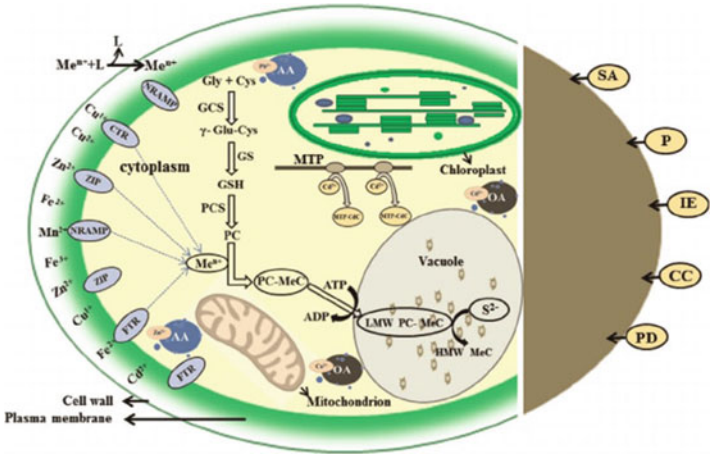

Fig. 1 Schematic representation of several mechanisms of heavy metal translocation, sequestration and uptake in living (*left*), as well as nonliving (*right, brown shaded*) microalgae. (Adapted from Kumar et al. 2015)

3 Factors Affecting Algal Sequestration of Heavy Metals

The binding, accumulation and removal of metals by microalgae depend on several biotic and abiotic factors. Therefore, metal uptake and removal efficiency are specific for specific metals and also may vary with algal genus or species. For instance, *C. miniata* and *C. vulgaris* were found excellent in removing hexavalent cation Cr; *C. vulgaris* and *S. platensis* performed comparatively better in the removal of trivalent metals, i.e. Fe and Cr; and freshwater green microalgae, *C. vulgaris*, *C. miniata* and *C. reinhardtii* were excellent for the removal of Hg, Cd, Pb, Ni, Cu and Zn (González et al. 2011). Microalgal cell density also plays an important role in its sensitivity to metal toxicity, and the smallest microalgae are thus most effective in sequestering metals (Khoshmanesh et al. 1997; Quigg et al. 2006). *Micromonas pusilla* are approximately twice as tolerant compared to *Minutocellus polymorphus* due to its smallest surface area, while the largest dinoflagellate species *Heterocapsa niei* was notably sensitive to Cu (Levy et al. 2007). In an independent study, increased biomass concentration of microalgae apparently improved the metal removal although beyond a certain threshold, it caused decreased metal binding per unit cell mass (Esposito et al. 2001). Dead *Spirogyra* species showed decreased Cu uptake with increased biosorbent concentration (>0.5 g/L) (Bishnoi et al. 2004), while in *Spirulina maxima* a noticeable reduction in Pb^{2+} uptake (from 121 to 21 mg/g) was observed by increasing the biomass concentration from 0.1 to 20 g/L (Gong et al., 2005). Algal tolerance and defence mechanism to heavy metals include reduced uptake, responses against oxidative damage, chelating compounds exudation capacity and metal ions efflux of primary ATPase pumps (Gaur and Rai 2001).

Abiotic factors that affect metal remediation in algae include pH, salinity and hardness of the medium, size and concentration of metal ions, its speciation, atomic weight and/or the reduction potential and interactions of a specific metal, temperature, organic substances, ligands, and many other properties as well (Wang 1987; Dönmez et al. 1999). Various functional groups present on the microalgal cell wall influence the acid-base properties of the solution, and the chemistry of metal affects the pH-dependent metal uptake (Monteiro et al. 2012). The chemistry of the pollutants, the properties of the functional groups of the biosorbents and the competition and interaction with coexisting ions are directly affected by pH of the solution (Vijayaraghavan and Yun 2008). Further, with the increase in pH, negatively charged functional groups (amino groups, carboxyl, imidazole and phosphate in ligands) get exposed more resulting to an attraction of positively charged metallic ions onto the negatively charged algal cell surface via a process of biosorption (Dönmez et al. 1999). Higher ionic strength of the solution showed lesser ion removal as increasing ionic strength results decrease the sites available for metal ion uptake (Dwivedi 2012). Schiewer and Wong (2000) observed that increasing pH and ionic strength resulted to reduction in proton binding, whereas increasing pH and decreasing ionic strength increased binding of Cu and Ni. The metal ion biosorption decreases at higher temperature due to increased solubility of metal ions (Elder 1989; Lau et al. 1999). Metal speciation also plays a significant role in binding of metal cations with microalgae (Monteiro et al. 2012; Pagnanelli et al. 2003; Doshi et al. 2007). Various studies also concluded that instead of a single metal, multi-metal solutions are the better representative of the effects of metal cations and thus can represent actual environmental problems (Fraile et al. 2005; Aksu and Dönmez 2006; Monteiro et al. 2012). In addition, pretreatment steps like treatment with calcium chloride ($CaCl_2$) enhance metal adsorption capacity of algae (Mehta and Gaur 2005). Commercial success of algal-based metal removal technology depends on (i) selection of suitable algal strain; (ii) adequate knowledge of sorption mechanism; (iii) development of cost-effective cell immobilization techniques; (iv) metal sorption prediction, by improved and advanced models; (v) manipulation of the metal binding sites of algal cell surface by genetic engineering; and (vi) economic feasibility of such type of remediation technologies (Mehta and Gaur 2005).

4 Energy Production from Metal Sequestered Algae: Advantages, Issues and Challenges

For long terms, utilization of fossil fuels is not an environmentally sustainable energy option due to rapid depletion as well as limited stock of fossil fuel, greenhouse gas emissions and energy security (Subhadra and Edwards 2010; Rawat et al. 2013; Gupta et al. 2013). Microalgae as a renewable energy feedstock are presently getting huge attentions for their dual role, i.e. pollutant remediation and production of biomass for sustainable biofuels. Algal biomass is an excellent

feedstock for the production of various types of biofuels such as alcohol through fermentation, syngas through gasification, biohydrogen, biomethane, biodiesel, etc. (Kushwaha et al. 2014). Numerous algal species are exceedingly rich in lipids and oils (Khan et al. 2009; Demirbas 2011), which can be increased up to 60–70% by manipulation of culture conditions. The type and the quantity of lipids produced (saturated fatty acids, polyunsaturated fatty acids, glycolipids or triacylglycerols) depend on the type of microalgae and the growth condition (Chisti 2007; Hu et al. 2008; Griffiths and Harrison 2009). Thus, for the optimized biodiesel production from algae, the screening of oil-producing microalgae species is very important (Rawat et al. 2013). Cultures with higher lipid productivities but moderate lipid accumulation levels (20–50%) are preferred for mass cultivation (Mata et al. 2010; Amaro et al. 2011). Various studies have reported various marine and freshwater microalgae species having substantially high lipid yields (Mata et al. 2010). The biodiesel can be produced from the algal lipids by transesterification of the fatty acids, and such biodiesel can be used for the transportation with or without little modifications (Miao and Wu 2006; Hu et al. 2008; Pittman et al. 2011; Nautiyal et al. 2014). Compared to petro-diesel, net CO_2 or sulphur emission is very less in algal biodiesels; therefore, overall net toxic gas exhaust is very less in the atmosphere (Hu et al. 2008; Williams and Laurens 2010; Hulatt and Thomas 2011). As per estimates, with 50 g/m^2/day productivity of a dry algal biomass having approximately 50 % lipid contents can theoretically produce up to 10,000 gallons of oil/acre/year (Pienkos and Darzins 2009). Application of microalgae for biodiesel has multiple advantages such as very short harvesting life and has higher solar energy yields, comparatively 20 times higher lipid yield than oil crops, neutral lipids produced have a high level of saturation and no requirement of arable land and more importantly it can be grown on various types of wastewater and hence requires less freshwater than oil crops making algal biomass a suitable feedstock for biodiesel production (Chisti 2007; Schenk et al. 2008; Rawat et al. 2013). However, sustainable production of algal biofuels is only possible with integrated phycoremediation of wastewater and production of biomass to be used for biofuels (US DOE 2009; Brune et al. 2009; Rawat et al. 2013). Numerous studies demonstrated the excellent treatment potential of various microalgae species for different types of contaminated wastewaters (Rawat et al. 2011; Gupta et al. 2016 and references within). Gupta et al. (2016) explored and reported excellent phycoremediation potential of two freshwater microalgae, *Chlorella* sp. and *Scenedesmus* sp., for wastewater treatment and lipid production. Voltolina et al. (1998) reported 33% of protein and 27% of carbohydrates along with 12% lipid yields in *Scenedesmus* sp. cultivated in artificial wastewater. In another study, Orpez et al. (2009) reported 17% lipid yield in *Botryococcus braunii* used for the tertiary treatment of secondarily treated sewage. Based on study using a cyanobacterial consortium, Kushwaha et al. (2014) reported the effect of Cr (VI) on biomass production as well as its removal from simulated wastewater using a consortium of two cyanobacteria species (*Gloeocapsa atrata* and *Oscillatoria subbrevis*). The maximum lipid production (0.081 g/g of dry biomass) was found at a concentration of 35 mg/L of Cr(VI) at pH 9 after 14 days of

incubation. The findings suggested that such species can be used for effective bioremediation of heavy metals as well as for the production of biomass for biofuel purposes. In similar studies, lipid content and inorganic elements such as metals, P and N were analysed in microalgae and used for treating carpet mill wastewater. The findings showed substantially high biomass production which was estimated ranging from 16.1 to 28.1 tons ha^{-1} $year^{-1}$ with an approximate lipid yield (3260 to 3830 L ha^{-1} $Year^{-1}$) during phycoremediation of a carpet mill wastewater (Chinnasamy et al. 2010). Lipid productivity of 45.49 mg/L/day (Ebrahimian et al. 2014) and 10.4 mg/L/day (Ji et al. 2014) was reported for *C. vulgaris* and *Micractinium reisseri*, respectively, when grown on mine wastewater. Another cost-effective option for harvesting algal biomass for biofuel purpose was reported by growing algae on metal ion-containing wastewaters (Zhou et al. 2012). Patidar et al. (2015) explored the bioaccumulation of metals and lipids for sustainable utilization in biofuel production and suggested for the simultaneous utilization of microalgal mats for biodiesel production and the bioremediation of heavy metals from contaminated sites. Raikova et al. (2016) reported remediation of metal from acid mine drainage by *Spirulina* sp. and significance of hydrothermal liquefaction (HTL) for biofuel production. Results showed that heavy metal appeared to catalyse the conversion to bio-oil and did not affect the higher heating value of the bio-oil. Although algal potential for heavy metal sequestration is widely explored, their postremediation potential for lipid production needs more research as some metals such as Cd are known to reduce lipid biosynthesis (Gillet et al. 2006). Thus, improvement in the economics of production of microalgal diesel will also depend on genetic and metabolic engineering (Roessler et al. 1994; Dunahay et al. 1996).

5 Conclusion

Algae possess several appreciable mechanisms to sequester heavy metals and various other contaminants from the culture medium. Moreover, the biomass produced during the phycoremediation can serve as an alternative source for bioenergy. Therefore, combined use of algae for metal remediation and energy production in a well-planned manner can provide an excellent mechanism of pollution control and substantially can contribute in meeting the future energy demand in a sustainable way. Although extensive work have been done on metal remediation using various species of microalgae, however, there is more scope of extensive research in order to make combined use of metal-remediated microalgae for energy production as a feasible option on pilot-scale basis. Such integrated approach may result in improving the economics and concurrently reduce the environmental burden of CHGs while performing valuable remediation services. The algal technologies are improving day by day, innumerable research advances in this field will certainly derive microalgae as a potential bioremediation agent for the treatment of varying strength and types of wastewater, and the algal biomass will be an energy source in the future world.

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Critical Evaluation of Algal Biofuel Production Processes Using Wastewater

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Abbreviations

AIWPS	Advanced Integrated Wastewater Pond System
ATS	Algal turf scrubbing
BOD	Biological oxygen demand
COD	Chemical oxygen demand
DAF	Dissolved air flotation
DW	Dry weight
FIMP	Flat inclined modular photobioreactor
GHG	Green house gas
HRP	High-rate pond
HRT	Hydraulic retention time
IPB	Integrated photo-bioelectrochemical
MFA	Monounsaturated fatty acids
MFCs	Microbial fuel cells
N	Nitrogen
OP	Open pond
P	Phosphorus
PAR	Photosynthetically active radiation
PBRs	Photobioreactors
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acids
SS	Suspended solid
TFF	Tangential flow filtration
TOC	Total organic carbon
VF	Vacuum filter
WSP	Wastewater stabilization pond

1 Introduction

Declining fossil resources, energy insecurity, and global warming issues are the major driving forces behind the search for alternative and sustainable biofuels to meet increasing demand for fuels (Jacobson 2009; Pienkos and Darzins 2009; Lü et al. 2011; Zhou et al. 2011; Zhou et al. 2012a, b). Microalgae, encompassing eukaryotic microalgae and cyanobacteria, are considered as one of the prospective clean and economical energy resources (Metting 1996; Spolaore et al. 2006; Thajuddin and Subramanian 2005; Tan 2007). Biomass produced from microalgae can provide energy using various energy conversion processes (Amin 2009; Brennan and Owende 2010; Mata et al. 2010). Microalgae production requires huge amount of nitrogen fertilizers, which raises questions about their environmental impacts and also causes high production cost of biofuel (Sialve et al. 2009; Lardon et al. 2009; Venkataraman et al. 1982). Also, wastewater coming out from the process causes serious environmental problems (Hoffmann 1998; Arora and Saxena

2005; de-Bashan and Bashan 2010). Eutrophication is the primary effect of release of phosphates and nitrates containing wastewater to the water bodies (Olguin 2003; Pizarro et al. 2006; de-Bashan and Bashan 2010; Mulbry et al. 2008; Godos et al. 2009). This pollution can be solved by using wastewater as feed for microalgae. The advantage of this approach is that, on the one hand, the microalgae will remove excess nutrients from the wastewater and, on the other hand, biomass will be produced which can be used for energy generation (Pizarro et al. 2006; Munoz and Guieysse 2008; Godos et al. 2009). Here wastewater works as cultivation medium for microalgae, while microalgae are used as an agent to reduce the pollution load (Martin et al. 1985; Cañizares and Domínguez 1993; Mulbry et al. 2008; Posten and Schaub 2009; Hoffmann 1998; Mallick 2002; de-Bashan and Bashan 2010; Olguín et al. 1997). These days algal research is primarily focused on lipids rich microalgal cultivation in order to produce economical and sustainable biofuel (Brennan and Owende 2010; Demirbas 2010; Demirbas and Fatih Demirbas 2011; Rawat et al. 2011). It is well reported by different researchers that microalgae can be used to extract energy efficiently using different energy conversion's processes besides biodiesel production technology (Sialve et al. 2009; John et al. 2011; Pittman et al. 2011). Furthermore, *Chlorella, Scenedesmus, Phormidium, Botryococcus, Chlamydomonas*, and *Spirulina* are some of the most widely used microalgae species for treating domestic wastewater (Olguin 2003; Chinnasamy et al. 2010; Wang et al. 2010; Kong et al. 2010).

Considering potential use of algal based technology for sustainable energy production and wastewater treatment. The primary objective of this article is to critically review different cultivation and harvesting processes pertaining to simultaneous algal production and wastewater treatment. The detail objectives are to:

- Assess feasibility of future applicability of algal production in wastewater.
- Evaluate different cultivation systems, their advantages, and disadvantages.
- Evaluate different harvesting processes, their advantages, and disadvantages.
- Identify the major issues in economically feasible cultivation and harvesting of microalgae in wastewater.
- Identify bottleneck in applicability of algal production in wastewater.
- Identify recent research and development to address major challenges in algal production in wastewater.

2 Wastewater and Microalgae

Nutrient rich municipal wastewater stream is one of the most suitable medium for cultivating microalgae for high biomass productivity while wastewater nutrient removal efficiently (Wang et al. 2010). Also, total organic carbon (TOC) which is present in high concentration in the wastewater could be used as food by some of the algal species for fast growth under different light conditions (Wang et al. 2010; Li et al. 2011). Xu et al. (2004) observed 55% lipid content on algae grown

in heterotrophic condition. The success of algal based treatment process for wastewater containing high organic content depends on the microalgal ability to assimilate organic carbons (heterotrophic growth) (Burrell et al. 1984), inorganic nutrients, and inorganic carbons (Lau et al. 1995).

The biomass and lipid productivity in various studies using different wastewater streams as substrate is listed in Table 1. Maximum biomass productivity was found in Zhou et al. (2011). It has been previously reported that heterotrophic algae consume organic carbons more efficiently under light condition than in dark (McKinley and Wetzel 1979; Boichenko et al. 1992). It was also reported that the growth rate for microalgae grown in mixotrophic condition is almost the sum of the growth of the microalgae grown in autotrophic and heterotrophic conditions (Lee 2007).

Furthermore, some recent review papers mentioning the importance of algal production using wastewater concluded that biofuel production using algae is not economically viable without use of wastewater considering existing algal production technologies (Pittman et al. 2011). Therefore, simultaneous wastewater treatment and biofuel production are an attractive option (Pittman et al. 2011). Lundquist et al. (2010) reported that algal production using wastewater was able to produce economical biofuels, they concluded that industrial scale algal biofuels production is not promising if it is not grown in wastewater.

2.1 Lipid Content

Microalgae biomass is considered as a promising energy source because of oil content present in the microalgae. Oil productivity of microalgae could be sometimes 100 times higher than that of some agricultural crops, such as canola, soy, and palm (Chisti 2007; Mata et al. 2010). Microalgal strains having high oil content are considered suitable for biodiesel production. These microalgae primarily belong to Chlorella, Dunaliella, Nannochloris, Nannochloropsis, Neochloris, Porphyridium, and Scenedesmus genera contain 20–50% of lipids by weight (Mata et al. 2010). The type and quantity of the lipids accumulate depends on the microalgae species and the growth conditions (Chisti 2007; Griffiths and Harrison 2009; Hu et al. 2008). Usually higher lipids concentrations are found either in photobioreactor-grown cells or batch culture-grown than that of open pond grown microalgae (Griffiths and Harrison 2009). However, it was observed that biomass productivity of these high lipid containing microalgae is less because of environmental stress and limited nutrient availability (Dean et al. 2010; Rodolfi et al. 2009).

It was observed in a recent study that *Chlamydomonas reinhardtii* shows very good growth with high lipid content in strong wastewater (Table 1), (Kong et al. 2010). Similarly, biocoil-grown microalgae show 25.25% DW lipid content with high biomass productivity. Woertz et al. (2009) observed lipid content ranging from 14% to 29% DW in mixed algae cultures grown in dairy manure wastewater after anaerobic digestion. Similarly Wang et al. (2010) found 9% to 13.7% DW total fatty acid content including phospholipids and glycolipids in culture-grown

Table 1 Comparison of biomass and lipid production by microalgae using different wastewaters (adopted from Pittman et al. 2011; Zhou et al. 2011)

Wastewater type	Microalgae species	Biomass productivity (mg/L/d)	Lipid content (%DW)	Lipid productivity (mg/ L/d)	References
Carpet mill	*Chlorella saccharophila*	23	18.1	4.2	Chinnasamy et al. (2010)
Carpet mill	*Scenedesmus* sp.	126.54	12.8	16.2	Chinnasamy et al. (2010)
Dairy wastewater, 25X	Polyculture of *chlorella* sp., *Micractinium* sp., *Actinastrum* sp.	NA	29	17	Woertz et al. (2009)
Primary clarifier effluent	Polyculture of *chlorella* sp., *Micractinium* sp., *Actinastrum* sp.	NA	9	24.4	Woertz et al. (2009)
Activated sludge extract	*Chlorella pyrenoidosa*	11.55	NA	NA	Cheung and Wong (1981)
Digested sludge extract	*Chlorella pyrenoidosa*	51.82	NA	NA	Cheung and Wong (1981)
Settled sewage	*Chlorella pyrenoidosa*	275	NA	NA	Tam and Wong (1989), Tam and Wong (1990)
Activated sewage	*Chlorella pyrenoidosa* and *Scenedesmus* sp.	92.31	NA	NA	Tam and Wong (1989), Tam and Wong (1990)
Secondarily treated sewage	*Botryococcus braunii*	35	NA	NA	Sawayama et al. (1992)
Artificial wastewater	*Scenedesmus* sp.	126.54	12.8	16.2	Voltolina et al. (1999)
Concentrated municipal wastewater	*Hindakia* sp.	275	28.3	77.8	Zhou et al. 2011
Concentrated municipal wastewater	*Scenedesmus* sp.	247.5	30.09	74.5	Zhou et al. 2011
Concentrated municipal wastewater	*Chlamydomonas reinhardtii*	2000	25.25	505	Kong et al. (2010)
Agricultural (dairy manure with polystyrene foam support)	*Chlorella* sp.	2.6 (g m^2/1/day)	9	230 (mg/m^2/day)	Johnson and Wen (2010)

microalgae (*Chlorella* sp.) in different wastewater concentration. Johnson and Wen (2010) compared the growth of the *Chlorella* sp. in suspended culture and attached polystyrene foam support system. They found similar total fatty acid content in the both of the growth systems. In one recent study Piligaev et al. (2015) observed that strains *Chlorella vulgaris* A1123 and *S. abundans* A1175 have a high total content of saturated (SFA) and monounsaturated (MFA) fatty acids (67.0% and 72.8%, respectively). *S. abundans* A1175 also had low polyunsaturated fatty acid (PUFA) content that would allow for its use as a source of high quality biofuels.

2.2 Nutrient Removal

Along with biofuel production microalgae are considered as very efficient way to remove nutrients from wastewater. Concentration of nutrients like N and P is one of the major differences between wastewater and other growth media as far as algal production is concerned (Ip et al. 1982; Konig et al. 1987; Wrigley and Toerien 1990). It is reported that various species of *Chlorella* and *Scenedesmus* can remove >80% nitrogen and in many cases they can almost remove ammonia, nitrate, and total phosphorus completely from the secondary stage treated wastewater (Martinez et al. 2000; Ruiz-Marin et al. 2010; Zhang et al. 2008). Lau et al. (1995) reported that *C. vulgaris* removes over 90% of nitrogen content and 80% of phosphorus content from the primary treated sewage. Green algae *Botryococcus braunii* is reported to grow well in piggery wastewater and removed 80% of the initial NO_3 content (An et al. 2003). Comparative nutrient removal in different suspended and attached systems is presented in Table 2. Several studies reported around 99% nitrate removal in tubular reactor, while 96% phosphate removal was observed in raceway pond (Hoffmann 1998; Shen et al. 2009; Lundquist et al. 2010; Chisti 2007; González et al. 2008; Shen et al. 2009). In the algal biofilm systems total nitrogen removal ranging from 87% to 100% and total phosphorus removal ranging from 98% to 100% (Wei et al. 2008; Przytocka-Jusiak et al. 1984; Guzzon et al. 2008; Johnson and Wen 2010, except rotating aluminum disks (60% nitrogen removal), algal turf scrubber (36–92% total nitrogen and 51–93% total phosphorus removal)) were observed (Torpey et al. 1971; Wilkie and Mulbry 2002).

3 Cultivation Systems

Cultivation is one of the main steps of algal biofuel production processes. Primarily there are four types of cultivation conditions for microalgae, i.e., photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic (Chang et al. 2011). There are basically two types of cultivation systems, i.e., suspended and attached, which are discussed here. Basic process with advantages and limitations of different cultivation systems is summarized in Table 3.

Table 2 Biomass productivity and nutrient removal in various algal-wastewater treatment systems (adopted from Logan and Ronald 2011)

Design	Nutrient loading (mg/L/day)	Nutrient removal	Biomass production (g/m^2/day)	Scale	References
Biomass productivity and nutrient removal in suspended culture systems					
Raceway pond	P: 1.2–7.5	P: 96%	10–20	Pilot and demonstration	Hoffmann (1998), Shen et al. (2009) and Lundquist et al. (2010)
Tubular reactor	N: 17.3	N: 99%	20–45	Pilot and demonstration	Shen et al. (2009), Chisti (2007), González et al. (2008)
Biomass productivity and nutrient removal in algal biofilm culture systems					
PVC brushes	TN: 5.5 TP: 1.7	TN: 87% TP: 98%	Not reported	Lab	Wei et al. (2008)
Rotating styrofoam disks	P: 1.7–3.3	N: 100%	2.2	Lab	Przytocka-Jusiak et al. (1984)
Rotating aluminum disks	N: 312	N: 60%	Not reported	Bench	Torpey et al. (1971)
Polycarbonate flow lanes	P: 1.2	P: 100%	2.9	Lab	Guzzon et al. (2008)
Algal turf scrubber	TN: 160–1030 TP: 80–160	TN: 36–92% TP: 51–93%	5.3–5.5	Bench	Wilkie and Mulbry (2002)
Polystyrene rocker system	N: 30.9	N: 100%	2.59	Lab	Johnson and Wen (2010)

Table 3 Process description, advantages, and limitation of main methods for algae cultivation (adopted from Kligerman and Bouwer 2015)

Method	Description	Advantages	Disadvantages	References
Wastewater Stabilization Pond (WSP)	Biological treatment where the symbiosis of bacteria and algae stabilizes wastewater and reduces pathogens. Key factors: sunlight, temperature, retention time, and BOD loading rate. The most common is a sequence of ponds: anaerobic (primary treatment), facultative (secondary), and maturation (tertiary)	• Cost effective (US \$7.8 m^{-2}) • Simple and cheap construction • Simple operation • Low maintenance cost • Low energy demand • Sludge removal unnecessary • Treats domestic and industrial wastewater • Well suited for tropical and subtropical countries • Maturation pond removes nematodes, helminth eggs, and viruses • Satisfactory removal of BOD	• Needs available land • Requires regular maintenance to avoid odor • Low SS removal • NH4+ levels >25 mg L-1 may affect BOD removal • In absence of wind, the algae population tends to stratify in a narrow 20 cm band during daylight hours	Ramadan and Ponce (2015) WHO (2015) Arthur (1983) Arar (1985) Pescod and Mara (1985) Al-Hashimi and Hussain (2013) Silva and Mara (1979) Victoretti (1964) Zhao and Wang (1996).
Advanced Integrated Wastewater Pond System (AIWPS)	WSP variant with four ponds in sequence: advanced facultative pond with fermentation pits, algal high-rate pond, algal settling pond, and maturation pond. Provides primary, secondary, and tertiary treatment	• Minimizes sludge accumulation • Maximizes algae production • Cost effective • Little maintenance • Satisfactory performance in BOD and solid removal • Lower odor	• Requires area similar to the conventional ponds • Setup costs higher than WSP • Sludge has to be removed twice a year • Energy costs higher than WSP	Ramadan and Ponce (2015) Oswald (1995) Oswald (1988). Muir et al. (1995) da Silva Nascimento (2001). Azov and Shelef (1982).

Algal High-Rate Ponds (HRP)	Shallow pond (0.3–0.6 m) operating at shorter hydraulic retention time. Raceway shape with a large paddle wheel vane pump to create a channel velocity (10–30 cm/s) for gentle mixing	• Algae productivity up to 50 t ha-1 y^{-1} • Productivity in commercial production as high as 40 g dry wt m^{-2} day^{-1} • Can receive a high loading rate (190 kg BOD ha^{-1} day^{-1}) • High removals of N–NH4 (48%) and P–PO4 (42%) • Mixing makes algae come to surface and capture more sunlight	• Light penetration limited by increase in algae cell density • Evaporative losses affect culture growth • Loss of CO_2 can contribute to lower production • Presence of predatory viruses, zooplankton, protozoa, and fungi can affect productivity	Rawat et al. (2011) Dalrymple et al. (2013) Rawat et al. (2013) El Hamouri et al. (2003) Brennan and Owende (2010)
Open Pond (OP)	Open system of cultivation utilizes paddle wheels or other devices for mixing and aeration. Common types: raceway, circular, inclined, and unmixed	• Relatively inexpensive • Easy to construct • Low costs of operation and maintenance • Low costs of mixing, aeration and nutrient dispersion • Can be used in large scale	• Limited to areas where water is inexpensive • High evaporative losses	Leite et al. (2013). Rawat et al. (2013)
Photobioreactor (PB)	Indoor or outdoor enclosure system using sunlight and/or artificial lights. Mixing provided by airlift or mechanical stirring/ pumping	• Artificial light stabilizes intensity and enhances total oil yields by 25–42% • Reduced light path increases the light received by each cell • Allows algal monoculture growth for extended periods and produces large amounts of biomass	• Energy requirements for artificial light • Not used on large scale • High capital and operational costs	Rawat et al. (2013) Leite et al. (2013). Rawat et al. (2011) Pittman et al. (2011)

3.1 Suspended Cultivation

Suspended cultivation system is the most preferred method of algae cultivation. In this process microalgae are kept free in the medium and additional mixing is provided to insure a good distribution (Katarzyna et al. 2015). Open pond systems and closed photobioreactors (PBRs) are the main types of suspended cultivation system.

3.1.1 Open Pond System

Open pond system (Fig. 1) consists of shallow channel pond equipped with paddle wheel to mix the suspension (Brennan and Owende 2010). This system is also called raceway pond. Nutrients and microalgae are circulated continuously. More than 90% of cultivation systems in the world are open pond system (Sing et al. 2011). The range of volumetric capacities is 102–106 L, depth of open pond system is up to 30 cm to ensure efficient sunlight penetration (Chisti 2007), and the normal hydraulic retention time (HRT) ranges between 4 and 10 days. (Rawat et al. 2011). Generally, CO_2 is used from surface air. Some open pond systems are equipped with submerged aerators to enhance CO_2 supply (Terry and Raymond 1985).

Such type of system generally has a low productivity (Borowitzka 1999). Christenson and Sims (2011) stated that the theoretical biomass productivity ranges from 50 to 60 g/m^2/d. However, Shen et al. (2009) concluded that 10–20 g/m^2/d efficiency is not practically achievable. It was shown that theoretical biomass productivity of open pond system is over estimated. Tam and Wong (1989) suggested that algal pond with high cell density should be installed as a secondary

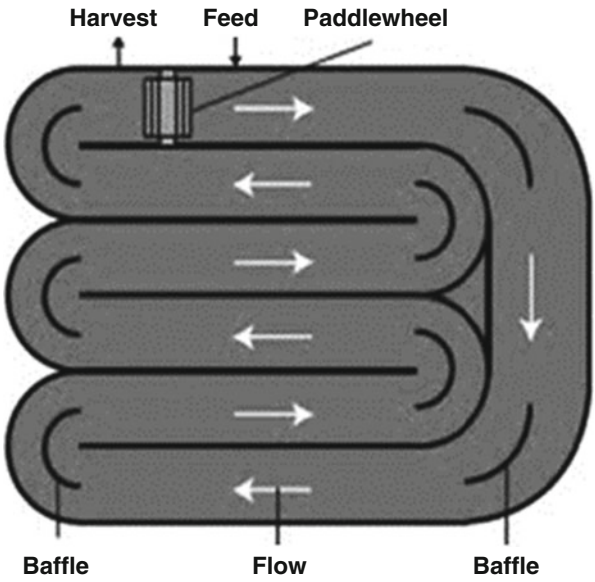

Fig. 1 Schematic representation of open or raceway pond (adopted from Singh and Sharma 2012)

treatment, since alga growth is better in wastewater stream after primary settling. Munoz and Guieysse (2006) observed 35 g/m^2/d BOD removal rate under the optimal operation conditions in pretreated municipal wastewater treatment. Phosphate removal can reach up to 96% in open pond system (Table 2) (Hoffmann 1998; Shen et al. 2009; Lundquist et al. 2010).

Though, open pond system is considered as relatively cheap and easy to operate (Oswald 1995, Li et al. 2008). However, the disadvantages are land requirement, dependency on climate conditions (Fallowfield and Barret 1985), possibility of contamination, and water loss by evaporation. As a consequence, productivity is low. Perez-Garcia et al. (2011) concluded that maintaining pure culture for the treatment of wastewater in an open pond is very difficult because of air contamination. Also, only few species are capable to maintain themselves in an open pond system (Carlsson et al. 2007; Chisti 2007; Pulz 2001; Rodolfi et al. 2009). Kagami et al. (2007) observed that viral infection significantly reduces algal population. An alternative to enhance the treatment performance is algal inoculum concentration (Su et al. 2012). The additional limiting factor in open pond system is low biomass concentration. Biomass productivity in this kind of system is affected essentially by hydrodynamic conditions which are controlled by mixing. Despite their raceway shape, open pond systems are poorly mixed (Chisti 2007).

There are three general types of waste stabilization ponds used in wastewater treatments, i.e., facultative ponds, anaerobic ponds, and aerobic ponds (Rawat et al. 2011). Facultative pond presents aerobic conditions on the surface and anaerobic conditions in the bottom. Anaerobic pond is sequestered from free dissolved oxygen and is usually used for wastewater with a high BOD load (Horan 1996). The organic load is superior to 100 g BOD m^{-3} d^{-1} (equivalent to 3000 kg/ha d). The depth ranges from 2 to 5 meters as light penetration is unimportant (Ramadan and Ponce 1999). Retention time is short and BOD removal ranges from 60 to 85% in warm climate (Alexiou and Mara 2003). Aerobic pond also known as algal high-rate pond (HRP) or also raceway pond is the type designed to promote algae growth. Figure 2 represents a side elevation of a HRP with a CO_2 addition.

Oswald and Golueke (1960) proposed first large scale HRP using wastewater for algal biofuel production. Lundquist et al. (2010) restated that HRP is the only

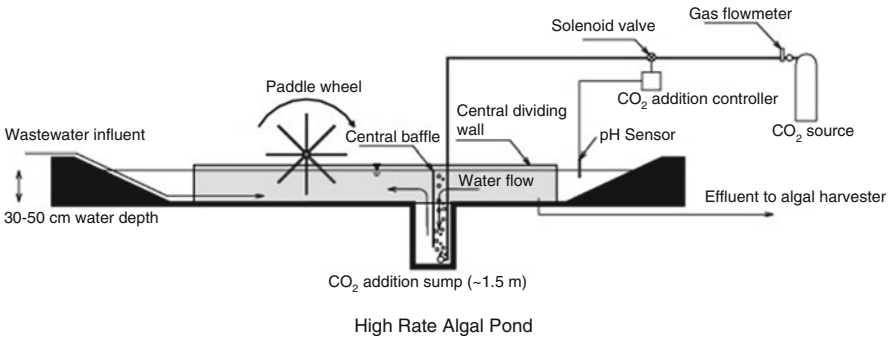

Fig. 2 Side elevation of a high-rate algal pond with CO_2 addition to enhance algal growth (adopted from Park et al. 2011)

feasible cultivation system for algae cultivation in large scale at low costs. In HRP cultivation system using wastewater as medium for the growth of microalgae, addition of CO_2 remains indispensable since the medium is a poor carbon source and cannot support the microalgae growth (Benemann 2003). Nevertheless, CO_2 addition is currently not used in large scale wastewater treatment HRP except few small pilot-scale plants (Park et al. 2011).

3.1.2 Closed Photobioreactor System

Photobioreactors are closed systems which made from transparent tubes, plates, bags, or hemispherical domes (Darzins et al. 2010). Different photobioreactor systems were developed for the purpose of biofuel production. Closed configuration allows growth in photoautotrophic, heterotrophic, or mixotrophic conditions. There are several types of closed photobioreactor. However, tubular PBR is the only type used at the large scale (Brennan and Owende 2010; Tredici and Zittelli 1998). Various factors were taken into account to overcome the problem faced in open pond systems and improve the efficiency of such system. In this kind of system, mixing, temperature, and CO_2 supply can be better controlled. Generally, CO_2 is supplied by mechanical pump or a bubbling system (Eriksen 2008). The volumetric productivity is higher since the surface-volume ratio is high. The biomass productivity is estimated to 20–40 $g/m^2/d$ (Shen et al. 2009). Tubular PBR presents productivities that range from 20 to 45 g $m-2$ $day-1$ (Chisti 2007, Shen et al. 2009, González et al. 2008). Su et al. (2011) used microalgae culture in a pilot stirred tank photobioreactor fed with municipal wastewater to assess carbon and nutrient removal. The average removal efficiency of COD, total kjeldahl nitrogen, and phosphate was 98.2%, 88.3%, and 64.8%, respectively. With different nutrient loading, several studies demonstrated that N and P removal can reach up to 99% and 86%, respectively (Chisti 2007, González et al. 2008 and Shen et al. 2009).CO_2 supply and oxygen removal are highly correlated to each other and further with the productivity of PBR (Yoo et al. 2013).

Closed photobioreactor systems have some shortcomings that affect the scaling-up of the system. Cooling devices and degassing zone increase the cost of such system and reduce scaling-up feasibility (Weissman et al. 1988 and Benemann 1989). The cost of such systems is also affected by material cost and high maintenance costs (Mata et al. 2010; Molina-Grima et al. 1999). There are different forms of PBR: tubular reactors, flat plate reactors, and column reactors (bubble or airlift columns).

Tubular Reactor

Tubular reactor consists of several clear transparent tubes placed outdoors and aligned with the sun's ray (Fig. 3). Mixing is realized by the mean of a mechanical pumping which provides a turbulent flow inside the vessels (Carvalho et al. 2006).

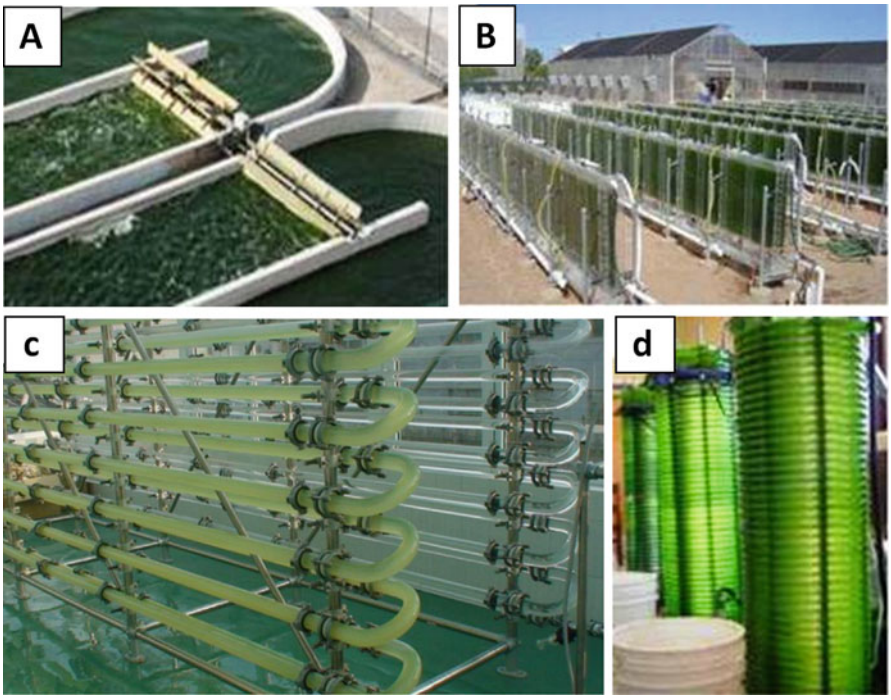

Fig. 3 Suspended microalgal cultivation (**a**) raceway pond (**b**) flat plate reactor (**c**) tubular photobioreactor (**d**) coil bioreactor (Adopted from Zhou et al. 2014)

The surface to volume ratio is considered high. The range of volumetric capacity is 101–104 L. Moreover, continuous harvesting is possible, generally after algae passage through the vessel (Brennan and Owende 2010; Yen et al. 2014).

In this kind of cultivation system, many improvements have been done to overcome the shortcomings of open pond system, like land requirement, evaporation, and contamination are reduced. However, some others problems are encountered. Oxygen is accumulated in the system rather than be returned to the atmosphere leading to inhibition of algae cells, since many algal species cannot withstand exposure for 2–3 h to oxygen concentration above air-saturation (7.5 mg/1 at 30°C) (Tredici and Materassi 1992), hence a degassing zone is indispensable. Carvalho et al. (2006) mentioned that, when considering scale-up, a complex or modular design has to be set to overcome oxygen accumulation and provides a suitable degassing zone. Carbon dioxide has to be supplied to the cultivation system and temperature should be controlled.

Many variations have been developed for tubular reactor: vertical, horizontal, and helical tubular reactor. Vertical tubular reactor consists of transparent vertical tubing fed by CO_2 by bubbling system, while horizontal tubular reactor consists of transparent horizontal tubing (Fig. 4). Helical tubular consists of a flexible plastic tube coiled in a circular framework (Fig. 5).

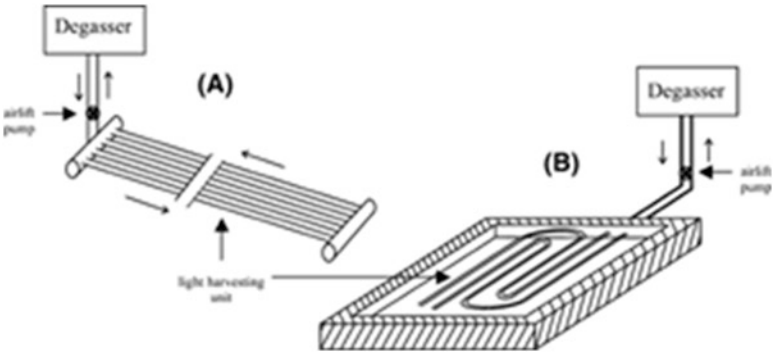

Fig. 4 Horizontal tubular reactor with a degassing unit and a light harvesting unit consists of parallel sets of tubes (**a**) or a loop tube (**b**) (adopted from Carvalho et al. 2006)

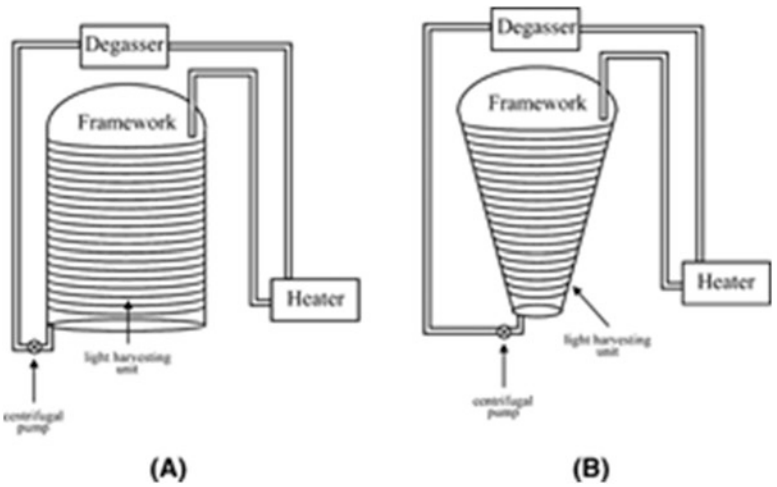

Fig. 5 Helical tubular reactors: biocoil (**a**) and conical framework (**b**) (adopted from Carvalho et al. 2006)

Flat Plate Reactor

Flat plate reactors are a large plane transparent material generally glass, plexiglass, or polycarbonate (Zhang 2015; Singh and Sharma 2012). Agitation is provided by bubbling air through perforated air tubing. A closed system of water spraying is employed to control temperature; the sprayed water from water sprinklers is then collected and recirculated through a cooling water pipe for refrigeration (Fig. 6). The main criterion considered in design is the maximum exposure to the sun light. It has been reported that high photosynthetic efficiencies can be achieved with flat-plate photobioreactors (Hu et al. 1996; Richmond 2000). First study on microalgae

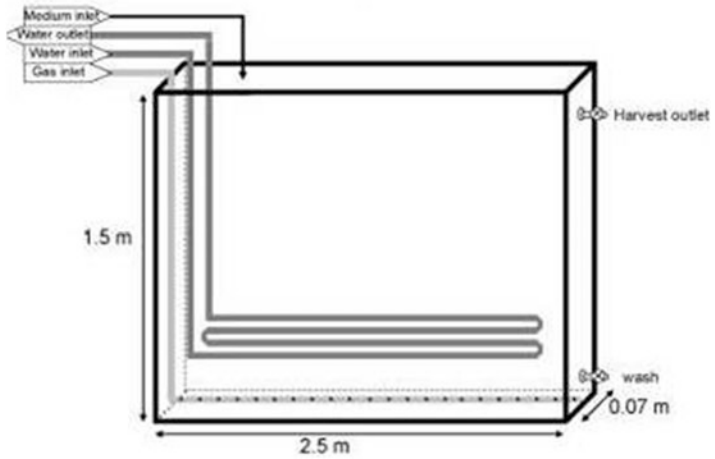

Fig. 6 Flat plate reactor (adopted from Molina-Grima et al. 2008)

cultivation using flat plate reactor was done by Davis et al. (1953). Samson and Leduy (1985) and Barbosa et al. (2005) developed a flat reactor equipped with fluorescence lamps, while Ramos de Ortega and Roux (1986) developed an outdoor flat panel reactor using thick transparent PVC materials. Recently, several studies have been conducted on flat plate reactors. Tredici and Materassi (1992) developed alveolar panels for biomass cultivation. Hu et al. (1996) developed a flat inclined modular photobioreactor (FIMP) for outdoor mass cultivation. Zhang et al. (2002) designed a vertical flat-plate photobioreactor for outdoor biomass production and carbon dioxide biofixation. Among the limitations of flat plate reactor is the possibility of algae growth on walls and then the light limitation.

Column Photobioreactor

There are two type of column reactor: bubble and airlift reactors (Fig. 7). In the bubble column reactor, air is provided through bubbling. At large scale, perforated plates are used (Doran 1995). In the airlift reactor, only one side called riser is fed with air. The other region called downcomer is connected.

While different forms of PBR were developed in the pilot-scale, only few of them were used on a large scale (Zhou et al. 2014). High cost is the major factor that reduced scaling-up feasibility. Table 4 shows recapitulation of scaling-up feasibility of various cultivation systems.

Since land-space requirement of microalgal wastewater treatment is one of the most constraining factors, various improvements are being done on PBRs such as multi-layer and airlift bioreactor, tubular, bag and floating reactor. Those systems have high growth rate combined with high light efficiency.

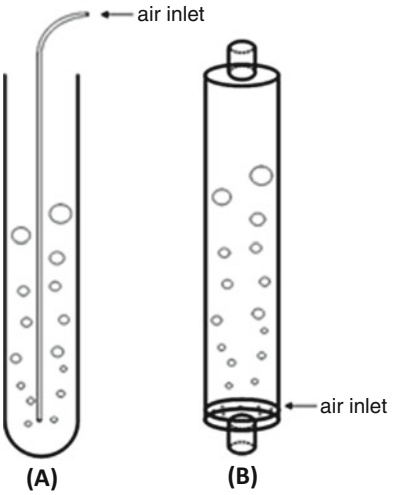

Fig. 7 Airlift (**a**) and bubble column (**b**) reactors (adopted from Carvalho et al. 2006)

Table 4 Comparison of properties of different bioreactor systems (adopted from Zhou et al. 2014)

Bioreactor type	Scaling-up feasibility	Cost	Land requirement	Growth rate	Light efficiency	Contamination issue
High-rate pond	Easy	Low	High	Low	Low	High
Lagoon	Easy	Low	High	Low	Low	High
Multi-layer bioreactor	Easy	Middle	Low	High	Middle	Middle
Coil bioreactor	Difficult	High	Low	High	High	Low
Airlift bioreactor	Difficult	High	Low	High	High	Low
Bubble column reactor	Difficult	High	Low	High	High	Low
Tubular reactor	Difficult	High	Low	High	High	Low
Bag reactor	Middle	Low	Low	High	High	Low
Floating reactor	Easy	Low	Low	High	High	Low

Lavoie and De la Noüe (1985) observed that hyper concentrated algal cultures have high removal efficiency of N and P within a short period of time. Hashimoto and Furukawa (1989) and Morales et al. (1985) investigated on hyperconcentrated algal cultures and observed high removal efficiency of N and P at a pilot-scale. They confirmed that the efficiency is proportional to cell density and independent of light efficiency. Investigations on feasibility of scaling-up of such system could open wide scope of improvement. The huge amount of water used in suspended cultivation systems is problematic. The biomass volume in such culture is composed of 99% water with 1% remaining to be extracted, which increases the cost of harvesting (Lehr and Posten 2009, Cao et al. 2009, Katarzyna et al. 2015).

3.2 Attached Cultivation

Attached culture is a new cultivation method with growing interest of researchers nowadays, since it provides a good solution for microalgal harvesting (Hoffmann 1998). High cell density and lower water and land requirement are the main advantages of attached cultures leading to higher interest and new researches in this field (Mulbry and Wilkie 2001, Wilkie and Mulbry 2002, Kebede-Westhead et al. 2006, Johnson and Wen 2010 Katarzyna et al. 2015, Lin et al. 2003, Ozkan et al. 2012). Attached cultivation can be classified into two types: matrix-immobilized system and biofilm system. Attached cultivation is based on the growth of algae on the surface of a solid support. Algal cells are allowed to grow on a surface of a material to form a biofilm. This type of cultivation is widely used in wastewater treatment industry (Wuertz et al. 2003, Jensen 1996, Adey 1982, Adey 1998a, b). Compared with ordinary suspended cultivation system, the attached systems offer higher biomass yields, easy to scale-up with better light distribution within the reactor, and better control of contamination (Katarzyna et al. 2015). It was shown in the study that the effect of substrate material on biomass productivity and lipid content of microalgae is significant.

Depending on the system type, biomass productivity ranges between 15 and 27 g m^{-2} day^{-1} for algal turf scrubber (Adey et al. 1993). Black polyethylene screen support used for algal growth presents a biomass productivity from 5 to 20 g m^{-2} day^{-1} (Mulbry and Wilkie 2001; Mulbry et al. 2005; Wilkie and Mulbry 2002). The attached system achieved 2.8 times higher biomass productivity and total lipid productivity of 9.1 g m^{-2} day^{-1} and 1.9 g m^{-2} day^{-1}, respectively, than the suspended system (Lee et al. 2014). *Scenedesmus obliquus* used in the suspended and attached cultivation systems developed high biomass productivity of 70.9 g m^{-2} day^{-1}, which is 5 to 8 times of productivity achieved in suspend culture (Liu et al. 2013a, b). The ability of attached system to reach a higher productivity was also confirmed by the study of Cheng et al. (2013) with the assessment of the growth of *B. braunii*.

It was shown in the study of Katarzyna et al. (2015) the effect of substrate material on biomass productivity and lipid content of microalgae. Biomass productivity increases from a value of 0.58 g m^{-2} day^{-1} to 2.57 g m^{-2} day^{-1}, respectively, when it grows in polyethylene fabric or polystyrene foam (Johnson Michael 2009). The effectiveness of substrate material and his influence on biomass growth have been studied by many researchers (Johnson 1994, Johnson 1994, Sekar et al. 2004, Gross et al. 2013, Christenson and Sims 2012).

Several researchers studied the use of immobilized microalgae in urban wastewater treatment (Lau et al. 1997, Chevalier and De la Noüe 1985a, Jimenez-Perez et al. 2004, Ruiz-Marin et al. 2010). Ruiz-Marin et al. (2010) compared the nutrient removal using two types of microalgae, *S. obliquus* and *C. Vulgaris*, cultivated in artificial and urban wastewater. And they found that *S. obliquus* shows better nutrient removal capacity (around 96%) of ammonia. Chevalier and De la Noüe (1985b) reported similar results of 100% for immobilized *S. obliquus*. *C. Vulgaris*

cultivated in the same condition presented a lower nutrient removal around 65% and 80%, respectively, in artificial and urban wastewater (Ruiz-Marin et al. 2010). Solid support medium having an influence on microalgae growth, *C. vulgaris* growing on alginate-immobilized medium achieved N removal of 82% and 95%, respectively, in urban wastewater and artificial wastewater (Travieso et al. 1996 and Lau et al. 1997). Nutrient removal ranges from 36 to 92% for total nitrogen and from 51 to 93% for total phosphorus (Wilkie and Mulbry 2002).

The main disadvantages of attached systems are limitation of diffusion, development of boundary layers, and light shielding. A diffusion gradient is necessary to carry nutrient to algal surface. A minimal shear velocity of 15 cm/s is required (Whitford 1960, Whitford and Schumacher 1961). Development of boundary layer is the second factor that limits nutrient uptake. Improvements were done in attached culture to overcome this problem. Addition of tipping bucket was adopted by Adey (1998a, b) and Wilkie and Mulbry (2002). Light shielding issue, caused by high density of cells at the surface of the solid supporter, limits the growth of the cell inside but not the pigment production (Chevalier and de la Noüe 1985a and Lau et al. 1997). Liu et al. (2013a, b) proposed a new elaborated cultivation system to mitigate the problem of light shielding of interior portions of the algae. Liu et al. (2013a, b) developed a new attached cultivation method to facilitate high photosynthetic efficiency (Fig. 8).

Microalgae cells grew on the surface of glass plate and filter paper and wastewater was circulated in the filter paper. The solar radiation can be perpendicular to

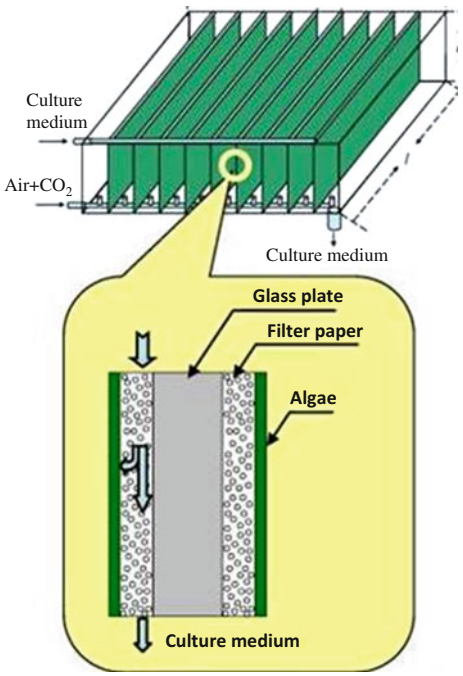

Fig. 8 Schematic diagram of the attached photobioreactors (adopted from Liu et al. 2013a, b)

the top or to one side of the bioreactor. One bottleneck for the use of attached system is the lipid productivity limitation. The study of Gross et al. (2013) compared lipid content in microalgae cultivated in suspended and attached system (open pond and rotating algal biofilm). It was shown that algae grown on biofilm system has higher carbohydrate and protein content and less lipid content than algae grown in suspended culture (8% of lipid content besides 22%). Selection of suitable cultivation system for algae based biofuel production should be based on biomass production as well as lipid productivity.

One of the several criteria for the selection of cultivation system for algae based biofuel production is directly correlated to the processing parameters which are gas transfer, mixing, and light requirement (Christenson and Sims 2011). Expansion of closed photobioreactor was the consequence of the need of a best degree of control especially when using wastewater medium for the growth of microalgae (Borowitzka 1999). Scalability is another important criterion for the selection of cultivation system since it is considerably related to the cost of the system and then affects the price of produced biofuel. Closed photobioreactors outperform open pond systems but are relatively costly (Mata et al. 2010, Ho et al. 2011).

In brief, the main challenge of algae production is how to achieve a high productivity with a minimum cost that can compete with the price of petroleum. Nevertheless, the interest in microalgae as second generation biofuel sources would become a valuable approach since it contributes to the goal of renewable energy production. Scope for research and development remains very wide.

4 Harvesting Methods for Microalgae

Harvesting of algal biomass is the second step which is necessary for concentrating algal biomass to produce biodiesel. The microalgae harvesting process poses challenges because of the small size of algal cells (generally 1–20 mm) and also to handle large liquid volumes to harvest relatively low density algal cells grown in open ponds (Lam and Lee. 2012; Pittman et al. 2011). Algal based wastewater treatment is not applied extensively by the wastewater industry primarily because of inefficient and costly harvesting methods (Pittman et al. 2011). There are several methods of harvesting, and they all use one or more solid–liquid separation steps to concentrate the biomass. The harvesting processes are energy intensive (Gao et al. 2014), accounting for 2–60% of the total cost of biodiesel production (Udom et al. 2013), depending on the method.

No single harvesting method is universally accepted. The suitable harvesting method is chosen according to algal species, growth medium, and end product (Shelef et al. 1984). Current harvesting methods include sedimentation, centrifugation, filtration, flocculation, flotation, and immobilization systems (Yanyan 2012). Comparative evaluation of these methods is given in Table 5 (Uduman et al. 2010; Zhou et al. 2014; Debora and Edward 2015).

Table 5 Methods for harvesting algae (adopted from Debora and Edward 2015; Zhou et al. 2014)

Method	Description	Advantages	Disadvantages	References
Coagulation/ Flocculation	This process aggregates the cells and increases their size before the flocculation. The flocks can be removed by sedimentation. The most common coagulants are aluminum sulfate and ferric iron chloride. Polymers and other natural coagulants (moringa, chitosan, and cactus mucilage) have also been tested.	• Established and easy to use • Can achieve more than 90% algae recovery	• Expensive • Each coagulant has an optimum dosage • Some coagulants contribute to greenhouse gas emission (polymers) and others have impact on health (aluminum sulfate) • Aluminum sulfate could cause some cell lysis • Ferric chloride causes salt discoloration.	Pittman et al. (2011) Udom et al. (2013), Skoronski et al. (2014), Mezzari et al. (2014)
Electro-coagulation	This process uses reactive metallic electrodes to produce positively charged ions that induce coagulation of negatively charged microalgal cells. The aggregated cells settle to the bottom by sedimentation. There are three modalities: electrolytic coagulant, electrolytic coagulation, electrolytic flotation, and electrolytic flocculation.	• 98% algae recovery • Low current sufficient for coagulation • Lower cost than coagulation • Reduces the contamination of residual algae biomass • Can be scaled up	• If the resulting solution has less than 10% total solids, a second method is needed to separate the algae from the liquid solution	Richardson et al. (2014)
Flotation	This process combines a chemical flocculent to gather the algae with bubbling air to bring the flocks to the surface, where they are removed by conventional skimming methods or by dissolved air flotation (DAF).	• Ozone increases algae flocculation from 31% to 55% • DAF is cheap method at large scale, handling more than 10, 000 m3/ day • DAF contributes to flock buoyancy, resulting in a concentrated cell foam (7–10% dry weight)	• Flotation based on ozone is expensive	Rawat et al. (2011) Richardson et al. (2014), Rubio et al. (2002)

Sedimentation/ Gravity	This physical process uses gravity to remove suspended solids from the liquid.	• The most common harvesting method • Easy to control and low cost • Amenable to large scale biomass harvesting • Recycled water rich in nutrients can be returned to the algae growth chamber due to the absence of chemicals	• Very slow process (0.1–2.6 cm /hr. • Deterioration of biomass can occur at high temperatures	Rawat et al. (2013), Pittman et al. (2011)
Immobilization	This process uses polymer encapsulation of microalgae. These substances can be added prior to or during the cultivation. Alginate is the most common immobilizing agent.	• Algal biomass can be recovered from an immobilized matrix	• It is unclear whether immobilized algae could be used for oil production • Efficiency of lipid extraction from the algal-polymer matrix is undetermined	Pittman et al. (2011), Moreno-Garrido (2008)
Filtration	A mechanical or physical process that uses a medium through which only the fluid passes, while oversize solids are retained. A Vacuum Filter (VF) is used for larger algae ($\geq$70 µm). For smaller cells, membrane microfiltration or ultrafiltration should be used.	• VF and polymer membrane can handle 0.1% solid content • Polymer membrane filtration consumes less energy ($\leq$500 kW h/ton dry algae) than other dewatering processes	• VF is energy intensive (5900 kW h/ton dry algae) • Microfiltration requires pumping and replacement membranes; expensive • Final solids content is only 30% (VF) and 10% (polymer membrane)	Rawat et al. (2011) Pittman et al. (2011), Udom et al. (2013),
Centrifugation	A mechanical process using an apparatus that rotates at high speed, separating substances of different density.	• Rapid and efficient • Works for a wide range of microalgae • High algae recovery ($\geq$95%) • Concentrates biomass into a cake	• Highest energy consumption (700 MJ/ton dry algae). • Highest GHG emissions (>80 kg CO2 eq./ton dry algae) • Infeasible on large scale due to high energy consumption	Rawat et al. (2013) Udom et al. (2013), Pittman et al. (2011)
Ultrasonic aggregation	Acoustic interaction forces and particle–particle interaction forces	• Continuous operation	• High cost, specific machine needed	Zhou et al. (2014)

4.1 Sedimentation

Sedimentation is low cost and probably most preferred biomass harvesting method which is used in wastewater treatment system (Brennan and Owende 2010). However, slow, unreliable, and gravity dependent rate of settling are considered as some of the main drawbacks of the method (Zhang and Hu 2012; Christenson and Sims 2011; Nurdogan and Oswald 1996).

4.2 Centrifugation

Centrifugation is the most widely used harvesting method and is based on particle size and density. Separation efficiency is dependent upon the size of desired algal species. This method is based on density difference which makes them stable and fast method. Molina-Grima et al. (2003) reported 95% harvesting efficiency for this method. Several centrifugal techniques are used such as tubular centrifuge, multichamber centrifuges, imperforate basket centrifuge, decanter, solid retaining disc centrifuge, nozzle type centrifuge, solid ejecting type disc centrifuge, and hydrocyclone (Shelef et al. 1984). High energy requirement makes this method uneconomical for large scale application (Pittman et al. 2011).

4.3 Filtration

Filtration is usually used for solid–liquid separation. There are several types of filtration, such as dead end filtration, microfiltration, ultrafiltration, pressure filtration, vacuum filtration, and tangential flow filtration (Harun et al. 2010). Membrane filtrations such as microfiltration and ultrafiltration are used for algal harvesting (Rawat et al. 2011). However, high energy consumption, high operational cost, membrane fouling, and clogging are the obstacles in field application (Uduman et al. 2010).

4.4 Flocculation

Flocculation process is used to make aggregates known as algae flocs. This process is often used as a pretreatment to destabilize algae cells from water and to increase the cell density by natural, chemical, or physical means. Negatively charged microalgae cells surface is neutralized by addition of chemical flocculants that leads to increase in the size of particles (Zhang and Hu 2012). Flocculation is a pH sensitive process, which is affected by concentration of flocculants, ionic strength, and characteristics of cellular surface (Oh et al. 2001). Chemical flocculants improve the efficiency of the process, however, they increase heavy metal

concentration which causes pollution (Christenson and Sims 2011; Munoz 2005). These days organic polymers such as chitosan are used as alternative flocculants (Christenson and Sims 2011). Munoz (2005) reported around 90% of algal biomass removal using 15 mg/L chitosan. Organic flocculation has edge over chemical flocculation as it could minimize changes in the culture medium by pH adjustment; however, pH adjustment itself is not economical for large volume (Yanyan 2012).

4.5 Flotation

In flotation harvesting microalgae cells are make to float on the surface of water by induced microair bubbles and are removed (Brennan and Owende 2010), microalgae cells are trapped on microair bubbles and float at the surface of water (Sharma et al. 2013). Dissolved air flotation (DAF) is a most preferred flotation method for algae removal (Christenson and Sims 2011). However, high every demand makes this method uneconomical (Greenwell et al. 2010; Henderson et al. 2008; Wiley et al. 2009).

4.6 Immobilization Systems

Immobilization system is considered as one of the most efficient harvesting methods (Yanyan 2012). In this process, cells are immobilized naturally or artificially to prevent them moving from their original location (de-Bashan and Bashan 2010; Tampion and Tampion 1987). Covalent coupling, entrapment immobilization, affinity immobilization, and adsorption are the main types of immobilization system (de-Bashan and Bashan 2010; Mallick 2002). Entrapment immobilization is one of the most used immobilization methods (Pittman et al. 2011). Although algal growth is occurred after immobilization, however, the nutrient removal efficiencies decrease after several cycles (de-Bashan et al. 2002).

4.7 Ultrasonic Separation

In this process ultrasound is used with sedimentation to increase the harvesting efficiency. Bosma et al. (2003) reported 92% algal biomass harvesting efficiency at low harvest flow rate and low algal biomass concentration.

5 Challenges in Implementation of Large Scale Integrated Wastewater-biofuel System

There are some basic challenges in implementing large scale integrated algae-wastewater system for simultaneous biofuel production and nutrient removal, which includes the cost effective production and harvesting of algae. Primarily nutrient supply and recycling, gas transfer and exchange, photosynthetically active radiation (PAR) delivery, culture integrity, environment control, land and water availability are the challenges in large scale algal production (Molina-Grima et al. 2003; Uduman et al. 2010). Few specific challenges are discussed below.

5.1 Harvesting Process

Inefficient and costly algal biomass harvesting technology is another bottleneck in implementing the commercial algal biofuel using microalgae based wastewater treatment systems. A variety of harvesting and dewatering technologies have been extensively studied including as given in Table 5 (Uduman et al. 2010, Debora and Edward 2015; Zhou et al. 2014). Filtration method is only used for harvesting microalgae with long length or formation of large-colony (Salim et al. 2010). The economic viability and potential environmental safety issues caused by these polymers limit the use of flocculation (Uduman et al. 2010). Among these processes, centrifugation is considered to be the most efficient method (Salim et al. 2010). However, high capital cost, energy input, and operational cost obstruct its large scale application. The main disadvantage of flotation is its environmental and economic viability. Another alternative is to immobilize or entrap microalgae cells in suspended media (De-bashan and Bashan 2010). However, these polymers are too costly and hence limit their applications in large scale (Smidsrod and Skjak-Braek 1990).

5.2 Night Biomass Loss

Energy costs associated with lipid extraction in algae based biofuel production (drying biomass, solvent use, etc.) accounted for 90% of the process energy consumption (Lardon et al. 2009; Lundquist et al. 2010; Clarens et al. 2011). Apart from metabolic variability among different algae, environmental factors such as temperature have been demonstrated to have marked effects on algal respiration (Langdon 1993; Grobbelaar and Soeder 1985; Ogbonna and Tanaka 1996; Le Borgne and Pruvost 2013; Torzillo et al. 1991; Ryther and Guillard 1962). Additionally, the physiological state of the culture (growth phase/cell density) can influence respiratory losses at night (Hu et al. 1998; Grobbelaar

and Soeder 1985; Ogbonna and Tanaka 1996; Michels et al. 2014; Beardall et al. 1994). Previous work on characterizing algae respiration in the dark relied heavily on changes in oxygen concentrations as a proxy for biomass losses in the dark (Burris 1977; Geider, and Osborne 1989; Grobbelaar and Soeder 1985). Furthermore, pond temperatures at night can vary significantly from daytime pond temperatures (Torzillo et al. 1991). Knowing the actual change in biomass concentration under variable night temperature conditions is a critical parameter that is often neglected in predictive phycological modeling attempts (Béchet et al. 2013). Moreover, it is reported in several studies that maximum loss rates are highly variable between species (Langdon 1993; Falkowski and Owens 1978; Geider and Osborne 1989; Edmundson and Huesemann 2015). Night biomass loss remains an underappreciated aspect of optimizing algae productivity in outdoor pond cultivation and, as suggested by Hu et al. (1998), potentially represents one of the most important limitations to productivity (Edmundson and Huesemann 2015). However, recent developments in hydrothermal liquefaction (HTL) of algae biomass may improve the energy balance of algae generated biofuels when compared with biodiesel production after lipid extraction (Elliott et al. 2013; Venteris et al. 2014; López Barreiro et al. 2013; Zhu et al. 2013; Liu et al. 2013a, b). Several studies report that greater than 30% of the biomass fixed during the day in outdoor, sunlit algae cultures (both ponds and photobioreactors) can be lost at night (Guterman et al. 1989; Hu et al. 1998; Torzillo et al. 1991).

6 Recent Research and Developments in Algal Biofuel Production

There are several key areas in which researchers are recently interested to get solution for problems faced by algal biofuel production. Some of them are discussed below.

6.1 Attached Immobilization Systems

Attached immobilization system is showing better prospects for biomass harvesting. Attached algal system is one of the immobilization methods which promotes the biofilm formation on the surface of the medium (Zhang and Hu 2012). Phototrophic biofilm reactor with polycarbonate slides was developed by Guzzon et al. (2008) to remove phosphorus from wastewater. He and Xue (2010) developed a system having fiber-bundle as carrier for attached growth of microalgae for secondary wastewater treatment. Shi et al. (2007) designed algal turf scrubbing (ATS) system to let filamentous algal community grow on a turf scrubber (screen),

which reduced harvesting cost. 100% immobilization efficiency could be achieved with more than 90% ammonium, nitrate, and phosphate removal efficiencies of ammonium, nitrate, and phosphate within 9 days (Shi et al. 2007).

6.2 Electrophoresis Harvesting System

In this alternative method compressor and saturator are replaced and surfactants are used to create small bubbles (Henderson et al. 2008; Wiley et al. 2009). The efficiency of the surfactants adsorbed at the bubble interface governs removal efficiency of the algal biomass (Henderson et al. 2008). Also, electric field induced motion of dispersed microalgae can be used to concentrate and harvest algal biomass (Christenson and Sims 2011). The basic principles of this harvesting method are the electrophoresis phenomenon and the negative charge of algal cells (Poelman et al. 1997). However, high energy requirement and costly electrode are the main obstacle of this method (Uduman et al. 2010). Zhou et al. (2012a, b) reported a natural metal ion mediated self-sedimentation/flocculation method by growing algae on metal ion containing wastewaters, which could be another option for cost-effective harvesting algal biomass for biofuel purpose.

6.3 Integrated Photo-bioelectrochemical System

Integrated photo-bioelectrochemical (IPB) systems are a newly emerging technology for sustainable wastewater treatment through synergistic cooperation between microbial fuel cells (MFCs) and algal bioreactors (Xiao et al. 2012 Xiao et al. 2012). MFCs are a bioelectrochemical reactor in which exoelectrogenic bacteria oxidize organic compounds and produce bioelectricity (Li et al. 2014). The direct energy recovery from wastewater makes MFCs a promising approach for simultaneous wastewater treatment and bioenergy production. The electricity-generating processes in MFCs promote the oxidation of organic compounds (Zhang et al. 2010); however, nutrients such as nitrogen and phosphorus are not effectively removed, unless special processes are linked to MFCs (Kelly and He 2014). While MFC energy production and algal nutrient bioremediation have been studied separately, there lack information about how different algae and bacteria might affect functions of integrated algal bioelectrochemical reactors (Xiao et al. 2015). In one study Xiao et al. (2012) found that the IPB system effectively removes both organic and nutrient with algae in the cathode, during the one-year long operation: more than 92% of organics, 98% of ammonium nitrogen, and 82% of phosphate were removed from synthetic wastewater. In the recent study Xiao et al. (2015) observed that organic removal was less influenced by the algal and microbial composition related to the distinct algal inocula. Also, energy production, in the form of both electricity and biomass, was significantly affected by algal sources

(Xiao et al. 2015). Further, they conclude that with a synthetic solution, the IPB system could achieve more than 90% removal of solution organic compounds and nearly 100% of ammonium nitrogen (Xiao et al. 2015).

7 Conclusion

The potential use of microalgae for simultaneous biofuel production as well as wastewater treatment has received considerable interest, but to make it economically viable more research and development is needed in cultivation and harvesting processes. Much of the research related algal cultivation and harvesting is currently confined to the laboratory, only limited studies are reported for pilot and few up to field scale. There are still major challenges in implementing algal based biofuel production using wastewater. There are various issues in cultivation systems like scaling, low productivity, low oil content, high land requirement, dependency on climate conditions, oxygen accumulation in the system leading to inhibition of algae cells, night biomass loss, etc. In harvesting system high energy demand, less efficient, and slow process are the common drawbacks. In brief, the main challenge of algae production is how to achieve a high productivity with a minimum cost that can compete with the price of petroleum. However, as stated by Pittman et al. (2011) the high biomass productivity of wastewater-grown microalgae suggests that this cultivation method offers real potential as a viable means for biofuel generation and is likely to be one of the many approaches used for the production of sustainable and renewable energy. Moreover, more research is required to overcome the above-mentioned issues that too in the coordination with researchers and industries.

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Advancements in Algal Harvesting Techniques for Biofuel Production

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1 Algae as a Biofuel Feedstock

The sufficient biofuel yield depends upon the type of feedstock employed for the production. Microalgae as biofuel feedstock have gained considerable importance due to several factors favoring it to be a potential candidate for biofuel production. Microalgae are unicellular photosynthetic micro-organisms, which are important for economical and easy cultivation resulting in high biomass yield. It has a complex and robust cell wall mainly composed of cellulose and hemicelluloses. Large scale cultivation of algae can be conveniently carried out in open systems like raceways, HRAPs without any requirement of arable land using energy from sunlight as well as in covered systems including photo bioreactors of several designs. Moreover, it is also ubiquitously present in lakes, ponds forming algal blooms and biofilms. Algae have also been reported to grow well in different wastewaters from sources like sewage treatment plant, agricultural fields, live-stock/cattle/dairy farms, and effluent from industries (Pittman et al. 2011; Prajapati et al. 2013a; Woertz et al. 2009). The major problem of these wastewaters is its inconsistent nutrient level usually with high nitrogen and phosphorus content. Algal growth in these wastewaters is an advantage as it utilizes these high levels of nitrogen and phosphorus for its growth simultaneously bringing the nutrient levels into the discharge limits. Several studies on wastewater treatment by algae in terms of nutrients removal like nitrate, phosphate, chemical oxygen demand (COD), ammoniacal nitrogen (Prajapati et al. 2013a) and metal removal like lead, copper, and others (Jalali et al. 2002; Mallick 2002; Vilar et al. 2008) have been reported.

The first generation and second generation biofuels are being generated using plant sugars/lipids and complete plant biomass, respectively, whereas its generation from algae is known to be third generation biofuel, overcoming the problem of competition for food or land (Eisentraut 2010; Lee and Lavoie 2013). Algae show 30–100 times higher biofuel yield as compared to terrestrial crops (Demirbas 2010). Different types of biofuels can be produced by microalgae on application of various bio-thermo-chemical processes, for example, anaerobic digestion and anaerobic fermentation results in the production of biogas and bio-hydrogen, respectively; hydrothermal liquefaction gives rise to bio-oil/bio-diesel and fermentable products of biomass like carbohydrates lead to the formation of bio-ethanol (Demirbas and Fatih Demirbas 2011; John et al. 2011; Prajapati et al. 2014a). All these biofuel routes have their own advantages but the actual implementation on commercial level is still in an embryonic stage. This is due to high dependency on the commercial scale cultivation of algae followed by its economical biomass recovery and other processing.

2 Problems Related to Algal Harvesting

Due to high fuel value of algae, this organism has been on scientific priority. After the cultivation of algae by suitable method, the dewatering from the microalgal cells is the most important step to move ahead for its application. However, the recovery of algal biomass from the culture media or wastewater is much more expensive than its cultivation. This is due to extremely small size of microalgal

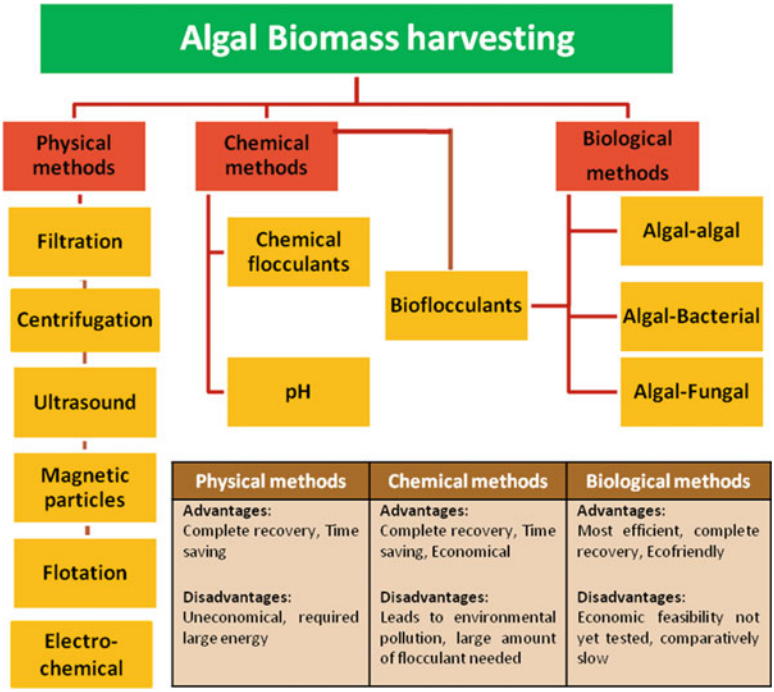

Fig. 1 Classification of methods used for algal harvesting with their advantages and disadvantages

cells ranging from 2 to 6 μm. Several studies have also shown a very high zeta potential of the algae, making it a stable suspension in aqueous medium. This high stability of algae further lowers the possibility of easy gravity separation under natural conditions. As a result, 40% of the total cost of algae biomass production is contributed in its cultivation and more than 60% cost has invested on the biomass recovery, which disturbs the overall economics of biofuel production (Molina Grima et al. 2003). Therefore, a comparative knowledge of existing harvesting methods, the advancements already made by the scientific community in this area and scope for betterment of these techniques may influence the researchers to optimize the critical conditions best suitable for maximum biomass recovery. The methods used for algal biomass harvesting are classified in Fig. 1.

3 Harvesting Techniques and Advancements

3.1 Physical Methods

Some of the widely employed physical techniques and its advancements have been discussed in the following sections.

3.1.1 Membrane Filtration

Membrane technology is one of the most successful and cost-effective technology being used for small scale as well as large scale harvesting of microalgae. This dewatering method has several advantages over other technologies, of which its energy efficiency is of prime importance. Apart from this, the complete biomass recovery and simultaneous removal of other contaminants like protozoa, bacteria, viruses, and other micro-organisms (Judd 2008) give this technology an edge over others. Furthermore, because of no chemical or coagulant usage, the resultant media/water after filtration can be recycled for other purposes like irrigation (Zhang et al. 2010).

Membrane filtration uses several types of permeable membranes, which is the only investment cost in this harvesting technique. The application of the membrane and manufacturing material determines the cost of the membrane. Before and during microalgal harvesting, there are few parameters, which need to be optimized for maximum biomass recovery, for example, membrane preparation, porosity, thickness, permeability, and flux (Bilad et al. 2012).

The recent studies are focussed on assessing the type of microfilters (MF, 0.2–0.8 μM) or ultrafilters (UF, different MWCO ~10–100 kDa) best suitable for harvesting and flux maintenance. The materials commonly used for microfiltration membranes are polyvinylidene fluoride (PVDF), a combination of PVDF and *N*,*N*-Dimethylformamide (DMF), mixed cellulose ester (MCE), etc. For ultrafilter, generally Polyether sulfone (PES) and Polyacrylonitrile (PAN) are being used (Rickman et al. 2012; Sun et al. 2013; Zhang et al. 2010). The concentration and combinations of these materials govern the pore size of membrane. Flux rate, defined as flow per unit area, is the most important parameter to judge the efficacy of the technique. The results of the critical parameters like flux determination by flux stepping test indicated that the fouling of member due to lower flux rate is more prone in ultrafilters. A comparative study on use of MF, UF, and a modified UF made up of fluoropolymers (PVDF) has been recently done (Sun et al. 2013). The results indicated that there was significant increase in permeate flux in the initial phase of experiment by MF as compared to UF, which steeply declined within 3 h similar to UF. The fast fouling tendency of MF due to bigger pore size was the reason given to this phenomenon. On the other hand, a modified UF made up of composite PVDF was the only membrane which was hydrophilic in nature and it showed the highest permeate flux. Therefore, apart from the membrane material, hydrophilic nature of the membrane is also one of the most important criteria for complete algal harvesting.

3.1.2 Centrifugation

Centrifugation is the most commonly used technique for dewatering algae. To avoid the prolonged process of common gravity sedimentation, sufficient energy input to increase the gravitation force as well as the centrifugal force leads to the quick settling of algal biomass. This technique is advantageous due to elevated harvesting efficiencies within considerably less time (Molina Grima et al. 2003).

But, high energy investment and non-feasibility of this technique at a very large scale is a bottleneck in the cost-effective application algae for biofuel production (Pienkos and Darzins 2009). Therefore, extensive researches and some recent advancement in this technique have led to its competitive use for algal harvesting.

A group of researchers have developed an economic model for harvesting algae by attaching a rotary vane pump to the centrifugation unit. This pump increases the flow rate of algae before pouring into the large scale centrifuge machine. The energy consumption per cubic meter tends to decrease on increasing the incoming flow of algal suspension. After the complete economic calculations, it was observed that there was approximately 82% decreased harvesting cost by using this technique (Dassey and Theegala 2013).

Similarly, a low cost modification in this method was also proposed, where, the use of membrane microfilters prior to centrifugation was estimated to lower down the energy consumption up to 0.169 kW h/kg of dry weight of algae as compared to the conventional centrifugation alone utilizing 0.5 kW h/kg (Baerdemaeker et al. 2013).

3.1.3 Ultrasound

Harvesting of algae by ultrasonic waves is a less commonly used technique, where the algal cells are continuously pumped into a resonator chamber, consisting of a transducer and a reflector, creating an ultrasonic field with high potential (bellies) and low potential (nodes). The algal cells experience an attractive force on the nodes of the chamber, which results into the agglomeration of cells on the nodal knot of the sonic waves. The algae aggregate then settle by gravity after stopping the ultrasonic field resulting in the ease of its separation (efficiency 93%). The major advantages of this technique are no fouling, lack of any kind of mechanical troubles, and can be run for longer operations continuously (Bosma et al. 2003).

3.1.4 Magnetic Particles

Another technique for harvesting is the use of magnetic particles, where the algal cells get adsorbed on these particles due to electrostatic force (Xu et al. 2011). Researchers have been trying to optimize the harvesting process through these magnetic particles due to its easy and quick separation with low energy and cost consumption (Hu et al. 2013; Xu et al. 2011). Magnetic particles have been used in the form of precipitated magnetite, silica coated hydrophilic magnetic particles (Cerff et al. 2012), and surface modified magnetic beads with diethyl aminoethyl (DEAE) and polyethylenimine (PEI) (Prochazkova et al. 2013). The separation of algae by these particles requires some critical pre-requisites like the modification of magnetic beads, its concentration, and pH. The results indicated a decrease in removal efficiency of magnetic beads with DEAE as compared to PEI modified magnetic beads in pH ranging from 4 to 10. It was also observed that the algal-magnetic bead complex was rigid enough to bear the shear force cause during agitation when kept in the external magnetic field. On the other hand, only algal

flocs tend to break on agitation and also showed non-functionality at extreme pH value from 4 to 12 (Prochazkova et al. 2013). Cerff et al. (2012) have proposed the mechanism of algae attachment to magnetic particles on the basis of the zeta potential results. Contradicting the previous results of electrostatic binding of algae to the particles, these researchers have reported that at elevated pH, the algal cells might have the affinity towards the deprotonated hydroxyl groups on the surface of magnetite. Therefore, these magnetite particles might be showing an ion exchange capability to the cells.

A recent advancement to this technology has been made by using the naked ferric nanoparticles (Fe_3O_4) for the recovery of microalgae like *Botryococcus braunii*, *Chlorella ellipsoidea* (Xu et al. 2011), and *Nannochloropsis maritime* (Hu et al. 2013). These nanoparticles have an advantage over surface modified magnetic particles as the production of these modified particles increases the cost of harvesting due to the use of functional materials for its coating. The separation mechanism of algae by the nanoparticles has been explained as an electrostatic interaction where the positively charged nanoparticles attract the negatively charged algal cells. Interestingly, there was an increase in aggregation of algal cells at neutral or alkaline pH. This was measured in terms of hydraulic diameter of algal-nanoparticle complex. This infers the low dosage requirement of the nanoparticles at alkaline pH with high recovery efficiency. The advantage of this method is its very high microalgal recovery efficiency rate up to 95–97% within very less time (4–5 min). Apart from this, the resulting bulk liquid nutrient media after separation were reused up to 5 cycles for algae cultivation (Hu et al. 2013). The magnetic nanoparticles after algae detachment were also tested for reusability, results of which showed that there was no loss of particle recovery activity of nanoparticles and 95–97% harvesting was observed even up to 5 cycles (Xu et al. 2011).

3.1.5 Flotation

Dispersed air flotation (DAF) is one of the oldest techniques being utilized for algae harvesting. This method incorporates the pumping of air into the liquid and the algae floats with bubbles to the surface of the liquid (Uduman et al. 2010). Chemical flocculants are now being used coupled with DAF, where cationic surfactants are used to flocculate algae (Liu et al. 1999). The major advantage of this technique is its efficiency to separate low density algal cultures in reduced harvesting time (Henderson et al. 2008). Vacuum gas lift has also been used for flotation but the harvesting efficiency was found to be 49% only (Barrut et al. 2013).

Recently, a pretreatment of algae with ozone (O_3) has been used prior to DAF, in which the algae gets oxidized, resulting into cell lysis and increased ability for cell flotation (Widjaja et al. 2009). The dispersion of ozone has been reported to have a better capability for algae to float as compared to simple oxygen. The experimental results of Cheng et al. (2010) have indicated that the bubbles of simple oxygen were small in size (1 mm), which were not able to make the algal cells float along with

bubbles, thereby no formation of algal froth on the top of the aeration column. This was not true in case of ozone, where the efficient algal flotation was observed. The reason behind this phenomenon has been discussed by Cheng et al. (2011) that the negative zeta potential was shown to be increased after ozonation making it slightly more hydrophobic, which helped in easy flotation on the surface. Another beneficial application of this advanced technique has been discussed by Nguyen et al. (2013) that cell disruption and easy lipid extraction from the algae after pre-oxidation by ozone also contribute to the high yield of biofuel production within less energy inputs.

3.1.6 Electro-Flocculation or Electrochemical Flocculation

There are some other physico-chemical flocculation techniques, which are not very popularly used for algae harvesting. The electrolytic flocculation was reported by Poelman et al. (1997), which used electricity to flocculate 95% of algae. A high energy investment in large scale open systems is the major constrain in this method. Alfafara et al. (2002) and Azarian et al. (2007) used polyvalent aluminum electrode for electro-flocculation of algae in continuous flow reactor. The purpose of using electrodes of this metal was the release of aluminum into the algal suspension, which also helped in flocculation. Therefore, the resultant removal of algae was simultaneously being done by electro-flotation and electrocoagulation. An integrated technology of electro-flocculation and dispersed air flotation was used by Xu et al. (2010) on *B. braunii* showing 98.9% harvesting efficiency after 14 min as compared to simple electro-flocculation, which could not show efficiency of more than 93.6% even after 30 min.

4 Chemical Methods

4.1 Chemical Flocculants

Chemical flocculation is a very quick and efficient process which has been tested and validated by many researchers at laboratory scale as well as large scale. Several coagulants or chemical flocculants have been reported to harvest algae from natural ponds, large scale cultivation units like PBRs and raceways. Some metal salts and polyelectrolytes have shown efficient algal floc formation, which could be separated easily either by gravity sedimentation or by applying physical techniques like filtration or low speed centrifuge (Brennan and Owende 2010). A comparative table depicting all the chemical flocculants with its harvesting efficiencies has been given in Table 1.

The inorganic metal salts like aluminum sulfate (alum) or polyaluminum or polyferric salts are very efficient in activity (Wyatt et al. 2012), but due to several

Table 1 Flocculants employed for harvesting microalgae

S. No.	Algae	Flocculant	Flocculant dose and optimum conditions	Flocculation efficiency (%)	Flocculation time	References
Natural/bio-flocculants						
1.	*Chlorella* sp. *CB4*	Cationic guar gum	40 ppm	94.5	30 min	Banerjee et al. (2013)
2.	*Chlamydomonas* sp. *CRP7*	Cationic guar gum	100 ppm	92.15	15 min	Banerjee et al. (2013)
3.	*Neochloris oleoabundans*	Chitosan	100 mg L^{-1}	95	>1 min	Beach et al. (2012)
4.	*Chlorella sorokiniana*	Chitosan	10 mg per gram algal dry weight below pH 7	99	15 min	Xu et al. (2013)
5.	*Microcystis aeruginosa*	Sepiolite modified with chitosan	0.011 g/L sepiolite with 0.001 g/L chitosan	90	10 min	Zou et al. (2006)
Inorganic flocculants						
6.	*Chlorella sorokiniana*	Ammonia	113.3 mmol L^{-1}	49.9	12 h	Chen et al. (2012)
7.	*Nannochloropsis oculata*	Ammonia	57.31 mmol L^{-1}	99	12 h	Chen et al. (2012)
8.	*Chlorella zofingiensis*	Ferric chloride	90 to 125 mg L^{-1} at pH 4.0±0.3	>90	2–3 min	Wyatt et al. 2012
9.	*Phaeodactylum tricornutum*	Polyaluminum chloride	30–70 ppm	100	30 min	Şirin et al. (2012)
10.	*Chaetoceros calcitrans*	Magnafloc®	0.1 mg L^{-1} at pH 10.3	98	–	Harith et al. (2009)
Synthetic organic flocculants						
11.	*Parachlorella*	Cationic starch	10–20 mg L^{-1}	90	30–35 min	Vandamme et al. (2010)
12.	*Scenedesmus*	Cationic starch	5–10 mg L^{-1}	80	30–35 min	Vandamme et al. (2010)
13.	*Chlorella vulgaris*	Poly (γ-glutamic acid)	22.03 mg L^{-1}	91	5 min	Zheng et al. (2012)
14.	*Chlorella protothecoides*	Poly (γ-glutamic acid)	19.82 mg L^{-1}	98	5 min	Zheng et al. (2012)
15.	*Scenedesmus* sp.	Polyamine polymer	8 mg L^{-1}	>90	5 min	Gupta et al. (2014)

disadvantages, the use of these metal salts affects the environmental quality by discharging the metal laden sludge into the open streams (Renault et al. 2009). Secondly, these inorganic flocculants are highly sensitive to pH, inactive against very small sized cells, and are only limited to few types of cultivation systems (Bratby 2016; Harith et al. 2009). Moreover, the concentrations required to flocculate algae increases on increasing the algal density, leading to a high consumption of these salts for the process (Granados et al. 2012; Şirin et al. 2012). The organic synthetic polymers like acrylamide, ethyleneimine, polyamine polymer, etc., are also known to have harvesting potential due to its low biodegradability and high consumption rate. Hence, these flocculants are also not encouraged to be used in large scale. Therefore, natural organic polymers like chitosan, guar gum, cationic starch, etc., have been reported to show a better flocculation (Beach et al. 2012; Vandamme et al. 2010; Zheng et al. 2012).

Apart from these chemical as well as bioflocculants, use of ammonia for algae flocculation has been discussed as a novel approach, which is also a low cost technology. Moreover, the ammonia concentration added to the algae suspension does not pose any harm but adds to the fertilizer value of the cultures. The algae were removed in 12 h at 99% harvesting efficiency (Chen et al. 2012).

4.2 pH

The pH induced flocculation is a highly feasible method for harvesting algae from large scale cultivation units. This method also solves the problem of getting the algae back to single cell unlike other methods, where its deflocculation is rather difficult (Knuckey et al. 2006). The increase in pH up to 9.3–11 using alkali like NaOH was able to show more than 80–90% settling of algal species like *Chaetoceros calcitrans*, *C. muelleri, Thalassiosira pseudonana, Attheya septentrionalis, Nitzschia closterium, Skeletonema* sp., *Tetraselmis suecica, Rhodomonas salina* (Knuckey et al. 2006), *Chlorella vulgaris, Scenedesmus* sp., *Chlorococcum* sp., *Nannochloropsis oculata,* and *Phaeodactylum tricornutum* (Vandamme et al. 2012; Wu et al. 2012).

The mechanism behind this pH-induced flocculation was explained by Wu et al. (2012), where the role of Mg^{2+} ions was found to be very important after ESEM-EDX analysis. According to the results, the Mg^{2+} ions in the medium tend to form magnesium hydroxide precipitate at high pH. The microalgal cells get enmeshed within the precipitate and then the algae settle by sweep flocculation forming algae-$Mg(OH)_2$ flocs. This phenomenon was further researched by Vandamme et al. (2012), in which the role of calcium along with magnesium was evidently presented. The calcium and magnesium were expected to precipitate in the form of calcium carbonate/sulfate, calcium magnesium carbonate, and magnesium hydroxide. For industrial application, Wu et al. (2012) have also suggested the use of $Ca(OH)_2$ as most feasible alkali because of its low cost and lower risk as compared to NaOH and KOH, which are highly corrosive in nature. Knuckey et al.

(2006) studied a combined flocculation method by using alkali induced flocculation followed by the addition of non-ionic polymer known as Magnafloc-LT-25 for algae harvesting.

5 Biological Methods

5.1 Biological Methods of Harvesting

Biological harvesting of microalgae means flocculating microalgae using biological agents such as bacteria, fungi, or other algal species. The flocculation is mainly mediated by extracellular polymer substances (EPS) and is often referred to as bioflocculation (Larkum et al. 2012). This process is of great interest since it is a chemical free method and flocculation occurs spontaneously with the addition of the biological agent without the input of external energy unlike physical flocculation processes. Bioflocculation has been used to harvest microalgae grown in wastewater treatment plants before (Craggs et al. 2012). However, since the process is poorly understood (Vandamme et al. 2013), it needs further research for its applicability in commercial scale. In the following sections different means of bioharvesting are discussed in detail.

5.1.1 Algal-algal Methods of Harvesting

Some naturally flocculating microalgal strains can be mixed with non-flocculating strains to induce flocculation (Vandamme et al. 2013). Flocculating microalgal species such as *Ankistrodesmus falcatus, Scenedesmus obliquus*, and *T. suecica* could be added to non-flocculating microalgal cultures like *C. vulgaris, Neochloris oleoabundans* to cause flocculation (Salim et al. 2011). In a more recent study, a harmful algal bloom forming dinoflagellate *Heterocapsa circularisquama* was used to harvest three microalgal species, namely *C. vulgaris, Nannochloropsis granulata,* and *Dunaliella salina* (Cho et al. 2016). The main advantage of using algal assisted algal harvesting apart from energy considerations is the ease of cultivation of flocculating and non-flocculating algae together. However, the main problem with such a method is that the flocculating algae start dominating over the non-flocculating algae which may cause a problem of algal blooms (Prajapati et al. 2013b). Extracellular polysaccharides have been mainly attributed to be the flocculating factor. Extracellular extract from an auto flocculating microalgal species *C. vulgaris* JSC-7 was able to flocculate *C. vulgaris* CNW11 and *S. obliquus FSP (*Alam et al. 2014). Similarly, in another study, extracellular biopolymers from the self-flocculating microalga *S. obliquus* AS-6-1 were able to flocculate *C. vulgaris* (Guo et al. 2013). In both the studies, the extracellular biopolymer mainly contained glucose, mannose, galactose, rhamnose, and fructose.

5.1.2 Algal-bacterial Methods of Harvesting

Paenibacillus sp. AM49 has been used to efficiently harvest *C. vulgaris* (Oh et al. 2001). Rodolfi et al. (2003) had observed that a high density culture of *Nannochloropsis* flocculated due to the presence of bacteria. In wastewater treatment plants, bacteria has been used to form algal-bacterial flocs ranging in size from 400 to 800 μm which could be then easily separated by gravity settling (Gutzeit et al. 2005). *Pleurochrysis carterae* was flocculated by a mixed bacterial culture grown in tap water media (Lee et al. 2009). *Microcystis aeruginosa* which forms algal blooms in water aggregates in the presence of heterotrophic bacterial community (Shen et al. 2011). Recently, a potent flocculant from *Solibacillus silvestris* W01 has been isolated which has been used to harvest *Nannochloropsis oceanica* (Wan et al. 2013).

5.1.3 Algal-Fungal Methods of Harvesting

Pelletization is a very common phenomena observed during the growth of certain fungi under continuous shaking or aeration (Kaushik and Malik 2013; Mishra and Malik 2014). Hence, the ability of pellet forming filamentous fungi (Fig. 2). However, the studies targeting fungal assisted bioflocculation of algae are scarce in the literature. Few recent attempts have been made in this direction (Xie et al. 2013). These studies indicate that algal-fungal growth (during co-cultivation) and pelletization are highly sensitive to culture conditions (autotrophic/heterotrophic) and the mechanism of the same is not very clear (Zhang and Hu 2012). Further, it requires high inputs of glucose to support fungal growth and the process time required to achieve significant harvesting is relatively longer (minimum 48 h). pH has been reported to play very important role in fungal pelletization. Higher fungal spore

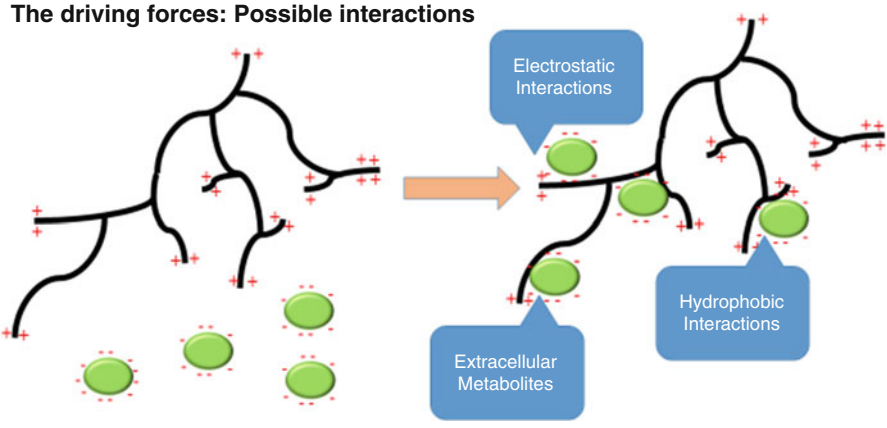

Fig. 2 Possible mechanism of algal-fungal interactions

inoculum and glucose inputs are supposed to shift the pH towards acidic resulting in efficient algal-fungal harvesting (Zhou et al. 2012). However, algal growth under autotrophic conditions usually results in strongly alkaline pH. Hence, to facilitate commercial applications, there is a need to devise a harvesting process that works at alkaline pH with minimum glucose inputs and faster harvesting kinetics. Recently Prajapati et al. (2014b) were able to cultivate *Aspergillus lentulus* in spent BG-11 media and achieved 100% harvesting with *Chroococcus* sp. 1.

5.1.4 Algal Harvesting Using Different Biological Agents

Apart from micro-organisms, seeds from plant *Moringa oleifera* have been used to harvest *C. vulgaris* cells (Teixeira et al. 2012). Biologically occurring polymer chitosan can also be used to harvest microalgal cells (Beach et al. 2012). Algal predators like the protozoan *Tetrahymena* have been used to rapidly flocculated microalgal cells (Jakob et al. 2016). Other than the conventional methods, algae have been genetically modified to increase its flocculation ability (Georgianna and Mayfield 2012).

6 Suitability of Harvested Biomass for Biofuel Application

The advancements made in the existing techniques are oriented towards obtaining the most suitable biomass for biofuel production. Few physical techniques like magnetic separation show high recovery efficiencies, but the separation of biomass from magnetic particles is rather difficult, making it unsuitable for biofuel routes (Hu et al. 2013). On the contrary, in ozone dispersion flotation technique, the exposure of ozone pretreats (pre-oxidizes) the algal biomass. This pretreatment helps in the lysis of the cells and lipid exposure to the surface. As a result, a better biofuel yield is expected by this process (Nguyen et al. 2013). Use of flocculants and alkali for harvesting has to be reversed or neutralized prior to take the biomass for fuel production (Knuckey et al. 2006; Şirin et al. 2012). Biologically harvested microalgal biomass can be used for biofuel production (biogas or biofuel). Al-Hothaly et al. (2015) harvested 500 L. of microalgal biomass of *B. braunii* using *Aspergillus fumigatus* and the resulted biomass was used for bio-diesel production by hydropyrolysis. Microalgal cells were co-cultivated and harvested with *A. fumigatus* and there was an increase in lipid content in the harvested biomass (Wrede et al. 2014). Also, unlike mechanical or other physical processes, there is minimal damage on the harvested biomass. When algal cultures were used to treat municipal wastewater, the bacterial culture present in the wastewater flocculated the microalgal cells with an increase in lipid content of the algal-bacterial flocs (Mahapatra et al. 2014). Hence, it can be seen that biological harvesting causes value addition of the microalgal biomass.

7 Techno-economic Feasibility

Techno-economics for biofuel production is the most important criteria to assess its feasibility at commercial scale. Using microalgae as biofuel feedstock has not been very successful till now for multiple reasons, one of which is large capital investment in harvesting. Many of the harvesting methods, as discussed in previous sections, show efficient harvesting with high rates of biomass recovery. However, due to high energy consumption and expensive consumables, these methods have not been validated at large scale. A comparison of various techniques along with their harvesting efficiencies, energy consumption, and cost has been tabulated in Table 2.

Among the physical techniques, centrifugation is the most efficient process with complete biomass recovery, but energy consumption up to 5 kW h/m^3 does not permit its practical application. Slight modifications in the technique by Dassey and Theegala (2013) and Bilad et al. (2012) have reduced the energy consumption by 40–82%. On the other hand, researchers have not done the techno-economic evaluation of magnetic separation, but a large amount of energy is consumed in the preparation of magnetic particles and flocculation–deflocculation process.

For chemical methods, the major capital investment is the cost of production and processing of flocculants. Due to environmental disadvantages of metal salts, bioflocculants have shown an ecofriendly approach for algal harvesting. Biological methods though are environmental friendly, the process is poorly understood which

Table 2 Techno-economic comparison of physical and chemical method for harvesting microalgal biomass

S. No.	Method of harvesting	Harvesting efficiency (%)	Energy investment	Cost	References
Physical methods					
1.	Centrifugation	94	0.80 kW h/m^3	$0.864/L oil	Dassey and Theegala (2013)
2.	Filtration (PVDF membrane)	98–99	0.91 kW h/m^3	ND	Bilad et al. (2012)
3.	Filtration + Centrifuge	80	0.169 kW h/kg DW	ND	Baerdemaeker et al. (2013)
4.	Ultrasound	93	4 W	ND	Bosma et al. (2003)
5.	Flotation	49.5	0.16–0.44 kW h/kg DW	0.02–0.4 € kg^{-1} DW	Barrut et al. (2013)
Chemical methods					
6.	Chitosan	92	ND	2–100 €/kg	Şirin et al. (2012)
7.	Metal salts	66–82	ND	0.4–2.1 $/kg	
8.	pH	90	ND	$US 0.13/kg	Wu et al. (2012)

ND Not determined

a major hindrance to its use is in commercial scale. Also, the use of different microorganisms for harvesting may contaminate the algae on prolonged usage. It has been observed that the biologically harvested biomass is richer in lipids making it suitable for the biofuel production; however, the studies are mainly on laboratory scale. Commercial companies like Sapphire Energy Inc., Algenol Biotech LLC, Genifuel/Reliance, Muradel Pty cultivate algae in large scale in open pond raceways or PBRs to generate biofuel. However, the method employed for algal harvesting is not well discussed and is the trade secret for each of these companies (Elliott 2016). There is a need to bridge this industry-academia divide in order to evolve field worthy harvesting techniques.

8 Conclusion

Harvesting of microalgal biomass is a critical step for biofuel production which constitutes more than 80% of the biofuel production cost. Various methods like physical, chemical, or biological methods have been employed for harvesting microalgal biomass. Although physical processes are rapid, they are energy intensive and hence significantly add up to the biofuel production cost. Chemical methods though less expensive than the physical ones contaminate the microalgal biomass with undesirable chemicals. Biological processes are poorly understood and are not yet applied in commercial scale. However, the biologically harvested biomass is more suitable for biofuel production since it increases the lipid content. More efforts need to be focused evaluating the mechanism, scale ups, and critical energy/economic demands of biological harvesting.

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Key Issues in Pilot Scale Production, Harvesting and Processing of Algal Biomass for Biofuels

Amritanshu Shriwastav and Sanjay Kumar Gupta

Contents

1 Introduction

It has now become a well-established fact that global dependence on natural petroleum reserves must decline and alternate energy sources must be identified and migrated to. This is due to two critical factors: (a) the remaining petroleum

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reserves are continuously getting depleted and, with current rate of extraction and use, may hardly sustain global energy demands for ~50–60 more years, and (b) the associated carbon emissions with their extraction and application contribute heavily towards the global warming. Lot of efforts are already in place to identify feasible alternate and renewable energy sources and include focus on solar cell, wind energy, geothermal and biofuels among others. Out of these, biofuels have garnered increasing attention as a direct replacement of petroleum products with the use of existing technology.

1.1 Biofuels

Biofuel or more specifically biodiesel is currently derived from organic biomass including plants and animal oils. Short-chain alcohol triglycerides or free fatty acids are transesterified to produce monoalkyl esters or biodiesel (Du et al. 2008). Common sources to produce biodiesel include soybeans, jatropha oil, waste cooking oil, animal fat, palm oil, corn oil and canola oil among others (Chisti 2007). Biodiesel have been observed to be less toxic, contribute less gaseous pollutants and effectively contain no CO_2 or sulphur in comparison with petro-diesel (Rawat et al. 2013). These factors significantly contribute towards acceptance of biodiesel as an alternative for conventional petro-diesel. Another important aspect is their suitability to be used with existing engines without (or minor) modification and ability to fit into existing infrastructure of distribution (Du et al. 2008). However, a major limitation in the commercial applicability of these biodiesels has been their low yield and unrealistically high arable land requirement (even for high yield palm oil) for meeting the demand for transport fuel (Chisti 2007).

1.2 Suitability of Algal Biofuels for Commercial Applications

The third-generation biodiesel derived from algal biomass, in general, addresses the limitations of other biodiesels. The high lipid content and biomass productivity essentially translate to reduced and achievable requirements of non-arable land and feasible commercial applicability. Hence, biodiesel from microalgae is increasingly being perceived as the only viable alternative of petro-diesel (Chisti 2007). In addition to biodiesel, other biofuels can also be generated from microalgal biomass, e.g. hydrogen, methane, etc. (Sivakumar et al. 2012). Commercial suitability of algal biofuels is due to the following factors (Brennan and Owende 2010):

1. The production of algal biomass with high lipid contents can be continued throughout the year on non-arable lands, thus avoiding the debate of food vs fuel.

2. Though the microalgae are aquatic species, the overall water requirement is very less in comparison to crop-based biofuel production and thus substantially lowers the dependence on freshwater resources.
3. Photosynthesis during autotrophic growth of algae is capable of biofixing the atmospheric CO_2.
4. The biomass growth can effectively be obtained using nutrients present in wastewater, thus also acting as a means of tertiary treatment.
5. In addition to biofuels, algal biomass is also a source of other useful products, viz. protein, pigments and many compounds of pharmaceutical nature, etc.
6. The chemical and physical properties of algae-derived biodiesel, viz. viscosity, density, flash point, heating value, solidifying point, cold filter plugging point, etc., have been found to be similar to those of petro-diesel (Xu et al. 2006). Most of these parameters for algal biodiesel also meet the international limits of biodiesel for automotive sector (Ahmad et al. 2011).

1.3 Techno-Economic Concerns with Algal Biofuels

Despite being perceived as the possible alternative for petroleum fuels, algal biofuels (and algal biodiesels) have yet to achieve techno-economic sustainability. The algal biomass production for primary objective of algal biofuel is at present economically unsustainable (Lundquist et al. 2010). Two approaches have been advocated by researchers for achieving the favourable economics: (a) to utilize the algal biomass as a byproduct for producing algal biofuel with a primary objective of wastewater treatment (e.g. nutrient removal) (Lundquist et al. 2010) and (b) adopting biorefinery concept in which a range of useful products (viz. protein, carbohydrate, pigments, etc.) are extracted with lipids from produced biomass in order to maximize the value recovery (Subhadra and Grinson 2011). In addition, the biomass production at pilot scale still faces many technical issues before the lipid production could be optimized. Such issues remain at three distinct stages of biomass production, harvesting, and processing for lipid and biofuel production and are discussed in detail in subsequent sections of this chapter.

1.4 Environmental Concern with Algal Biofuels

The rapid exploitation of natural reserves of fossil fuels and environmental concerns with the burning of fossil fuels lead the path of the development of carbon neutral biofuels. However, the environmental sustainability aspects of renewable biofuels are of prime concerns. The first-generation biofuels which were obtained from the energy crops and oilseed were phased out due the debate over food vs fuels. The second-generation biofuels were based on biomass. However, the use of the water and arable land resources for the production of biomass was again of the

major environmental concerns. The production of lignocellulosic biomass requires substantial amount of water resources and arable lands. In the past few decades, numerous studies evaluated the extensive requirement of water for second-generation biofuels (Varis 2007; Hoekstra and Chapagain 2008; De Fraiture et al. 2008). Therefore, the production of such biofuels was much needed which should be free from environmental concerns. In this regard, the microalgae seem to be the potential contender for the production of third-generation biofuels. The microalgal biomass has been recognized as a versatile feedstock for biofuel purposes which fulfil all the basic prerequisites of environmental sustainability. Numerous studies have demonstrated the excellent use of microalgal biomass for the production of various types of algal biofuels at small scales. However, the pilot scale production of liquid algal biofuels such as algal biodiesel, ethanol, etc. as well as the gaseous fuels such as algal biohydrogen or biogas is still in infancy stages. The major environmental and economic concerns are the high production cost and high energy demands in the production as well as conversion of the algal biomass to biofuels. The energy demands for the harvesting and dewatering of microalgal biomass and oil extraction from algal biomass through the conventional processes as well as its conversion require substantial energy which is carbon intensive.

The carbon and water footprints are generally used to major the environmental sustainability of various commodities including biofuels. The major concern with the production of biofuels is the water footprint, as huge amount of water is required in production of both the algal and lignocellulosic biomass. As per estimates, almost 86 % of the global water is used in the agriculture alone (Singh et al. 2015). Therefore, the additional demand of water, which is already a scarce resource, for the production of algal biofuels may worsen the scenario. Numerous studies have shown environmental concerns due to extensive water used for biomass energy production. Berndes (2002) estimated almost double water loss in large-scale biofuel production through the evapotranspiration by 2100. Gerbens-Leenes et al. in 2012 reported 70–700 times larger water footprint for the biomass-based biofuels compared to the fossil fuels. Approximately tenfold increase in the water footprint is expected only for the biofuels used in transport in ten largest biofuel-consuming countries. Therefore, the requirement of the huge water could be a limiting factor for algal biofuels. The water and land use patterns, type of the feedstock, its productivity, climatic condition, geographical locations, etc. also regulates the carbon and water footprint for any biofuels (De Fraiture and Berndes 2009).

Similar to the water footprints, Johnson and Tschudi in 2012 recommended the measurement of CF of the biofuels. The carbon footprint of the biofuels were approximately 0.248 billion global hectares in 2010, and as per estimates, it will be increased by twofold by 2020. Fahd et al. (2012) reported that in 1 g of algal biodiesel production, around 1.72 g CO_2 is emitted which is substantially high compared to the biofuel production from the oilseeds. The environmental sustainability of algal biofuel depends on several other factors as well. However, the water and carbon footprint can be reduced by applying advanced tools and techniques. Substantial reduction in WF and CF is possible by use of flue gases as well as use of

wastewater for culturing and production of algal biomass, selection of high oil-yielding algal species, etc. Similarly, the energy production from algal biomass in a biorefinery concept is one of the best environmentally sustainable option.

2 Issues with Algal Biomass Production

The commercial production of lipid-rich algal biomass is a complex process depending on many factors. The key concerns during the pilot scale biomass production are summarized in this section.

2.1 Selection of Suitable Algal Strain

Griffiths and Harrison (2009) summarized some suitable characteristics of algal strain for mass production as listed in Table 1.

One of the primary aspects for successful pilot scale algal production is the selection of suitable strain which can (a) have higher lipid productivity and (b) counter the culture contamination and environmental variability invariably occurring in such application. Bioprospecting for suitable cultures having these characteristics is one of the most important and challenging issue for pilot scale algae production (Mutanda et al. 2011). Indigenous cultures from native locations are considered better for such large-scale production since these are already adapted to the environmental conditions. Further investigations in selecting species having higher lipid productivity depend on both the biomass productivity and lipid content (Griffiths and Harrison 2009). These two objectives are often counteracting to each other, since conditions for higher biomass productivity are

Table 1 Suitable traits in algae for mass production (Griffiths and Harrison 2009)

Characteristic	Benefit
High growth rate	• Less area requirement • Outcompeting the contaminants
High lipid content	High value product suitable for further processing
Growth in extreme condition	Reduced probability of competition and contaminants
Large cell size, filamentous or colony formation	Easy harvesting at lower cost
High tolerance to variation in environmental conditions	Reduced effort on maintaining growth conditions
Tolerance to various contaminants (viz. NO_x, SO_x, etc.)	Applicability to contaminated wastewater

known to suppress the lipid content and vice versa. Hence, selection of culture with high lipid productivity becomes challenging in nature. Another factor that controls the selection of the strain is suitability of produced lipids for further processing (Mutanda et al. 2011).

The ability of algae to sustain the environmental stresses and variability becomes important during their large-scale production. Also, the possibilities of establishing symbiosis with existing bacteria increase the chances of successful application of these algal strains. In addition, the ability to grow in conditions hostile to other contaminants may help in identifying suitable operating conditions and hence tackling the issue of contamination in these systems.

2.2 Growth Media and Reactors

Once the proper algae culture is selected, the growth media for such large-scale production becomes important. Though artificial growth media have been utilized for low-scale biomass production, any effort for pilot scale production can only be sustainable with wastewater rich in nutrients, i.e. domestic sewage. However, utilization of wastewater presents some unique challenges during the biomass production. First are the variability of nutrient levels and the ability of selected algae to cope with it without any negative impacts (Shriwastav et al. 2014). Second, the presence of high organic substrates in this sewage may either pose additional stress to algae culture (Gupta et al. 2016) or promote bacterial growth. Both of these impacts require careful investigation for their ultimate impact on quantity and quality of the produced lipids.

Another important factor remains the mode of biomass growth, i.e. open systems or photobioreactors. Historically, open systems such as aerobic oxidation ponds have been used for algal growth. However, inefficient mixing and light distribution results in suboptimal growth and hence lipid productivity (Arceivala and Asolekar 2007). The development of high-rate algal ponds (HRAP) with paddles resulted in better growth conditions for microalgae. These systems (HRAPs) are widely utilized for large-scale biomass production. However, dependence of growth on direct sunlight and prevalent environmental conditions (e.g. temperature) result in variable growth. Also, these open systems are prone to contamination and hence require careful operation. To address these issues of open systems, closed systems as photobioreactors have also been investigated. However, only tubular photobioreactors are deemed suitable for large-scale biomass production (Chisti 2007). Although costlier than open systems, these photobioreactors provide higher biomass productivity and fewer contamination issues. The choice of open or closed systems depends on multiple factors and requires careful selection.

2.3 Effects of External Factors

Major concern during pilot scale production of algal biomass is the effect of various external factors. The variable nature of sunlight and the intensity critically govern the growth characteristics in open systems, and because of inefficient light utilization, they have low productivity than closed systems. The application of artificial illumination is yet to be investigated at such large-scale production, though it has been deemed suitable at low scale. Also, the temperature affects the growth of algal culture. Hence, selecting proper strain having optimal temperature within the prevailing conditions would invariably result in better performance. In addition, evaporative losses with open system are directly dependent on temperature. The issue of contamination by protozoa and other algae in open systems is a serious concern which could eventually lead to system failure. This can be dealt with by providing highly selective growth conditions at additional cost. However, this strategy limits the choice of suitable algal strain considerably (Rawat et al. 2013).

3 Issues with Biomass Harvesting

The harvesting of produced algal biomass proves to be a highly complex process due to many inherent factors and may account for significant fraction of the total production cost (Chisti, 2007).

3.1 Key Parameters and Available Technology

Major difficulties in devising efficient harvesting processes arise from low culture density in open systems, and small cell size (Li et al. 2008). Since closed systems such as tubular photobioreactors are more efficient in biomass production, cell density may be up to 30 times higher in them than open systems. This leads to easier harvesting (Chisti 2007).

Over the years, different technologies have been applied for biomass harvesting. These include flocculation, flotation, sedimentation (gravity or centrifugal) and filtration among others. Detailed description and critical comparison of these processes are available (Chen et al. 2011; Rawat et al. 2013). Table 2 lists few of their traits. Since these processes are dependent on different characteristics of algal culture, a proper selection of algal strain and a suitable harvesting process are critical (Rawat et al. 2013).

In general, harvesting is a two-stage process. Bulk harvesting is the first step towards efficient biomass recovery, where 2–7% solid concentrations are achieved from the bulk broth depending on the initial levels and the process adopted. Then

Table 2 Some aspects of the harvesting processes in practice (Rawat et al. 2013)

Process	Advantage	Disadvantage
Filtration	Low cost	Slow, membrane fouling, cell damage
Centrifugation	Fast, efficient	Highly energy intensive
Gravity sedimentation	Low cost, low energy requirement	Slow, applicable only with high cell density
Chemical flocculation	Low cost, low cell damage	Risk of degrading the biomass quality and yield
Dissolved air floatation	Low cost, easy upgrade to pilot scale	Energy intensive, may degrade product quality
Bio-flocculation	High efficiency	High energy requirement than other flocculants
Electrolytic flocculation	High efficiency	High energy requirement, electrode fouling, high system temperature
Submerged membrane filtration	Low cost, less shear to the cells	Membrane fouling

this slurry is further thickened using more energy-intensive processes, viz. centrifugation and filtration for use during downstream processing (Brennan and Owende 2010).

Not all methods are efficient for biomass harvesting, and their application is governed by many other factors such as cost and energy requirement. For example, gravity sedimentation is only efficient for harvesting algae which inherently have good settling characteristics. However, this provides with one of the cheapest and very low energy-intensive techniques available. Hence, processes such as these are suitable with biomass production in HRAPs, where algae with better settling are favoured. Similarly an informed decision for a suitable method should be based on relative urgency between efficiency, cost and input energy in each case.

3.2 Major Precautions and Concerns

Since harvesting is dominantly a physical process, concerns of damage to biomass and the lipid quality are very real. Addition of chemical agents for flocculation may have an impact on the acceptability of harvested biomass for downstream processing and hence requires serious care during their selection. One of the major factors during various harvesting processes is the requirement of skilled or semi-skilled personnel to sustain the operation. Also, desired solid concentrations in the thickened slurry govern the selection and overall cost of harvesting and hence must be optimized. Main precautions and concerns for selecting and implementing a harvesting process are listed below:

- Suitability of the harvesting process with the algal strain is important for optimal recovery. However, maintaining a single species (of desired qualities) in these large-scale systems (especially open systems) is very difficult, and more often

than not, a population of diverse species is obtained whose dominance is governed by prevailing conditions. Hence, the selection of a harvesting protocol should also account for changing dynamics of these species during the operation.
- Though many of the harvesting processes can achieve very high recovery efficiencies (viz. microfiltration and centrifugation), their applicability is finally governed by the economics of the whole process. A proper analysis between recovery efficiency and the operation cost should help in selecting suitable processes.
- Also, as discussed earlier, the impact of harvesting on the quality of biomass and lipids decides acceptance of that process. Further investigations are necessary if other products are to be extracted in addition to lipids.

4 Issues with Processing of Algal Biomass

Once the algal biomass is produced and recovered, it requires further processing for lipid extraction that is finally utilized for biodiesel production. The processing of algal biomass for these objectives is highly complex and poses some serious concerns for product quality. In addition to the quality of extracted lipids, the overall extraction cost also plays an important role for feasibility of this approach.

4.1 Suitability of Harvested Algae and the Extraction Process

As the thick biomass slurry is obtained, various downstream processes are utilized for converting it to biofuels. However, the nature of the biofuel (lipids, biogas, etc.) inherently depends on the biomass quality after harvesting. Although, algae cultures are selected for production based on their suitable lipid profile and productivity, the mechanical and/or chemical processing during various upstream processes may either denature the accumulated lipids within the cells or altogether destroy cells leading to lipid loss. Both of these cases render the recovered biomass unsuitable for downstream extraction.

Once thickened slurry of suitable biomass is recovered, further drying is often practised to increase its viability. Many ways to achieve this have been investigated and include sun drying, low-pressure shelf drying, spray drying, drum drying, freeze drying and fluidized bed drying among others (Brennan and Owende 2010). Many of these are highly energy intensive in nature and considerably add to the cost of production. Hence, recent focus is on developing processes, where wet algal biomass can directly be utilized for lipid extraction (Patil et al. 2011; Sathish and Sims 2012).

Further, cell disruption is needed before lipids can be extracted using solvents. This is achieved by processes, viz. microwave digestion, ultrasonication, autoclaving or osmotic shock among others (Ansari et al. 2015). However, the efficiency

and cost of individual process vary. The liberated lipids are then extracted using solvents such as mixture of methanol and chloroform. The choice of solvents also plays an important role for acceptability of the lipid as well as reuse of remaining biomass.

4.2 End Use of the Biomass: Single or Multiple Product Extraction

The final justification of using microalgae for biofuel can only be achieved with favourable economics. Conventional approach has been to extract the lipids only. This simplifies the operational details considerably, and process optimization is needed only for lipids. Use of the residual biomass for animal feed may be undertaken depending on the residual toxicity of extracting solvents. However, such an approach has not been able to achieve favourable economics so far. Hence, recent investigations are directed towards extracting plethora of useful compounds from algal biomass in addition to lipids and thus maximize the benefit. This approach is fairly reasonable since algae host many valuable compounds, viz. proteins, carbohydrates, pigments, medicinal compounds, etc. (Pulz and Gross 2004). Efforts towards this follow the algal biorefinery approach, where cumulative value extraction is optimized (Subhadra 2010). However, simultaneous extraction of multiple compounds poses unique challenges. The effect of extraction of one compound on the yield and quality of other metabolites needs careful investigation. This requires proper selection of extraction processes for all targeted compounds as well as their sequence of extraction (Ansari et al. 2015). Hence, the current focus of the community is towards developing greener extraction protocols for target compounds with minimal effect on yield and quality of other products.

5 Summary and Conclusions

The importance of algal biofuels as a feasible alternative of petroleum products has resulted in ever-increasing attention towards developing technologies for their large-scale production. However, such an undertaking is highly complex and faces multiple issues during each intermediate steps. These issues are discussed in detail in this chapter and are also summarized in Fig. 1. Achieving sustainability in this requires tremendous efforts. Though the large-scale production for algal biofuel is technically feasible, it is yet to achieve the economic sustainability. The efforts to achieve are focussed on two distinct themes, (a) to develop better processes at each step to maximize the biomass productivity and minimize the operational cost and (b) to maximize the value extraction once the biomass is harvested by developing more efficient and greener technologies for each

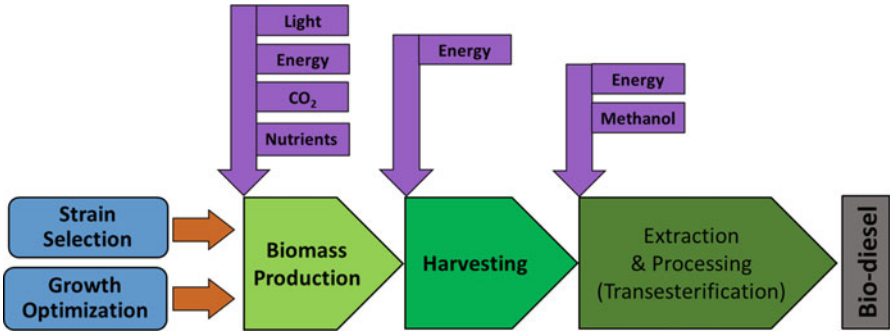

Fig. 1 Summary of the key processes and involved issues during algal biodiesel production, modified from Scott et al. (2010)

compound with algal biorefinery approach. This again highlights the importance of further research towards achieving the overall objective of feasible and commercially viable production of algal biofuels.

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Algal Biomass Pretreatment for Improved Biofuel Production

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1 Introduction

Algal biomass contributes significantly in the biomass based renewable energy generation. Algae are photosynthetic aquatic microorganism, which utilize CO_2 for synthesis of biomass and other metabolites. Algae have ability to utilize nutrients from range of wastewaters and CO_2 from the various gaseous streams including industrial flue gas (Prajapati et al. 2013). Simultaneous biomass production and wastewater treatment further improve the potential of algal biomass as feedstock for biofuel production (Chinnasamy et al. 2010; Choudhary et al. 2016; Prajapati et al. 2016). Major biofuels produced using algal biomass include: biodiesel, bioethanol, biooil, biohydrogen and methane (Prajapati and Malik 2015). However, irrespective of the biofuel production route, the recalcitrant nature of the algal cell wall is the major hurdle. The recalcitrant nature of the algae is due to the presence of complex biopolymers such as microfibrillar polysaccharides, matrix polysaccharides and proteoglycans. Hence, pretreatment of the algal biomass usually becomes necessary to improve the biofuel extraction. Pretreatment of algal cells deals with extraction process and yield of biomass/biofuel. This phase includes the cell wall disruption mediated by physical (mechanical), chemical and enzymatic methods.

In the light of the above discussion, the present chapter is focused upon various aspects of algal cell wall and its role in decreasing the biofuel yield. Subsequently, various pretreatment technologies utilized for improving the biofuel yield are described. Some of the pretreatment methods such as enzymatic lysis, homogenization, milling, ultrasonication, osmotic pressure shock and pretreatment of algal cells mediated solvent extraction method are presented in this chapter. Moreover, the quantification of the pretreatment effects is crucial in order to identify the best possible pretreatment methods and also for the optimization of a particular method. Hence, qualitative and quantitative analysis of algal biomass pretreatment is also included. Finally, some recent report on algal biomass pretreatment resulting in improved biofuel yield is discussed to cover the current and future prospects of the algal based biofuels.

2 The Algal Cell Wall

The cell walls of algae are composed of different fibrillar, matrix and crystalline polymers, proteins and other compounds. The carbohydrate fraction of cell wall primarily consists of cellulose, hemicellulose and glycoproteins along with limited quantity of rhamnose, fucose, mannose and glucose. The generalized structure of algal cell wall consists of the plasma membrane over which a cell wall consisting of celluloses and glycans is present. However, in some algae an additional ‘encrusting layer’ is present which constitutes of significant amount of silica, calcium carbonate or a resistant biopolymer called as ‘algaenan’. The protein content of the algal cell

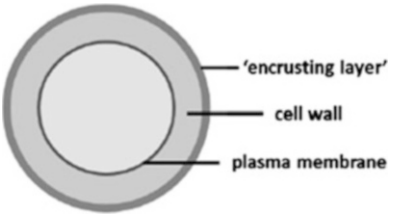

Fig. 1 Generalized trilaminar structure of algal cell wall

wall is also significantly higher. The complex amino acid and carbohydrate patterns of the algal cell wall determine its shape and integrity and exhibit functions such as signaling, defense, cell recognition, expansion and differentiation.

Microalgal cell wall has the ability to alter their cell wall composition with environmental condition (Fig. 1). This property is more prevalent in symbiotic algae which make it difficult to study their cell wall. Free-living algal cells have significantly thicker cell walls, increased storage reserves and modified chloroplast structure as compared to symbiotic algae (Sanders et al. 2005).

Fluorescent stains and dyes have been helpful in distinguishing different algal groups wherein the sugar composition of cell wall has been used as a means of algal classification. However, many algal groups (e.g. *Chlorella* spp., *Scenedesmus* spp., *Haematococcus* spp.) respond unusually to stains and dyes that make the study of cell wall profiling difficult. The inability of fluorescent dyes and stains to penetrate the microalgal cell wall resulting in unusual staining is due to the characteristic cell wall structure and composition of algae. The action of fluorescein diacetate (FDA), a vital stain, was reversed in algal cells wherein >90% of the dead cells were stained and the viable cells were impenetrable by FDA stain. Another dye, propidium iodide which is an indicator of cell death is reported to give the same red fluorescence in dead cells as is emitted by the autofluorescing healthy cells of algae rendering it to be non-functional as well. The culture environment also affects algal cell wall structure and cellular metabolism and hence affects the staining of many microalgae. Cell wall staining in *Coelastrum cambricum* with calcofluor-white was dependant on cell age whereas in *Scenedesmus acuminatus, Synechocystis* sp. and *Anabaena* sp. it was non-functional with the exception to broken, dividing or dead cells. Many other stains recommended for algal use have also been reported to produce unexpected results (Markelova et al. 2000). Nile blue oxazone, a lipophilic stain, was also rendered problematic for staining non-polar lipids due to autofluorescence.

Algal cell wall preparation has been obtained using a 'cocktail' of enzymes for studying its characteristic features. Enzymes such as cellulase, macerozyme, chitosanase, pectinase, xylanase, achromopeptidase and naturally occurring enzyme mixtures have been used along with surfactants to rupture algal cell wall. However, it has been observed that different algal strains (e.g. *Chlorella vulgaris*) have the ability to regenerate their cell walls following complete enzymatic cell wall degradation to protoplast (Honjoh et al. 2003). The problems associated with enzymes for cell wall degradation are that they exhibit strong strain and growth

stage specificity particularly when a weak inner layer sensitive to cellulose is layered with an outer layer sensitive to pectinase (Jaenicke et al. 1987).

Culture environment also plays its role in enzymatic cell wall degradation as an increase in pH exhibits cell aggregation and also restricts the release of autospores. These effects are observed as the changes in cell wall metabolism result in stretching of cell wall rather than its rupture (Malis-Arad and McGowan 1982). Certain metabolites and biopolymer are synthesized in microalgal cells upon exposure to varying environmental conditions. Mycosporine-like amino acids (MAAs) are secreted and gradually released to the extracellular sheath whereas internal carotenoids are produced under radiation induced stress. In many of the microalgae cells it has been linked to the synthesis of metabolites forming a strong biopolymer in the outer walls which is also known as algaenan. It prevents the permeation of the cell wall in many common algal strains (Pattanaik et al. 2008).

Resistant biopolymers are synthesized throughout the cell cycle in many algal cells and were previously mistaken as sporopollenin. This strong biopolymer is now known as alagenan. Algaenan, an Acetolysis Resistant Biopolymer (ARB), is a polyhydrocarbon with characteristic features analogues to sporopollenin such as hydrophobicity, tolerance to high temperature, resistance to chemical treatments such as detergents, orthophosphoric acid, alkali hydrolysis and acetolysis, resistance against biological agents, high temperature and insolubility to polar and non-polar solvents (Zych et al. 2009). Algaenan exhibits a strong correlation with the ultra-structure of the algal cell wall which is described as a 'Trilaminar Structure (TLS)' (Burczyk et al. 1999). However, the information on structure of algaenan is limited yet it is believed to be similar in chemical composition to suberin, cutin and sporopollenins (Kontkanen et al. 2009). Algal cells containing algaenan exhibit greater physical strength along with high chemical and microbial resistance (Allard et al. 2002; Cooney et al. 2009). However, it is not ecologically widespread with predominance in green algae while it is mostly absent in marine microalgae (Kodner et al. 2009). The true structure and anabolic pathways leading to the synthesis of resistant biopolymers in plants and algae haven't been defined properly (Dobritsa et al. 2009). Structure of algaenan exhibits strong correlation with the plant cuticular membranes (Allard and Templier 2001).

A comprehensive structure of algaenan has been presented by Allard et al. (2002). Algal cells comprising of algaenan mostly constitute of linear polyesters in the outer cell walls that are rich in long chain fatty acids, dicarboxylic acids and alcohols. Limited understanding of chemical structure of algal cells and the linkages present (chemical degradation and low yields of algaenan during extraction) reveals the lack of ether groups, and presence of aldehyde and ester linkages (Salmon et al. 2009) (Fig. 2). The macromolecular structure of algaenan provides protection to algal cells from enzymatic and chemical attack. Lipid pathways, e.g. oleic acid biosynthesis, has been linked to algaenan biosynthesis and is found to be inhibited by metazachlor (Couderchet et al. 1996; Vandenbroucke and Largeau 2007). Therefore the macromolecular structure of algaenan varies with different algal strains. Sugar content of cell wall in algae increases with growth yet the content of algaenan remains unchanged. However, the characteristic trilaminar

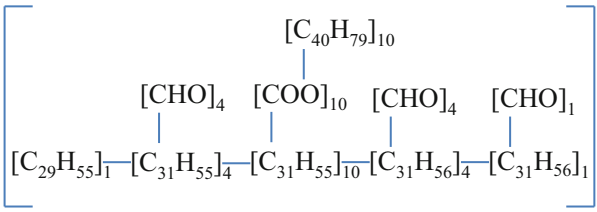

Fig. 2 Macrostructure of algaenan

structure is lost due to metabolic disorders leading to biosynthesis of unusual sugars (Burczyk et al. 1995).

3 Effect of Algal Cell Wall on Biofuel Production

Algal cell wall poses a noteworthy barrier to lipid and other metabolite extraction from most algal species (Razon and Tan 2011). Developments in the field of algal biofuels have largely been restricted due to the rigid cell wall structure. The major contributors to the resistive nature of the algal cell wall are presence of algaenan and other complex carbohydrates. The carbohydrate polymers in the algal cell wall are mainly composed of cellulose, xylose, glucose fructose and mannose. Algal biomass has been vastly studied and projected as the most viable alternative against fossil fuels. However, for this to succeed, significant advancement in genetic manipulation for optimum production and processing of biomass, lipid extraction and downstream processing is essentially required. To this, biofuel production is restricted owing to the complex cell wall characteristics of microalgae. Algal cell wall restricts biofuel production as the elevated content of cell wall polysaccharide in algal cells significantly complicates the process for extraction of lipids and algal metabolites. The complex nature of cell wall interferes with the pretreatment of algal biomass towards improving biofuel yield. In pretreatment step, the algal cell wall is disrupted to increase the availability of bioactive compounds to be converted to biofuels (Harun et al. 2014; Huang et al. 2014). Thus efficient pretreatment is highly desirable for increasing biofuel yield which is adversely affected by the rigid algal cell wall (Wiltshire et al. 2000).

4 Pretreatment Methods for Algal Biomass

4.1 Physical Methods

Physical pretreatment of algal cells is usually performed to disrupt the cell wall and to improve the effectiveness of the lipid extraction process by increasing the contact of solvent with the intracellular lipid content. Dismantling of the algal cell wall

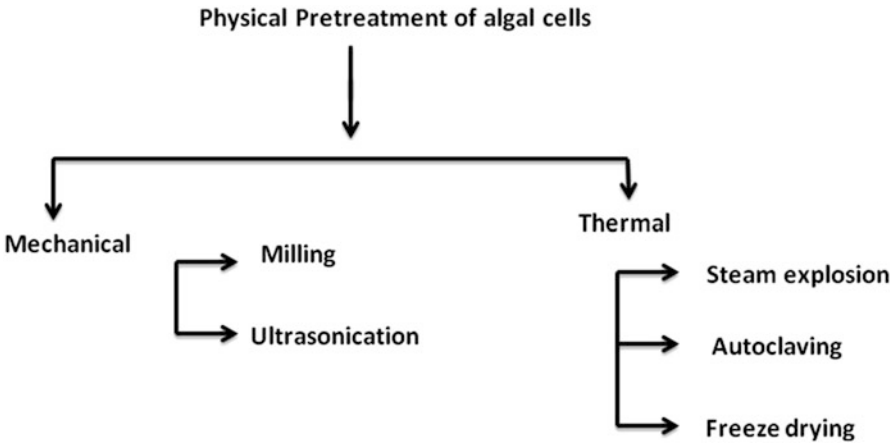

Fig. 3 Classification of physical pretreatment of algal cells

permits coherent recovery of the intracellular lipids resulting in fast and enhanced efficiencies in lipid extraction (Lee et al. 2010; Gouveia et al. 2007; Greenwell et al. 2010; Cooney et al. 2009). Depending upon the device or agent used for disrupting the algal cell wall the physical pretreatments have been classified in Fig. 3.

4.1.1 Milling of Algal Cells

Bead mills have been used extensively in past to disrupt the cell wall of the algal cells. In this process the extracellular wall of microalgae is breakdown by grinding and agitation of the cells on a solid surface of the glass beads (Mercer and Armenta 2011). Bead mills use the exhilarating bead to generate a high level shearing which can tear down the cell walls of microalgal cells (Munir et al. 2013). The diameter of the shearing beads typically ranges from 0.3 to 0.5 mm (Doucha and Vansky 2008). The beads of bead mills are made up of silica and oxides salts of zirconia and zirconium and carbide salt of titanium. There are two types of the vessels which are used in milling operation: shaking vessel and agitated beads.

Shaking Vessel

The vessel containing algal cells shakes on the vertical axis in order to disrupt the cell wall. The vessel or several vessels are mounted over platform which vibrates along the horizontal axis. The beads come in contact with algal cells during vibrations which result in the disruption of cell wall. However, this type of bead mill is only applicable at laboratory scale and is not efficient as compared to agitated vessels bead method. The extraction of intracellular lipids from *Chlorella vulgaris* using bead mills with shaking vessels was performed by Zheng et al.

(2011). The authors reported that the maximum extraction of lipids was only 11% which was inferior in comparison to the other methods. In case of *C. protothecoides* around 18.8% recovery of lipids was obtained by Shen et al. (2009).

Lipid extraction of around 28% was obtained in case of *Botryococcus* (Lee et al. 2010). A slight increase in extraction of lipids (25–30%) from the cells of *Chlorella*, *Nostoc* and *Tolypothrix* was obtained by using shaking vessel mediated by bead beating (Prabakaran and Ravindran 2011). In an another case study involving the bead beating in the shaking vessel of *P. tricornutum* cells showed an elevation in recovery lipids by 40% (Ryckebosch et al. 2011).

Agitated Beads

This method involves the simultaneous agitation of algal cells with beads. The simultaneous agitation performed inside the vessel provides enhanced destruction of algal cells which increases the extraction efficiency in terms of lipid concentration. The better heat transfer in these vessels is assured by an internal rotating agitator. The optimum temperature of the vessel is maintained by external metal jacket filled with coolant. The arrangements of cooling jackets are more important for heat labile molecules. Cells of *Nannochloropsis* were agitated with beads and results indicated the recovery of 33% lipids per 100 g of cells (Gouveia et al. 2012). Similarly a recovery of around 17.5% of the lipids was obtained by simultaneously agitating the cells of Chlorococcum with beads in a common vessel (Halim et al. 2012). In another advancement of this technique Baldev et al. (2014) observed two fold elevations in lipid yield by agitating the cells of *Scenedesmus* together with mechanical grinding.

4.1.2 Ultrasonication

Another mechanical method mediated by waves for recovery of lipids and disruption of algal cell wall is known as ultrasonication. This method involves exposure of the algal cells to high intensity ultrasonic waves leading to the formation of cavitation bubbles surrounding the cells. These bubbles subside and generate shockwaves which break the cell wall resulting in leakage of lipids into the external environment. It has been observed that the lipid extraction assisted by ultrasonication in algal cells is significantly higher coupled with condensed extraction time (Lee et al. 2010; Mercer and Armenta 2011; Menendez et al. 2013). Pernet and Tremblay (2003) observed the elevation in rate of extraction of lipids from the cells of *Chaetoceros gracilis* by using ultrasonication. However, some controversies in the results of recovery of lipids from microalgal cells and scale up difficulty have been reported by Halim et al. (2012) and Mercer and Armenta (2011). Lipid extraction from the cells of *Botryococcus* has been done using autoclaving, bead milling, microwave and sonication (Lee et al. 2010). The results of investigation showed that the recovery of lipids was quite diminished by ultrasonication method.

However, recovery was significantly higher compared to single solvent extraction technique. Another controversial observation was made by Shen et al. (2009) during the recovery of lipid content from *Chlorella protothecoides*. The results of the experiments showed that the least recovery of lipids was obtained in case of ultrasonication compared to other mechanical disruption method. On the other hand, the extraction of lipids from *Chlorella*, *Nostoc* and *Tolypothrix* was maximum using ultrasonication compared to microwave, autoclaving and bead beating assisted cell wall disruption (Prabakaran and Ravindran 2011).

4.1.3 Thermal Pretreatment of Algal Cells

Pretreatment of algal cells thermally can be implemented to disrupt the cell wall which in turn enhances the recovery of the intracellular lipid. Broadly the thermal pretreatment of algal cell includes freeze drying, steam explosion and autoclaving.

Freeze Drying

One of the most preferred thermal techniques for disintegration of algal cell wall is freeze drying due to its placid working conditions and flexibility in recovery of lipids (Grima et al. 2003; Pasquet et al. 2011). Another advantage of freeze drying is that it fluidizes the difficulty in extracting the lipid from wet biomass. The microalgal cells are sensitive to degradation under freeze drying which leads to loss of lipids by evaporation (Lardon et al. 2009; Pourmortazavi and Hajimirsadeghi 2007).

Milling of the freeze dried pretreated algal cells has showed the enhanced recovery of intracellular lipids. The enhanced recovery is due to the reduction of diffusion gradient and enhanced specific area (Halim et al. 2012). The efficiency in terms of recovery of lipids can also be improved by the removal of water during freeze drying pretreatment (Guldhe et al. 2014). The experimental outcomes of freeze drying of *Dunaliella tertiolecta* indicated that integrity of cell was intact (Pasquet et al. 2011). Experimental finding of Pourmortazavi and Hajimirsadeghi (2007) proved that the freeze drying of algal cell enhances the effectiveness in recovery of lipid compared to liquid–liquid extraction.

Autoclaving

An additional category of thermal pretreatment is autoclaving of algal cells at 121°C and 15 lbs of pressure (Surendhiran and Vijay 2014). Such condition of high thermal stress induces the rupture of algal cell wall which results in release of lipids from intracellular sites. Surendhiran and Vijay (2010) and Lee et al. (2010) observed the fact that the recovery of lipids from autoclaved cells of *Nannochloropsis oculata* and *Chlorella vulgaris* was higher contrary to the other

methods of pretreatment. However, the autoclaved microalgal cells showed that the 15.4% and 33.7% of the recovery of intracellular lipids using autoclave and microwave assisted pretreatment, respectively (De Souza Silva et al. 2014). The major disadvantage of this technique is scale up of the process at large scale. In addition to this, adapting this technique at large scale may not be cost-effective state of the art.

Steam Explosion

Explosion of high pressure steam can be done in the closed chamber to disrupt the cell wall of the algal cells which results in easy recovery of intracellular lipids. The operating temperature and pressure of the steam during explosion with biomass range from 160 to 260°C and from 1.03 to 3.45 MPa, respectively. The sudden shift of the pressure to the standard ambient pressure leads to destruction of cell wall. However, it is preferential to use this method at lower temperatures so as to prevent the degradation of intracellular lipids. The depressurization of the vessel takes place in the collection tank of the steam explosion unit and the flash valve is used to restore atmospheric pressure. This technique of algal cell wall destruction is quite efficient and cost-effective method. Steam explosion mediated extraction of lipid from algal cells has shown far more promising result as compared to other pretreatment methods like autoclaving, ultrasound and microwave technique. In addition to this, Montane et al. (1998) reported that steam explosion is an economically feasible methodology for processing of lignocellulosic material which adds value to the quality of feedstock. The major advantage of this technology is comparative lesser cost of operation, lower maintenance cost and corrosion resistance.

4.2 Chemical Pretreatment

Pretreatment of algal cells with alkali, acid and surfactant comes in the category of chemical pretreatments. The rationale behind attempting the chemical pretreatments of algal cells is to break the chemical linkages available in the cell wall. The additional advantage of this method is that it is lower energy demanding procedure compared to other mechanical operations of cell wall disruption.

A broad category of chemical agents used in various research works for the lysis of algal cell wall are liquid nitrogen, nitric acid, acetic acid, sodium chloride, hydrochloride, nitrous acid, osmotic shock and sulphuric acid. The chemical pretreatment of the algal cells using above-mentioned reagents resulted in more effective extraction of intracellular lipids, sugar, carotenoid, astaxanthin and agar. Another major breakthrough in chemical pretreatment of algal cell wall is hydroxyl radical mediated thermal pretreatment of algal biomass digestion. The results of this pretreatment showed increased digestibility of the algal biomass due to high rate conversion of cellular polysaccharides in to fermentable sugar.

4.3 Biological Methods

As discussed above, the major fraction of cell wall of green algae majorly composed of cellulose and other biodegradable components. Though, the physico-chemical pretreatment results in the significant solubilization of algal cell biomass, the techno-economic feasibility of such methods is seriously questionable. On the other hand, biological pretreatment of algal biomass offers great advantages over physico-chemical pretreatment. For instance, they require low energy inputs and relatively safe for environment (Prajapati et al. 2015a). Based on the bioagent being used, biological methods of algal biomass pretreatment may be classified into two groups. The first group includes the utilization of whole microorganism (usually bacteria) as bioagent to carry out the pretreatment, whereas, in the second approach, enzymes are used for hydrolysis of algal biomass.

4.3.1 Microorganism as Bioagent

Microorganisms require carbon and other nutrient sources for growth and reproduction. Microorganisms take up the carbon source (e.g. glucose) from the growth medium, which is then degraded by constitutive enzymes. However, if the free and preferred substrate (carbon and nutrients) is not available, majority of them may synthesize inducible enzymes for degradation of complex substrates. Diauxic growth of bacterial cells on media containing both glucose and lactose is the wonderful example of repression and production of inducible enzymes.

The algal cells contain significant amount of stored carbon (usually cellulose) and other inorganic nutrient which may support the growth of hydrolytic bacteria and other microorganisms. Non-viable algal cells are utilized by associated hydrolytic bacteria through production of inducible enzymes. This approach is now being used as efficient technique for pretreatment of algal biomass for improved biofuel production. For instance, Miao et al. (2013) reported biological pretreatment of algal biomass by naturally storing it at room temperature. Similarly, microaerobic biological pretreatment of algal biomass was carried out by Alzate et al. (2012). These reports include involvement of hydrolytic aerobic bacteria for degradation of algal cell wall through inducible enzyme production. Further, range of anaerobic bacteria may also be utilized for algal biomass pretreatment as they produce hydrolytic enzymes and are involved in the anaerobic degradation of waste and biomass.

4.3.2 Microbial Enzymes for Pretreatment

Hydrolytic enzymes have been evolved as most suitable tool for biological pretreatment of biomass including algae and other lignocellulosic wastes. Hydrolytic enzymes convert the algal cell and other complex compounds into simple low

molecular weight compounds such as soluble sugar. In the specific case of algal biomass, the enzymatic pretreatment weakens its cell wall and hence improves the efficiency of further steps involved in algae to biofuel conversion route. There have been some recent studies targeting pretreatment of algal biomass using commercial enzymes. For instance, Gerken et al. (2013) have tested various commercial enzymes including chitinase, lysozyme, pectinase, sulphatase, *b*-glucuronidase and laminarinase for pretreatment of *Chlorella vulgaris* biomass. Similarly, Ehimen et al. (2013) used mixture of commercial enzymes (α-amylase, cellulose, lipase, protease and xylanase) for pretreatment of *Rhizoclonium* biomass to improve its biomethane yield under anaerobic digestion. Though the pretreatment using commercial enzyme was technically better than the physico-chemical methods, the cost associated with the enzyme procurement makes it economically unviable for industrial scale biofuel production. Moreover, the algal cell wall is made up of complex biopolymer and enzymes are very specific for their target site, an enzyme cocktail is needed instead of single enzyme, for efficient pretreatment of algal biomass. Interestingly, fungi are good producers of range of extracellular enzymes under the induced conditions. Recently, effect of fungal crude enzyme was tested for pretreatment of algal biomass (Prajapati et al. 2015a). Microscopic images showing pretreatment of *Chroococcus* sp. due to cellulolytic action fungal crude enzymes are shown in Fig. 4. The added advantage of fungal crude enzyme based pretreatment is their very simple and low cost production using agro-residue under solid state fermentation.

The enzymatic pretreatment of algal biomass is very promising. Moreover, the crude enzyme based pretreatment further improves the economic viability of the

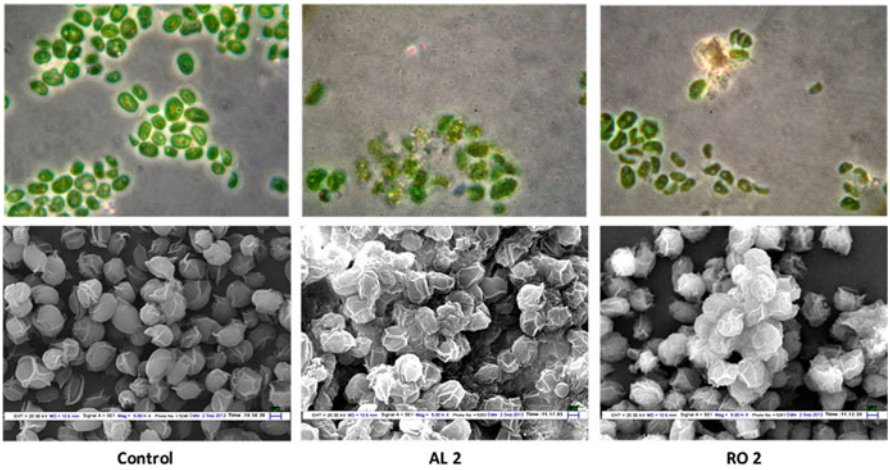

Fig. 4 Phase contrast and SEM images of enzyme treated algal cells. AL2: algae treated with *Aspergillus lentulus* crude enzyme and RO2: algae treated with *Rhizopus oryzae* crude enzyme at 20% dose (adopted from Prajapati et al. (2015a))

algal biofuel production. However, the process is under research and development stage and needs extensive techno-economic feasibility assessment before its commercialization.

5 Advancements in Pretreatment Techniques

The major obstacle which needs to be resolved for improving the efficiency of biofuel yield is (a) development of optimum method for degradation of algal cells and (b) well-organized procedure to convert algal digest into the specific compound mediated by highly precise catalysed reactions. This section discusses about techniques adapted or developed for the enhancement of biofuel yield from algal cells. In specific case of biodiesel, specific and correct lipid extraction technique should be chosen. The process parameters like pH, temperature, pressure, etc., should be optimized to have noteworthy control over extraction method and production of biofuel from algal cells. The novel extraction techniques (pretreatment methods) which have contributed significantly to the higher production of biofuel have been shown in Fig. 5.

The characterization features of an ideal extraction process include high throughput application, inexpensiveness, rapidness and eco-friendly product recovery. Contrary to this, conventional extraction processes involve huge cost, large volumes of extracting reagents (solvents) together with high time requirements. In addition to this, diminished productivity with non-specific selectivity has been observed in traditional methods. Traditional techniques are also not controlled fully by automatic controls. Against these drawbacks, usage of new technologies mentioned in Fig. 5 provides better alternative for fast, inexpensive, selective, eco-friendly and automatically controlled extraction.

5.1 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) (mediated by solvent) is performed on temperature and pressure higher than the critical temperature and pressure. The major

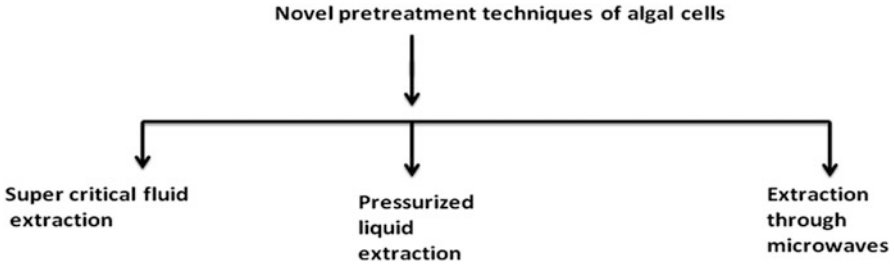

Fig. 5 Novel pretreatment techniques for enhanced biofuel production from algal biomass

advantage of this technique is its eco-friendly nature and swift extraction rate. Some applications of SFE have been observed in extraction of fatty acids (indolic derivative) and carotenoids from *Chlorella vulgaris* and *Dunaliella salina*, respectively.

5.2 Extraction Through Microwave

Microwave assisted extraction of intracellular compounds form algal cells is usually performed by vibration of water molecules within the cell. Vibrational energy generated in the liquid medium elevates the temperature of intracellular environment. Increase of temperature results in evaporation of water which in turn transfers high pressure swing on the cell wall. This pressure swing on the cell surface disrupts the cell's wall. EM mediated recovery of intracellular compound is superior to SFE in terms of process economics. EM mediated recovery is performed in extraction of polysaccharides, iodine, bromine and phytosterols and phytol from algal biomass.

5.3 Pressure Liquid Extraction

The pressure liquid extraction (PLE) of intracellular components is one of the most recent advancements that is yet in research and development phase. The major assistance of this technique over other traditional methods of pretreatment is its rapidness in extraction and smaller loads of solvents used for extraction. This method is dependent upon the combined (simultaneous) application of temperature and pressure. PLE has been found very efficient in extraction of antioxidants like phenols, carotenoids (fucoxanthin), fatty acids and fucosterol.

6 Analysis of Algal Biomass Pretreatment

Once the algal biomass is pretreated, it could be utilized for efficient biofuel production through relatively easier lipid extraction (for biodiesel) or enhanced methane production through anaerobic digestion. However, to select the most effective approach, the assessment and analysis of the resulting pretreatment in the algal biomass is highly desirable. Irrespective of the methods adopted, the pretreatment analysis can be carried out either qualitatively or quantitatively.

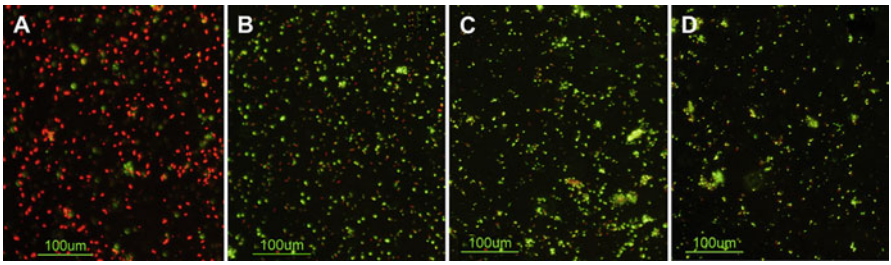

Fig. 6 Sytox staining *Scenedesmus* ecosystem of not thermally pretreated biomass (**a**) and thermally treated at 90 C after (**b**) 30 min, (**c**) 60 min and (**d**) 180 min. *Green colour* is due to the fluorescence of Sytox® Green/DNA complex whereas the *red colour* is due to the auto-fluorescence of chlorophyll present in the live cells (adopted from González-Fernández et al. (2012a))

6.1 Qualitative Detection of Algal Biomass Pretreatment

Qualitative analysis involves visual observations and microscopic imaging. It gives an indication whether the particular pretreatment method is causing any change in the properties of cells or rupturing the cell wall. Usually, microscopic images (Light, SEM or TEM) are sufficient to indicate pretreatment of the algal biomass. However, fluorescence microscopy analysis is gaining interest due to associated simplicity and more clear indication. Under this approach the cells are stained with some compound having fluorophore which specifically stains the cells with damaged cell wall and emits the light under visible radiation. Sytox® green is the wonderful example of such a stain (Prajapati et al. 2015a).

The Sytox® Green/DNA complex has excitation and emission maxima of 504 and 523 nm, respectively. This dye specifically binds the exposed DNA of algal cells having damaged cell wall and gives intense green colour under visible radiation (Fig. 6). The use of Sytox® green has recently been reported for primary detection of cell wall degradation during thermal pretreatment of *Scenedesmus* biomass (González-Fernández et al. 2012a), comparison of ultrasound and thermal pretreatment of *Scenedesmus* biomass, comparison of various commercial enzyme for pretreatment of *Chlorella vulgaris* biomass (Gerken et al. 2013) and action of fungal crude enzyme on algal cell wall (Prajapati et al. 2015a, b).

6.2 Quantification of Algal Biomass Pretreatment

Qualitative measure of the pretreatment of the algal biomass is not very informative. This approach only indicates the possible action of the pretreatment method being used on algal biomass. However, for comparison among different methods, the qualitative data is not sufficient. For comparative evaluation, quantitative data

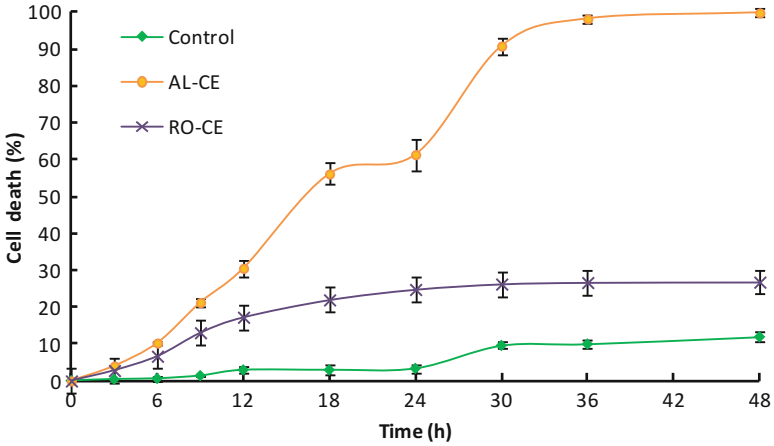

Fig. 7 Variation of cell death as a result of pretreatment with crude enzymes obtained from different fungal strains. AL-CE and RO-CE represent the cell death profiles obtained during pretreatment with crude enzyme of *A. lentulus* and *R. oryzae*, respectively

on different pretreatment method is highly desirable. One of the direct approaches for obtaining quantitative data during the pretreatment is the 'live and dead cell counting'. In a recent publication, automated cell counter equipped with fluorescent filters was used to count the live and dead cells during the enzymatic pretreatment of algal biomass (Prajapati et al. 2015a). The results were then presented in the form of % cell death given by the following equation:

$$\text{Cell death } (\%) = \left(\frac{X_0 - X_t}{X_t}\right) \times 100 \tag{1}$$

where X_0 and X_t are the live cell count (cells mL^{-1}) at time 0 and t during the enzymatic incubation, respectively. The variation of the cell death with incubation time during pretreatment with crude enzymes is illustrated in Fig. 7.

The indirect measure of pretreatment could be taken from the solubilization of algal biomass during the action of pretreating agent. Estimation of fermentable sugar concentration and the soluble chemical oxygen demand (sCOD) could be used a parameter for quantification of algal biomass solubilization. As a result of pretreatment and solubilization of algal biomass, the sugar concentration as well as the sCOD increases with pretreatment time. Alzate et al. (2012) have proposed the following equation for estimation biomass solubilization of algal biomass in terms of sCOD:

$$S_D = \left(\frac{\text{COD}_s - \text{COD}_{s0}}{\text{COD}_T - \text{CODs}_0}\right) \times 100 \tag{2}$$

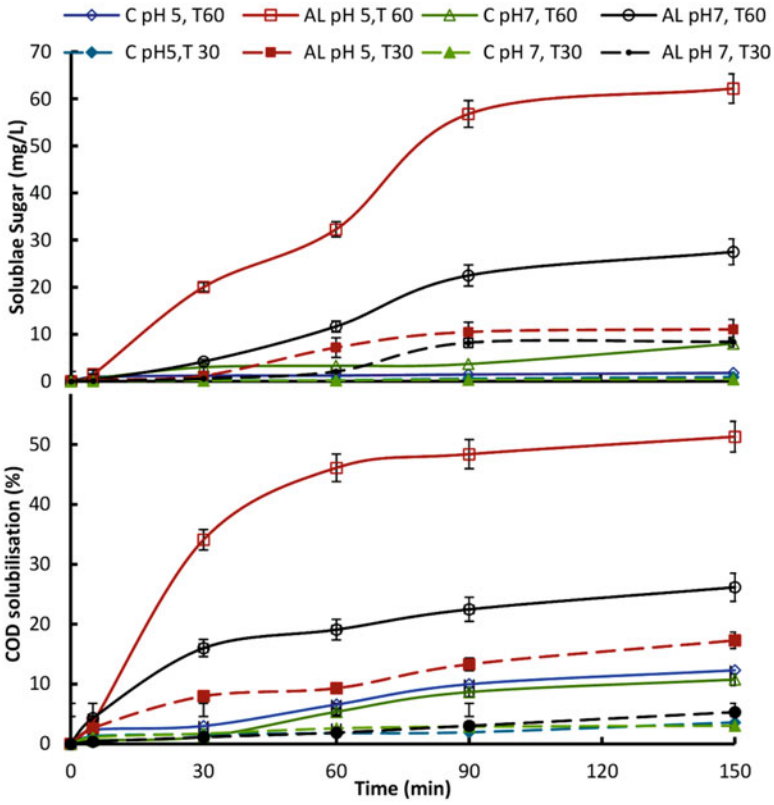

Fig. 8 Variation of sugar and COD solubilization from algal biomass with enzymatic pretreatment under optimal pH and temperature with elapsed time. AL-crude enzymes from *A. lentulus*, C-control; buffer only (adopted from Prajapati et al. (2015b))

where S_D is solubilization degree (%), COD_S is the soluble COD after pretreatment, COD_{So} is the soluble COD in the raw algal biomass and COD_T the total COD of the algal biomass. Similarly, in a recent report, sugar and COD solubilization profiles (Fig. 8) obtained during enzymatic pretreatment of algal biomass have been reported (Prajapati et al. 2015b).

The quantitative data obtained in terms of cell death, sugar release or COD solubilization, provides more information for establishing the efficacy of the pretreatment method. This information may be useful for optimization of a particular pretreatment method or for comparative evaluation of different pretreatment methods.

7 Enhanced Biofuel Production Through Algal Biomass Pretreatment

Pretreatments result in solubilization of algal biomass through rupture or alteration of the cell wall. As a consequence, the biofuel production becomes easy from the pretreated algal biomass. For instance, the lipid extraction from the algal biomass enhances that if the biomass is pretreatment. Similarly, there have been several studies reporting enhanced methane and hydrogen production through the pretreatment of algal biomass (Nguyen et al. 2010; Passos et al. 2014). The various reports on algal biomass pretreatment are summarized in Table 1.

As reflect from Table 1, significant work is being carried out worldwide on algal biomass pretreatment for improved biofuel yield. Various pretreatment methods are now available for improving the biofuel yield from the algal biomass. Moreover, techno-economic feasibility assessment reports are also available for comparative evaluation of different pretreatment alternatives. Nevertheless, only few attempts are being made on pilot scale and field scale validation of the pretreatment and algal biofuel production and the majority of presently available reports are based on the laboratory scale experimentation. Hence, there are several critical research gaps related to process scale up and validation, which needs to be worked out, in order to make algal biofuel economically viable.

8 Conclusion and Prospective

Algal biomass has evolved as an excellent raw material for biofuel production. However, recalcitrant nature of the cell wall makes the biofuel extraction difficult. The cell walls are basically composed of fibrous composites of microfibrillar polysaccharides, matrix polysaccharides and proteoglycans. Extracellular polymeric substances in limited quantity may also be present in the algal extracellular matrix. Hence, pretreatment of algal biomass is desirable in order to make the algal to biofuel conversion feasible. There are various physico-chemical methods available for algal biomass that has resulted in improved biofuel yield. Among the various alternatives, thermal pretreatment results in highest degree of algal biomass solubilization and biofuel extraction. However, this approach is not considered economically viable due to involvement of high energy intensive steps. On the other hand, fungal crude enzyme based pretreatment provides a low cost and highly effective approach to improve the biofuel yield from the algal biomass. Also, there have been some attempts of integrated pretreatment to make algal biofuel economically viable. Despite the research and advancements in the area of algal biomass pretreatment and biofuel extraction, it is still at the laboratory scale. Hence, further in depth assessment, techno-economic analysis and the pilot scale validation of the algal biomass pretreatment is highly desirable, to make the algal biofuel sustainable and economically viable.

Table 1 Effect of various methods employed in the pretreatment of different algal biomass

Algae	Pretreatment	Remark	References
Algal mixture	Thermal	Up to 63% COD solubilization	Alzate et al. (2012)
	Mechanical	Up to 60% COD solubilization	
	Biological	9–29% COD solubilization	
Scenedesmus sp.	Mechanical and thermal	Up to 10% COD solubilization	González-Fernández et al. (2012b)
Rhizoclonium	Biological	CH_4 yield improved by 20%	Ehimen et al. (2013)
	Mechanical	CH_4 yield improved by 7–13%	
Mixed biomass from wastewater	Thermal	CH_4 yield increased by 70%	Passos and Ferrer (2014)
Chlorella vulgaris *Scenedesmus* sp.	Low temperature autohydrolysis at 50°C	6–12% COD solubilization	Mahdy et al. (2014a)
	Alkaline (0.5, 2 and 5% w/w NaOH dosages)	20–43% sugar solubilization	
C. reinhardtii and *C. vulgaris*	Hydrolytic enzymes supplied by Novozyme	CH_4 increased by 14% (*C. vulgaris)* but no increase in *C. reinhardtii*	Mahdy et al. (2014b)
Chlorella vulgaris	Crude hydrolytic extracellular enzyme solution extracted	H_2 yield of 43.1 mL H_2/g dry cell weight	Yun et al. (2014)
C. vulgaris	High pressure thermal pretreatment	Up to 64% methane yield enhancement	Mendez et al. (2014)
Chroococcus sp.	Fungal crude enzymes (under non-optimal conditions)	44% and 46% of total sugar and COD of biomass during 48 h incubation at 30°C	Prajapati et al. (2015a)
Chroococcus sp.	Fungal crude enzymes (optimized conditions)	Up to 50% biomass solubilization in 150 min at 50°C 28% enhancement in methane yield	Prajapati et al. (2015b)
Chlorococcum infusionum	Alkaline pretreatment (NaOH)	Glucose yield: 350 mg/g Bioethanol: 0.26 g ethanol/g algae	Harun et al. (2011)
Lipid-extracted microalgal biomass residues	NA	Hydrogen production up to 30.03 ml/g (VS)	Yang et al. (2011)
Scenedesmus and *Chlorella*	Combined acid-catalysed pretreatment and extraction	> 90% sugar solubilization Up to 97% fatty acid recovery form wet algal biomass	Laurens et al. (2015)

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Environmental and Economic Sustainability of Algal Lipid Extractions: An Essential Approach for the Commercialization of Algal Biofuels

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Contents

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1 Concept of Sustainability

Advancements in science and technology have improved man's standard of living. Increased amenities are now affordable to the common man but at the expense of energy and the environment. Anthropological activities pose severe burdens to the environment while continuously damaging it with increasing incidences of pollution, waste accumulation, species extinction, etc. The alarming decline in fresh water resources, food, arable land and the undesirable changes in climate stress the need for the adoption of sustainable technologies.

The term sustainability, according to the Brundtland Report (1987), can be defined as 'meeting the needs of the present generation without compromising the needs of the future generation' (WCED 1987). It allows for the harmonious coexistence of man, animals and nature. Innumerable suggestions and recommendations for sustainability have been proposed. Despite these, operationalizing the concept into reliable measurable terms is still a challenge and highly debated. Nevertheless, some of the widely accepted assessment methods will be discussed in this chapter.

The prerequisite for anthropological activities is energy, which is commonly derived from fossil fuels. Fossil fuels are therefore extensively used to meet the demands of the exploding population. The rapid depletion of these fuels and the increasing problems of pollution owing to its rampant use have triggered the search for alternative sources of fuel that are both renewable and eco-friendly. The concept of biofuels was thus introduced as a sustainable fuel technology. First generation biofuels, i.e. various alcohols obtained from the fermentation of starch and sugar containing fuel crops (corn, sugarcane, etc.), suffer from carbon emissions, low productivity and fuel efficiency, competed for agricultural resources (land, water, nutrients, fertilizers, pesticides, etc.) and triggered an increase in food crop prices. Second generation biofuels derived from lignocellulosic biomass such as wood and agricultural waste have disadvantages such as technological barriers, feedstock availability and accessibility, introduction of invasive species like *Miscanthus* (Sayre 2010; Singh and Olsen 2011). Third generation fuels or algal fuels are claimed to be better alternatives owing to their higher lipid yields and productivities, lower land requirements, ability to decrease carbon emissions, etc. The cost of production of the fuel is, however, high. A major portion of the cost is attributed to the operations involved in downstream processing, viz., biomass harvesting, drying or dewatering, lipid extraction and its trans-esterification. Different approaches to these operations are discussed and their costs and benefits compared in Sects. 4, 5 and 6. Appropriate evaluations of costs, services and efficiencies of these algal fuels in various

dimensions (social, economic, ecological) are essential to determine the actual status of their feasibility and sustainability. Some of the most popular and widely accepted assessment methodologies suitable for algal fuels are also discussed in Sect. 8.

2 Algae: The Multi-Faceted Fuel Machinery

Algae may be prokaryotic or eukaryotic photosynthetic organisms found in a range of sizes and capable of surviving in diverse ecological habitats. There are over 40,000 identified species. The biochemical composition of algae varies widely across species as well as within species when grown under different conditions. Lipids, carbohydrates and proteins are the major biomolecules present in the algal cell and the first two have been exploited for multiple fuel applications.

Carbohydrates are produced within the cell to form structural components of the cell wall or are stored as energy reserves such as starch and glycogen (*Arthrospira platensis*). They serve as feedstock for the production of biofuels like bioethanol, biobutanol, biohydrogen, etc. Several species accumulate carbohydrates in high levels such as *Spirogyra* sp. (33–64%) and *Porphyridium cruentum* (40–57%) (Markou et al. 2012). Other algal species can be manipulated to overproduce carbohydrates by varying cultivation conditions such as with *Chlorella* sp (50–65%). Nutrition limitation is the most common strategy employed for overproduction of storage products in general.

Lipids in the cell are comprised of various neutral lipids like triacylglycerols (TAGs), waxes, sterols, polar lipids, prenyl derivatives (carotenoids, quinones, etc.,) and phytylated derivatives (chlorophyll). TAGs, composed of three fatty acids esterified onto a single glycerol backbone, are synthesized under unfavourable conditions to serve as storage reserves. Algae capable of accumulating high levels of TAGs are used as feedstock for the production of biodiesel. TAG accumulation has been extensively studied in a number of algae including several *Chlorella* sp., *Dunaliella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., etc. Most common algae like *Chlorella, Nannochloropsis*, *Nannochloris*, etc., are known to accumulate lipids up to 20–50% of its dry weight (Mata 2010). Algae commonly produce C14:0, C16:0, C18:1, C18:2 and C18:3 fatty acids, although the relative percentage of the different fatty acids is highly variable. It can also contribute to fuels such as bio-oil from pyrolysis and biohydrogen from its fermentation.

Owing to this biochemical composition, algae are considered promising, exciting and sustainable third generation feedstock that can convert CO_2 into biofuels. They have the potential to meet our transportation fuel needs, due to their ability to produce wide range of fuels, viz., biodiesel, biohydrogen and bio alcohols like ethanol and butanol that are discussed below. The fuel yield is typically high when compared to that produced from the first generation and second generation feedstock. The fuelling process from algae is continuous and renewable unlike the seasonal first and second generation feedstock (Dragone et al. 2010). Thus algae are believed to be an economically feasible alternative that can subtle the growing energy needs. The general classification of fuels is explained in Fig. 1

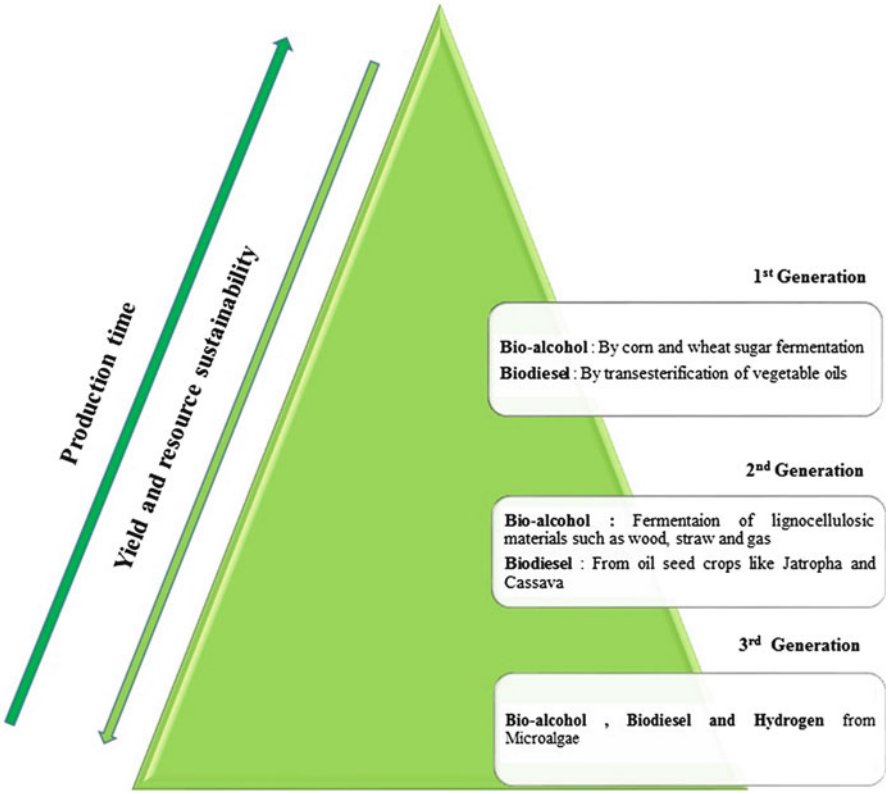

Fig. 1 General classification of fuels (modified from Nigam and Singh 2011)

2.1 Biodiesel

Biodiesel is a biodegradable and nontoxic mixture of fatty acid methyl esters (FAME) with energy content comparable to petroleum based fuel (Sheehan 1998). Algae are brilliant sources to make biodiesel, as oil accumulated by them has characteristics similar to that of vegetable oil from rapeseed and soy. The global market for biodiesel is increasing exponentially in recent times (Deng et al. 2009). The micro-algal cells accumulate the oil in the form of triglycerides (TAG), which are then trans-esterified to produce biodiesel. The fatty acids produced by algae are comprised of saturated (SFA), monounsaturated (MUFA) and poly unsaturated fatty acids (PUFA). The fatty acid profile greatly influences the properties of biodiesel produced. For instance, biodiesel with high content of SFA has decreased cold flow properties and can become viscous at ambient temperature. On the other hand, high PUFA content will make biodiesel susceptible to oxidation (Hu et al. 2008). Thus proper percentage of SFA and UFA is very important for a good quality fuel (Deng et al. 2009). Thus, while screening micro-algal source for biodiesel

production, not only the yield but the FAME profile also forms a criteria for strain selection. Among the lipid accumulating microalgae, *Chlorella* is a potential source as it can accumulate high lipid content and produces good quality biodiesel (Mallick et al. 2012; Xu et al. 2006). Albeit algae have obvious potential for an alternative fuel source, it lacks universal commercialization due to high cost associated with the downstream processing steps. Novel techniques are being developed so as to economically stabilize algal biodiesel production. Any progress in lipid extraction would have a considerable impact on the process economy (Wahlen et al. 2011).

2.2 *Bioethanol*

Bioethanol constitutes to the major proportion of biofuel produced globally. While lipids in algae can be utilized to produce biodiesel, the carbohydrates can be converted to ethanol. In fact algae can be a dedicated source for ethanol as they are built with carbohydrate rich cell wall. Algae are capable of synthesizing and accumulating large quantities of carbohydrate from cheap raw materials which can be further used for bioethanol production (Harun et al. 2010). The algal cake remaining after lipid extraction consists of starch and cellulose which can be further fermented to ethanol. The algal cake fermentation further releases CO_2 which can be fluxed into algae growth chambers to increase its production (Fig. 2).

In general, algae that accumulate starch or form filaments/colonies are suitable as they are often carbohydrate enriched. This includes species of *Spirulina*,

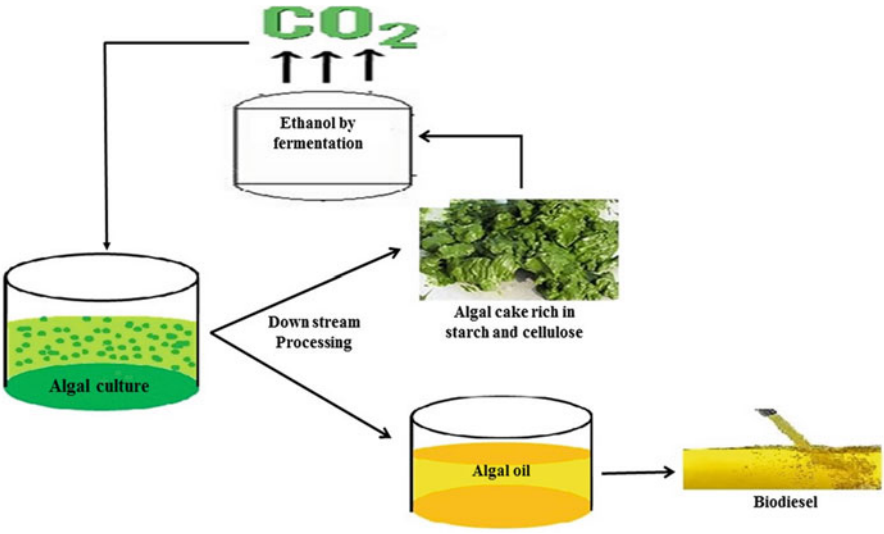

Fig. 2 The interlinked process of biodiesel and bioethanol production

Spirogyra, *Gracilaria*, etc. Apart from this, *Chlorella vulgaris* which can accumulate starch up to 37% of its dry weight can be a source for ethanol production (Minh and Hanh 2012). After lipid extraction, when the left over *C. vulgaris* cake was fermented with *Saccharomyces cerevisiae,* ethanol recovery of 65% was obtained. Bioethanol concentration of 38.3 g/g of algae was obtained from de-oiled *Chlorococcum* sp. (Harun et al. 2010). Though the production of bioethanol from algae concept is well known, the process technology is still in its infancy and needs comprehensive exploration with respect to energy and cost analysis.

2.3 Biohydrogen

Molecular hydrogen can be produced by certain species of microalgae and cyanobacteria under specific conditions. Biohydrogen production in microalgae was first observed in *Scenedesmus obliquus* (Schenk et al. 2008). *Chlamydomonas reinhardtii* is the microalgae now commonly used for studies on hydrogen production.

Production of biohydrogen can be brought about in two steps. The first step takes place in all photosynthetic organisms and involves the light induced photolysis of water to obtain protons, electrons and oxygen. Electrons so formed are carried down the electron transport chain to ferredoxin and finally onto the enzyme hydrogenase. The production of molecular hydrogen is ultimately brought about by hydrogenase using electrons from the reduced protein ferredoxin and protons from the media. Further, this step must be carried out anaerobically as oxygen inhibits hydrogenase (Markou et al. 2012). The second step thus requires the presence of hydrogenase—an iron containing enzyme in the chloroplast that ultimately confers upon microalgae the ability to produce biohydrogen.

Alternatively, the metabolic breakdown of carbohydrates such as starch, under anaerobic conditions also supplies the reducing energy required for hydrogen production by hydrogenase (Varfolomeev 2011). The gas so produced and released by the organism subsequently escapes into the gas phase and is thus separated from the media and easily recovered.

Microalgae containing high carbohydrate contents can serve as feedstock for biohydrogen production by anaerobic bacteria such as those of genus *Clostridium*. Algal biomass left over after the extraction of lipids and pigments are rich in carbohydrates and can be further exploited for biohydrogen production.

2.4 Biomass Pyrolysis Products

Pyrolysis of algal biomass is the process of heating it to high temperatures in the range of 350–700°C in the absence of oxygen leading to the decomposition of biomass to bio-oil, syngas and bio char. Pyrolysis of biomass is suggested after the

extraction of lipids. The bio-oil so obtained is the main product of interest and used to produce diesel and gasoline. Bio char can be further combusted and used for powering the algae combustion system (Mu et al. 2014). It is also a suggested fertilizer due to its high carbon content (Schenk et al. 2008) and acts as a soil conditioner and provides support for beneficial microbes. It can also serve as raw material for the production of carbon fibers, carbon nano-tubes and activated carbon (Maddi et al. 2011). The bio-oil content of pyrolysis products can be maximized with flash pyrolysis (Wang et al. 2008). Syngas can also be combusted for power generation or even converted to hydrogen by Fischer–Tropsch processes. Most studies report results obtained from the pyrolysis of lignocellulosic feedstock such as saw dust, cherry seeds and grape residue. The pyrolysis studies on microalgae have, however, indicated better bio-oil quality and stability (due to lesser oxygen content). Further, complete decomposition can be brought about at much lower temperatures in microalgae thus demanding lesser energy (Varfolomeev 2011). A study on *Chlorella* demonstrated a 28% yield of dry bio-oil from pyrolysis at just 400°C (Boer et al. 2012). Another study on heterotrophic *Chlorella protothecoides* claimed bio-oil yield of 57.9%. The oil yield, as seen in different studies, varies significantly across different microalgae species in the range 24–43 wt.% (Yanik et al. 2013). Nitrogenous compounds such as proteins present in algal biomass are undesirable as they are converted to pyrroles, indole derivatives and long-chain alkanes that decrease fuel quality. These ultimately constitute to nitrogen oxide emissions during combustion (Maddi et al. 2011). They also lead to the formation of pyrolysis products that later inhibit the catalysts in the subsequent biodiesel production. This problem has been addressed to some extent by catalytic pyrolysis where the use of zeolite catalysts such as ZSM-5 that decrease both nitrogen and oxygen content in the produced bio-oil (Thangalazhygopakumar et al. 2012). The primary disadvantage of pyrolysis, however, is the need for relatively dry biomass and therefore the high energy requirement for drying even prior to the pyrolysis process.

3 Impediments in Algal Fuel Commercialization

The production of biofuels, particularly biodiesel, from algae has a number of advantages over its production from plants. Despite these merits, commercialization of the fuel remains a task yet to be achieved. The major constraints to the technology are discussed below.

3.1 Economic and Operational Constraints

The technology is primarily limited by the overall cost of production. Chisti and Yan (2011) predict that algal biodiesel may be considered an option only when

crude petroleum will cost ≥$100 per barrel (Mata 2010). Major costs are associated with harvesting and further downstream processes.

Open raceway ponds and photobioreactors (PBRs) are used for the cultivation of microalgae. While raceway ponds are cheaper to maintain and operate, the biomass yield obtained is low. It is also characterized by operational difficulties since effective mixing, temperature and pH regulation, prevention of contamination, etc., are difficult. PBRs provide for a much more efficient mode of production and give rise to high biomass and lipid productivities but have high installation expenses (Chisti and Yan 2011). The cost of biomass recovery here is lower than that for raceway ponds (Chisti 2008). Elaborate studies are carried out to determine trade-offs. High fertilizer requirements of nitrogen and phosphorus and the water footprint constitute to high costs and offset the phototrophic advantage of algal cultivation.

Biomass harvesting following cultivation form 20 to 30% of the production cost (Kumar et al. 2010). Harvested biomass may still contain up to 90% (w/w) water. Dewatering is thus essential prior to lipid extraction and trans-esterification. Dewatering of microalgae is the most energy expensive process and thus contributes to a major portion of the production cost. Drying and hexane extraction of the lipids are said to contribute to 90% of the energy expenses in biodiesel production (Singh and Olsen 2011). Although cheaper alternatives like solar drying exist, this method yet again poses demands for large areas to facilitate drying on an industrial scale.

3.2 Ecological Constraints

Algae have the advantage of being cultivated on non-arable land. Ample sunlight, however, must be available over these areas to enable maximization of photosynthesis. Seawater can be used for cultivation. Nonetheless, freshwater requirement is still high on account of its use for other operations. Several studies have suggested the use of carbon dioxide from flue gases as a cheap source of carbon for cultivation. Further, literature often suggests coupling oil production with other applications for economic feasibility. All these advantages hold well only if there exists close proximity between the land available for cultivation, water and carbon source, as well as between the production plants. Further, although algae are not seasonal and can be cultivated all-round the year, variations in sunlight can result in a difference in productivity.

3.3 Social Constraints

The use of expensive and limited fertilizers for the cultivations of algae over food crops raises questions about the sustainability of the fuel (Klein-Marcuschamer et al. 2013). The production of biofuels is accompanied by rising food prices due to the lesser land area now devoted for food crop production (Kovacevic and Wesseler

2010). Although, this area attributed to micro-algal cultivation is theoretically claimed to be less, actual areas used may be higher than anticipated or desired. The lack of a competitive price of algal fuel discourages consumers from considering the biofuel option even for use as blends despite the rising problems of pollution and fossil fuel depletion. Biofuels are used only when required to conform to legislations. Although subsidies over the use of biofuels have been criticized (Webb and Coates 2012), well-informed policy making rather than a complete retraction of a subsidy could improve the biofuel scenario. Further, lack of large scale production data has often limited comprehensive economic assessments of the various biofuel production strategies and consequently discouraged prospective investors. Existing analyses suffer from disadvantages due to the use of outdated and incomplete information. Improved and accurate assessments based on technological advancements could thus improve the scope of biofuel commercialization.

With the goal of commercialization, several changes and developments that have been carried out in algal downstream processing, starting with biomass handling strategies followed by extraction and FAME production will be discussed in this chapter henceforth.

4 Biomass Processing Methods

When the algal cells have accumulated lipid to its estimated maximum possible, the foremost step in biomass processing is harvesting that involves water removal from algal culture. The most commonly employed techniques for harvesting include centrifugation, gravity sedimentation, filtration and flocculation. The harvested slurry is taken further for lipid extraction. The biomass recovery often accounts for 20–30% of the total algal production cost (Kumar et al. 2010). However, this fraction would be insignificant with respect to total biodiesel production cost. The major challenge would be to release the oil from the intracellular compartments via energy efficient and economically viable processes. Though oil yield from microalgae is comparatively higher than oilseed crops, the extraction procedure becomes tricky with algae when compared to the oil seed crops, such as Jatropha and Sunflower (Chisti 2008). Unlike oil seed plants, a commercially viable lipid extraction protocol is not yet available in market for algal biomass. The difficulty in extraction arises as microalgae are tough cell walled single cell organisms. For instance, the simple milling or mechanical pressing for oil seed extraction is not generally applicable for microalgae. The simple milling of oil seeds produces meal containing 90% solid and hence easily extractable oil. In contrast, the algal biomass is high in water content which would offer resistance to pressing or milling force. The dewatered slurry resulting after harvest contains only 5–25% solids and the rest is moisture. This slurry can be taken further for oil extraction, either directly (wet way extraction) or can be dried prior to extraction (dry way extraction). Both the methods are advantageous in their own way and depending on the procedure opted

specific pre-treatment steps have to be followed. Also the percentage of oil recovered and the FAME profile changes for dry and wet extraction

4.1 Dry Way Extraction

The conventional procedure of procuring oil from micro-algal biomass is to dry them first, followed by extraction procedures. Drying involves removing the rest of the moisture from the harvested slurry and is considered to be one of the expensive processes comprising about 20–75% of the total processing cost (Uduman et al. 2010). The advantage of drying is that it improves the shelf life of the biomass. In general, the biomass production will be high in summer compared to winter. The resourceful tactic is to allocate the upstream biomass production phase to summer and the downstream oil extraction phase to winter. Since the harvested slurry through the upstream process is perishable, it has to be processed immediately for extraction. But with drying, the biomass produced during summer time can be collected, stored and processed in bulk in the winter period. Thus drying helps in maintaining the production–extraction balance and general economic balance of the power plant. On the other hand, the drying process is highly energy intensive, thus making it less preferable from sustainability angle.

A number of techniques are available for drying the biomass, such as solar drying, spray drying, oven drying, freeze drying and fluidized bed drying (Shelef and Sukenik 1984). The selection of drying technique depends on the scale and speed of operation. Some of these are summarized in Table 1. Though solar drying is renewable and economically viable, it requires more time, produces less stable

Table 1 Summary of different drying techniques

Technique	Economic sustainability	Drying speed	Suitable scale of operation	Biomass quality	Influence on oil extraction
Solar drying (Seasonal)	Zero energy intense and hence highly sustainable	Slow	Large	Biomass is less stable as it is prone to contamination due to slow drying rate	Presence of free fatty acids is high which will decrease the trans-esterification efficiency
Convective or oven drying (Non-seasonal)	Less energy intense and moderately sustainable	Fast	Small	High	Comparatively better
Freeze, spray and drum drying (Non-seasonal)	High energy intense and zero sustainability	Rapid	Small	High	Better

biomass and affects the efficiency of trans-esterification due to the presence of high amount of free fatty acids in the oil extracted from sun dried biomass. These difficulties are overcome when using techniques like spray, oven and freeze drying, but are expensive and not suitable for industrial scale. Thus dry way of lipid extraction through conventional is not apposite as an eco-sustainable process. Several developments are in process so as to improve the eco viability of algal oil extraction and extraction from wet biomass is being explored as a potential alternative. But one should not neglect the fact that economic sustainability of drying process is also possible by utilizing the energy of the flue gas at the emission source outlet for heating the biomass. However, availability of space to set up a biodiesel production plant in close proximity to emission source outlet might be a constraint.

4.2 Wet Way Extraction

Wet way of oil extraction has greater advantage as it is a less energy intensive process. For instance, total energy required to produce 1 kg of biodiesel through wet route is 42.3 MJ, but the same from dry biomass consumes 107.3 MJ (Fig. 3, (Lardon et al. 2009)). Thus energy consumption of dry way extraction is almost 60% higher compared to the wet route.

The oil extraction by wet route requires essential pre-treatment procedure for disrupting the algal cell walls. Following disruption, the released intracellular oil can be recovered by several extraction techniques which are discussed in the later sections. Various techniques are available to disrupt the algal cell wall, which are generally classified into mechanical approach (microwave, ultrasonication and high pressure stress), chemical approach (acids and Enzymatic rupture). The lipid yield

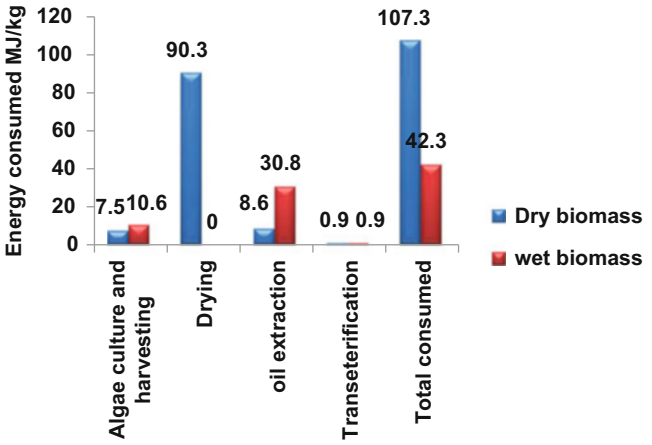

Fig. 3 Energy requirements for algal oil extraction at various stages (modified from Lardon et al. 2009)

Table 2 Comparison of various cell disruption techniques (modified from Byreddy et al. 2015)

Cell disruption method and process time (min)	Oil yield (% DW)	Energy consumed (MJ/kg biomass)	Advantage	Disadvantage	Scalability
Grinding (2)	44.6	Not determined	Quickest and efficient	Localized heating caused denaturation of molecules	Poor
Sonication (20)	31	1200	Faster extraction, suitable for all cell type	Damage chemical structure of molecules	Moderate
Shake mill (5)	30.5	690	Rapid method	High energy intensive, high heat generation	Moderate
Water bath (20)	20.8	2400	Maximum disruption	Increases the viscosity, energy intensive	Moderate
Osmotic shock (2)*	48.7*	4.8*	Lower energy consumption	Generation of waste salt water	Moderate
Bead vortexing (20)	22.8	48	Can be established easily	High heat generation, incomplete cell lysis	Moderate

*highest oil yield

and the fatty acid profiles are influenced by the method of cell disruption employed. A comparison in lipid yield and fatty acid profile through various disruption techniques in micro-alga *Thraustochytrids* showed that disruption by osmotic shock gave highest lipid yield. Also the process was very less energy intensive (Byreddy et al. 2015). The comparison among various disruption techniques is summarized in Table 2.

A good ratio of saturated, monounsaturated and poly unsaturated fatty acid was obtained with osmotic shock disruption. Sonication and grinding, on the other hand, resulted in higher percentage of PUFA and this would affect the oxidative stability of the fuel. The best method of disruption, however, cannot be decided based on tests on a single organism as the disruption technique providing optimum yield can vary across the species due to the difference in their cell wall structure and culture age (Table 3).

Though there are several techniques of cell disruption available, only few are suitable for scale up. If the technique is highly energy intensive it won't be applicable for pilot scale, as high energy intensity translates to high cost of production. Methods like microwave, sonication and grinding have been proved efficient for cell disruption, but at lab scale (Table 3). In reality, whether or not they are suitable for scale up to quantities required for process scale is still not clear (Mercer and Armenta 2011).

Table 3 Cell disruption methods across various strains (modified from Byreddy et al. 2015; Byreddy et al. 2015)

Organism used	Method studied
Botryococcus sp. *Chlorella vulgaris* *Scenedesmus* sp.	Autoclaving, Bead beating *Microwaves*[a], Sonication Osmotic shock (Lee and Han 2015)
Chlorella sp. *Nostoc* sp. *Tolypothrix* sp	*Sonication*[a], Osmotic shock Microwave, Bead beating Autoclave (Prabakaran and Ravindran 2011)
Chlorella vulgaris	*Grinding*[a], Sonication Bead milling, Enzyme lysis Microwave (Zheng et al. 2011)
Schizochytrium sp. S31 *Thraustochytrium* sp. AMCQS5-5	Grinding, Bead vortexing Sonication, Water bath, *Osmotic shock*[a] Shake mill (Byreddy et al. 2015)

[a]Efficient method of disruption

Table 4 Lipid yield in *Nannochloropsis* through different modes of trans-esterification

Trans-esterification mode	Method of oil extraction	Trans-esterification reaction mixture	Biodiesel yield (% of biomass)
Conventional or indirect	Sonication (5 min) with methanol–chloroform (1:2)	Methanol–chloroform (1:2) with strontium oxide (SrO) catalyst	18.9
Conventional or indirect	Microwave irradiation (5 min) with methanol–chloroform (1:2)	Methanol–chloroform (1:2) with SrO	32.8
IDT	–	Methanol–chloroform (1:2) with SrO	6.25
IDT	–	Sonication (5 min) with methanol–chloroform (1:2) and SrO	20.9
IDT	–	Microwave irradiation (5 min) with methanol–chloroform (1:2) and SrO	37.1

In general, at industrial scale, techniques like high pressure homogenizer (HPH), Hughes press and bead mills are employed. Chemical and enzymatic lysis is not preferred as they involve additional unit operation steps. For instance, though disruption by osmotic shock consumes less energy (Table 4), it requires the additional step of recovering the salt water. This would be energy intensive and consequentially affect the scalability of the process. The pilot scale feasibility aspects for a cell disruption technique include continuous processing of highly viscous biomass in a shorter time period with minimal energy consumption and minimal product degradation (Yap et al. 2015). In addition, the disruption

techniques should be less sensitive to the type of strain under use. Analysis by Yap et al. suggests HPH is scalable and energy efficient at process scale, when the operated biomass paste is high in solid content (5–25% W/W). Since the solid content in the harvested algal slurry also falls in the same range HPH can be effectively scalable. The energy consumed by HPH at 150 MPa pressure for 25% w/w algal slurry was approximately around 0.9 MJ/Kg biomass, which is significantly less when compared to energy consumed by osmotic shock lysis (Table 2).

A different approach to wet way of extraction has also been proposed. It is a one step process, in which the algal suspension is directly subjected to oil extraction doing away with an additional biomass recovery process and is discussed in detail in the later Sect. 6 of this chapter.

5 Algal Oil Extraction

As discussed earlier oil extraction can be carried out directly with dried biomass or the wet biomass is pre-treated for cell rupture and released lipids are recovered by extraction. Traditional method of extraction involves the application of organic solvents. The common and accepted methods include Soxhlet that uses non-polar solvent hexane, Folch (Folch et al. 1987) and Bligh and Dyer (Bligh and Dyer 1959) methods that involve the use of co-solvent system comprising a mixture of non-polar and polar solvents, viz., chloroform and methanol.

5.1 Evolution in Algal Oil Extraction

Though above mentioned methods are well established, they require high energy input and longer extraction time. For the same reason, achieving sustainability in algal fuel production is majorly dependent on oil extraction process. Lots of developments are being made so as to make the extraction process cost effective. A brief analysis of the same is discussed in the following section.

5.1.1 Extraction from Wet Biomass

As discussed in Sect. 4.1, directly processing the wet biomass for extraction can save energy consumption up to 60%. Unfortunately, extraction from wet biomass decreased the yield of lipid. In fungus *Mortierella alpine,* Bligh and Dyer extraction by wet way resulted in oil recovery of 21.6% whereas through dry way it was 41.1% (Zhu et al. 2002). When Soxhlet method was tried on *Chlorococcum* sp. with 70% water content, a 33% decrease in lipid yield was observed as compared with dry *Chlorococcum* sp. The lipid yield with dry biomass was 0.015 g/g algae, whereas with wet biomass it was 0.01 g/g algae (Halim et al. 2011). The decrease in yield is

attributed to the lack of miscibility between water and the non-polar organic solvents since this prevents the direct contact of algal cell with organic phase (Halim et al. 2012). Extraction from wet biomass has also been tried with miscible solvents which interestingly resulted in an improved oil yield. When 2-Ethoxy ethanol (2-EE) was used for oil extraction, for the same amount of biomass the TAG yield for wet biomass was ~3.8 mg and for dry it was ~2 mg. Thus 2-EE extracted double the amount of oil extracted from wet biomass as compared to dry (Jones et al. 2012). But it was failed to notice that this method also resulted in increased chlorophyll extraction that can affect the purity of the oil and can also lead to error prone estimates in lipid yield which is discussed in detail in Sect. 7.1. Also since 2-EE is miscible with water, it would make the solvent recovery cumbersome.

5.1.2 Heat Assisted Extraction from Wet Biomass

The major difficulty associated with direct solvent extraction from wet biomass is solvent penetration. To improve the contact between the alga cell and the solvent phase, extraction assisted by increase in temperature was tried. Heating could be achieved either by conventional thermal heating or by electromagnetic waves like microwave and ultrasonic wave. Wahidin et al. (2014) compared the oil yield from 80% wet *Nannochloropsis* sp. with thermal heat assisted and microwave assisted hexane-methanol (2:1) extraction (Wahidin et al. 2014). The reaction temperature was 65°C for both methods. But the reaction time was 40 min for conventional thermal heating and 5 min for microwave heating. Microwave irradiation assisted extraction was observed to yield higher lipid content (38.31%) as compared to water bath heating (23.01%). Also there wasn't significant change in FAME composition. Balasubramanian et al. (2011) showed that microwave assisted extraction in 84% wet *Scenedesmus obliquus* extracted 77% of the recoverable oil in 30 min at 95°C, but conventional heating could recover only 47% (Balasubramanian et al. 2011). It is obvious that microwave heating has achieved higher lipid yield in short span of time compared to conventional heating. This is because microwave heating is rapid, provides quick energy transfer by reducing the thermal gradients and aids in faster penetration of the extractant. For instance, what could be achieved with conventional heating in 80 min can be achieved in 1 min with microwave (Lühken and Bader n.d.). Microwave heating results in only one third of the average costs of conventional heating (Wahidin et al. 2014). As a simultaneous time and energy saver microwave assisted extraction looks exciting. Check Sect. 6.1 for few additional details on microwave extraction. Earlier Cheng et al. (2013) have also compared bath assisted and microwave assisted chloroform–methanol (1:1) extraction in *Chlorella pyrenoidosa*, but didn't find significant difference in lipid yield between the two (Cheng et al. 2013). Nevertheless, the authors found microwave assisted in-situ trans-esterification to be efficient.

5.1.3 Eco-Friendly Extraction

Most organic solvents employed in lipid extractions are toxic (Mercer and Armenta 2011). Also, handling large volumes of solvent and their vapours can represent risks of fire and explosion. Terpenes, natural solvents found in citrus fruits and many other plants, have gained attention as green solvent for extraction (Tanzi et al. 2012). They have already been demonstrated for industrial extraction of rice bran oil (Mamidipally and Liu 2004). In *Chlorella vulgaris,* extraction with terpene solvents *d*-limonene and p-cymene gave better yield when compared to extraction with hexane, whereas with solvent α-pinene, another terpene, the yield was comparable. p-*cymene* and d-limonene extraction resulted in 72.7% and 46.5% increase in lipid yield with respect to hexane extraction. No change in FAME composition was observed across different solvents used (Tanzi et al. 2012). In *Nannochloropsis oculata* also, p-cymene was proved to be efficient. The lipid yield was 21.5% of dry weight with p-cymene in, which was higher than that with hexane (8.31%). With *d*-limonene and α-pinene the lipid yield was 18.73 and 18.75%, respectively. The time of extraction for terpenes was 30 min, but for Soxhlet extraction it was 8 h. The energy analysis showed that Soxhlet method consumed 8.84 kWh, whereas with terpene extraction, it was 2.15 kWh. There is a considerable saving in time and energy, and hence the cost too (Dejoye Tanzi et al. 2013). Thus using terpene solvent offers a safe, efficient and cost-effective oil extraction. Further with microwave assistance, terpene solvent extraction can be successful with wet biomass too, thereby saving extra energy and time.

5.1.4 Supercritical Extraction

Extraction with organic solvent requires additional unit operation of evaporation, to recover the solvent from the extracted lipids. Solvent recovery can be energy intensive and recovery is not 100%. A 0.5 to 5% loss is possible. As an alternative, supercritical fluid extraction has been used. It results in a highly purified extract free of toxic solvents and doesn't require energy to recover the extractant (Sahena et al. 2009). Super critical fluids have increased solvating power above their critical temperature and pressure points. Supercritical carbon dioxide ($SCCO_2$) is the primary solvent used in the majority of supercritical fluid extractions. $SCCO_2$ offers a safe extraction due to its inertness, low toxicity and low flammability (Macías-Sánchez et al. 2007). Their advantages as a green extractant include the following:

- Their transition between liquid and gas phase allows faster fluid penetration across cellular matrices, and results in higher lipid yield in a short time span (Halim et al. 2012).
- The critical temperature of CO_2 is relatively low (31.1°C) (Macías-Sánchez et al. 2007) and allows for successful extraction of thermo-labile lipid fractions without the risk of degradation. Further, the cost of compression is also rather modest owing to its moderate critical pressure of 72.9 atm (Halim et al. 2012).

- The solvating capacity of $SCCO_2$ depends on its density. By changing the pressure of extraction the density can be adjusted to tune the solvating capacity such that it interacts primarily with neutral lipid fractions (Halim et al. 2012).
- At room temperature CO_2 decompresses to gas and precipitates out the extracted lipid (Halim et al. 2011). Thus complete solvent recovery is possible and is zero energy intensive.

In dry *Chlorococcum* sp., 80 min of $SCCO_2$ extraction resulted in oil yield of 0.058 g/g of algae, but even after 5.5 h of Soxhlet, it was 0.032 g /g of algae. It is a substantial improvement in a short span of time (Halim et al. 2011). It is said that $SCCO_2$ extraction will not work with wet biomass as the water content will act as a barrier for contact between alga cell and $SCCO_2$ (Mercer and Armenta 2011). Despite this fact Halim et al. (2011) achieved higher yield of lipid with 70% wet biomass of *Chlorococcum* sp. With $SCCO_2$ extraction, wet lipid yield was 0.071 g/g of algae, while that of dry was 0.058 g/g of algae. Hence $SCCO_2$ extraction can be efficient with wet biomass too. Further the efficiency can be improved when assisted with microwave irradiation for a shorter period. In dry *C. vulgaris*, 6 min of microwave treatment improved the $SCCO_2$ extraction efficiency by 2.6 folds (Dejoye et al. 2011). This will be applicable for wet biomass too. In addition $SCCO_2$ extraction produces equivalent FAME profile to those obtained with conventional organic solvent extraction (Xu et al. 2008).

The major demerit is that $SCCO_2$ extraction is considered costly due to the high energy associated with fluid compression and heating. In addition, installation expenses of the pressure vessel for $SCCO_2$ extraction are also high (Halim et al. 2011). However, this could be balanced by the zero energy intensive solvent recovery process. Further as discussed in Sect. 5.1.2, instead of conventional heating, if microwave heating is applied, efficiency can be improved and both time and energy can be saved as well. The installation of high pressure vessel is a onetime investment and considering the advantages of $SCCO_2$ extraction, it may be worth investing.

5.1.5 One Step Oil Extraction

Originoil, now known as Originclear, is a leading provider of water treatment solutions based in Los Angles. In the year 2009, they proposed a breakthrough process for extracting oil on continuous process from algae without killing it and obtained patent for the same in 2012. It is a one step process that combines harvesting and extraction. The algal cells in suspensions are subjected to Quantum Fracturing™, a process that combines electromagnetic field generation and modification in pH by CO_2 pumping, which causes the cells to release a portion of its lipids that can be collected. The cells, some or all are able to sustain the process and stay alive to go through additional lipid production in multiple cycles. The oil released floats at the top, thereby allowing the oil, water and biomass to be separated in less than an hour in a gravity settler (US 20120040428 A1). The

process was successful at lab scale and later was scaled up to 200 gallon tank size (report not available). This new technology reduces the time and energy expenditure to a greater extent and does not involve use of chemicals or heavy machinery.

Though some of the above discussed extraction techniques look efficient and cost effective, it also possible to directly trans-esterify the oil from whole biomass, by circumventing the extraction step and is discussed in the following section.

6 Modes of Trans-esterification

The oil from algae is not fit for direct use in engines and needs a conversion process called trans-esterification to produce biodiesel (Fuls et al. 1984). Traditionally biodiesel production from algae involve multiple steps, such as drying, oil extraction followed by trans-esterification (three-step approach). Though this method is industrially adapted, the whole procedure is energy intensive and the processing cost is high (Chisti 2008). With main research focus on cost reduction, improvised techniques were proposed. As discussed in Sect. 5 of this chapter, several improvements were brought about to make the lipid extraction cost efficient. Similarly, another approach of simultaneous lipid extraction and trans-esterification, known as direct trans-esterification or in-situ trans-esterification, was proposed as a cost-effective alternative (Johnson and Wen 2009). This method, by combining lipid extraction and trans-esterification into one step, simplifies the downstream pathway, thus decreasing costs and minimizing the solvent usage. In addition, direct trans-esterification produces biodiesel with high purity (>99%) as compared to the indirect process where purity was in the range of 91–98% (Vicente and Bautista 2009). The development in trans-esterification process is summarized in Fig. 4

6.1 In-Situ Dry Trans-Esterification

Several researches have tried direct trans-esterification with dry algal biomass, called in-situ dry trans-esterification (IDT), which is a two-step approach comprised of drying and trans-esterification. From Fig. 3, it can be seen that if the extraction step is eliminated for dry biomass, the energy consumption of 8.6 MJ/kg of biomass can be saved. Of course the number looks small, but at pilot scale this will contribute to significant amount of power saving. Unfortunately direct dry trans-esterification resulted in decreased yield. In *Chlorella vulgaris,* IDT gave a FAME yield of ~14% with respect to oil extracted whereas the conventional method resulted in FAME yield of ~27%. This might be due to the poor penetration of trans-esterification reaction mixture (TRM) through the thick cell walls of *C. vulgaris* (Prommuak et al. 2012). But in *Schizochytrium limacinum,* though it has thin cell walls (Honda et al. 1999), IDT resulted in decreased FAME yield as compared to the traditional method (Johnson and Wen 2009). However, when

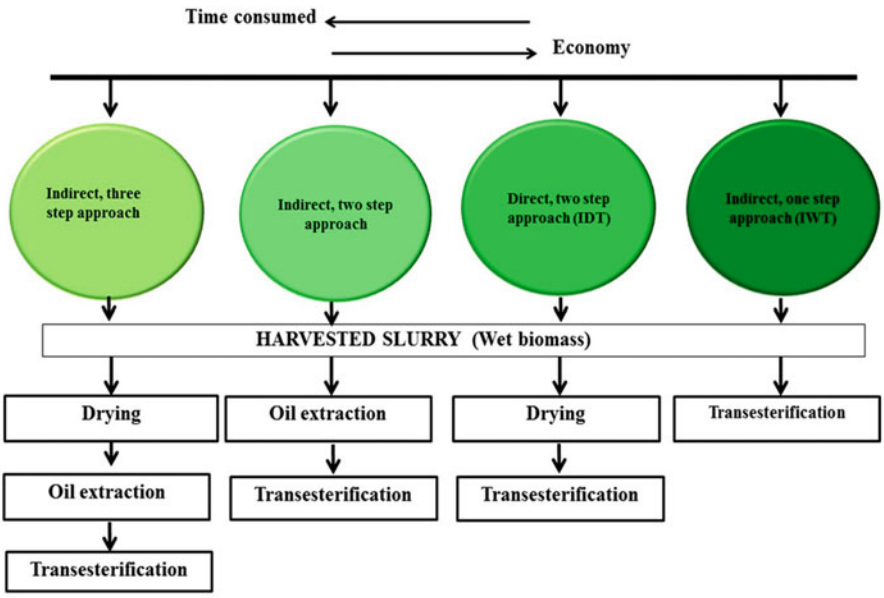

Fig. 4 Development in trans-esterification procedures

chloroform was included in the TRM for IDT, the obtained FAME yield was higher (72.79% of oil) when compared to indirect trans-esterification (63.7% of oil). In *Nannochloropsis* sp. unless the IDT is simultaneously assisted by sonication or microwave, there wasn't any improvement in the FAME yield when compared with traditional method (Koberg et al. 2011). The different methods studied by Koberg et al. and their respective lipid yield are summarized in Table 4. Similarly, improvement in fatty acid ethyl esters (FAEE) yield for IDT was obtained in *Chlorella* sp. when assisted with ultrasound or microwave. Thus IDT with additional assistance either with solvent or electromagnetic waves shows improvement in FAME yield and considerably reduces the time of biodiesel production. The reason behind microwaves improved efficiency in shorter time period is by rapid heating as discussed in Sect. 5.1.2. As for energy consumption, microwave assisted trans-esterification consumed relatively less energy when compared to the conventional heat assisted trans-esterification. For example, when waste cooking oil was trans-esterified by microwave heating, 94% conversion was achieved in 2 min with 288KJ of energy. On the other hand, conventional heating for same amount of oil (amount not mentioned) took 100 min for 92% conversion and ended up consuming 3150 KJ of energy (Gude et al. 2013). By reducing the reaction time microwave does a drastic saving in energy consumption. Thus microwave heating assisted extraction and trans-esterification possess scale up potential, but not to be concluded unless demonstrated.

6.2 *In-situ Wet Trans-Esterification*

Another approach to direct trans-esterification involves using the wet biomass (harvested slurry), called as in-situ wet trans-esterification (IWT), a single step procedure that eliminates both drying and extraction operations. Thus IWT is very less energy intensive and the process can be economically sustainable. For instance, as seen from Fig. 3 the total energy consumption for biodiesel production through IWT would be 10.6 MJ/kg of biomass which is significantly less when compared to conventional trans-esterification methods. However, as with IDT, the earlier studies with IWT were also unsuccessful. In *S. limacinum,* there was a tremendous drop in FAME yield from 72.79% of algal oil to 8.45%. With increase in residual water content in algal biomass there will be a progressive decrease in FAME yield. For 50% water content the FAME yield obtained would be half of that with 0% water content, i.e. through IDT (Wahlen et al. 2011). A modification in IWT was proposed by Cao et al. (2013) which resulted in significant improvement in FAME yield comparable to that through IDT (Cao et al. 2013). Initially, the presence of water in harvested slurry of *Chlorella pyrenoidosa* had negative effect on FAME yield when IWT was carried out at 90°C. With increase in water content from 0 to 90%, the FAME yield decreased from 91.4 to 10.3%. But when the temperature was raised to 150°C, the FAME yield became independent of water content. With 90% water content and at 150°C the FAME yield was almost 100% of algal oil, which is in fact higher than that obtained through IDT (91.4%) at 90°C. Thus the increase in trans-esterification temperature improved the efficiency of IWT. But at the same time IWT at high temperatures can be energy intensive. Instead if high temperature assisted IWT (HT-IWT) is performed with microwave heating, HT-IWT can be ideal energy saver.

7 Errors in Bio-Oil Estimations

The total oil yield obtained from a particular fuel production process may be lower than expected. The cost of the final product may also be more bloated than anticipated. More often than not, these discrepancies are a result of errors in the estimation of lipids and lead to inaccurate conclusions regarding the feasibility of algal fuel systems.

7.1 *Errors from Strain Selection*

The most often encountered error with the quantification of lipids is the measurement and reporting of total lipids instead of neutral lipids which are the actual precursors to biodiesel. The use of the Bligh and Dyer method is an example where

overestimation of the oil potential occurs, if it is used as the sole source of measurement.

Strain selections for large scale biodiesel production are based on screening them for oil production. The screening is traditionally carried out by determining the lipid yield using the conventional gravimetric method of Bligh and Dyer. This method is, however, error prone as it simultaneously extracts lipids as well as its derivatives such as chlorophyll leading to overestimations (Archanaa et al. 2012). The chlorophyll content can vary from 1 to 25% of lipid weight (Su et al. 2008). Thus, the strain so selected may not necessarily be a viable candidate for oil production. Consequently, the strain may not perform as well as expected thereby negatively impacting the feasibility of the production process

7.2 *Errors in Validation of Screening and Extraction Methods*

Further, although other lipid screening techniques have emerged, such as the use of Nile red fluorescence (Chen et al. 2009) and dielectric analysis (Higashiyama et al. 1999), these tend to be evaluated for their efficiencies using the Bligh and Dyer method as reference.

The same error also leads to incorrect prediction in efficiency of lipid extraction methods. As discussed earlier, several extraction methods have been developed for algae so as to cut down the biodiesel production cost. The efficiency of these newly developed methods is also determined based on its comparison with conventional Bligh and Dyer method and Soxhlet.

For example, in *C. vulgaris*, oil extraction with terpene solvents had lower yield when compared to Bligh and Dyer but higher than Soxhlet (Tanzi et al. 2012). Based on this, it cannot be concluded that oil extraction with terpenes is inefficient when compared with Bligh and Dyer as the latter extracts chlorophyll as well. Similarly, concluding that extraction with terpenes is more efficient than with hexane in the Soxhlet method is also unwise as the use of terpenes could have led to higher chlorophyll extraction.

The above views are also supported by the polar nature of chlorophyll. Chlorophyll shows greater affinity to the more polar methanol–chloroform mixture used in Bligh and Dyer, slightly decreased affinity to the relatively less polar terpenes and negligible affinity to non-polar hexane.

Thus, organism and lipid extraction method comparisons should be preferably carried out following the removal of chlorophyll. Better comparisons can be made based on the individual FAME yield obtained by GC-MS. By comparing FAME yield in *N. oculata* as already discussed in Sect. 5.1.3, Tanzi et al. (2012) found that the yield was not so different between Bligh and Dyer method and terpenes, but higher than the Soxhlet method (Dejoye Tanzi et al. 2013). Thus a conclusion based on FAME profile can be said to be more reliable.

Decolourization of the residual algal biomass after extraction is sometimes related to extraction efficiency. For instance, Jones et al. (2012) proved that 2-EE is a better alternative to hexane by estimating the amount of extracted TAG via HPLC (Jones et al. 2012). The authors, however, suggested that the degree of decolourization in residual algal biomass of *Chlorella* sp. can be used to indicate efficiency. This was concluded from the observation of the wet algal solid extracted with hexane being green, while it was completely decolourized with 2-EE. The decolourization was, however, a result of the loss of pigment (chlorophyll removal) and would not by itself serve as a good indication of extraction efficiency.

Such errors that are possible at the lab scale can lead to a negative notion on the feasibility of the process at the pilot scale. Knowledge of these errors is thus vital to performing lab scale validations.

7.3 Errors from Inappropriate Assumptions

A number of assumptions regarding the lipid content, lipid composition and oil yields are made by studies based on data available from literature on other algal strains or species. Assumptions for algae have also been made based on data from oil seeds and plants. These can often lead to large differences on scaling up the process. Extrapolation of laboratory data to large scale is yet another source of error.

8 Assessment of Sustainability

Studies on algal fuels present contradictory results. The global views on the use and sustainability of the fuel are ambiguous. While many authors report favourable economics and ecological impacts, others elaborate on shortcomings and the impracticality of existing strategies. If algal fuels are to serve as alternatives to fossil fuel, it needs to be economically, ecologically and socially sustainable. This requires a complete and comprehensive assessment of its sustainability. Most studies use too many assumptions, incorporate inadequate data and thus result in erroneous analyses. A number of methods to systematically assess sustainability of various processes are available. These can be used to evaluate individual stages like lipid extractions or extended to entire algal production pathways. As already discussed earlier in this chapter, several methods of biomass processing and oil extractions are available. The different techniques can be systematically evaluated for overall sustainability using these assessments and the suitable ones employed. In order to make an informed decision regarding the production of algal biofuels, a combination of these assessments may be required. Some of the most recommended assessments are discussed here.

8.1 Sustainable Process Index

The Sustainable Process Index (SPI) is a measure formulated by Krotscheck and Narodoslawsky in 1995 to evaluate the ecological impact and economic viability of various processes (Krotscheck and Narodoslawsky 1996). The concept is based on the assumption that solar energy is the primary input for all natural and consequently anthropogenic processes. This energy is converted to various products and services. The manifestation of solar energy into products and services, however, requires the use of land area which is finite. Thus area is considered the limiting factor for a sustainable economy and it forms the basic unit for SPI determination.

The burdens contributed by a process life cycle on the environment are first evaluated in terms of mass and energy flows. The flows induced by human activities are referenced to natural flows and subsequently aggregated to area. A number of activities constitute to the manufacture of a product or service. The total area (A_{tot}) must include area for raw materials (A_R), provision of energy (A_E), physical installation (A_I), staff support (A_S) and the area required for disposal of waste and dissipation of emissions and by-products (A_D), and is calculated as in Eq. (1) (Narodoslawsky and Krotscheck 2004):

$$A_{tot} = A_R + A_E + A_I + A_S + A_D \tag{1}$$

The impact of a single product or unit service (a_{tot}) is thus obtained by dividing A_{tot} by the number of products/services obtained from the process.

The SPI finally compares this area thus required for a product with what is actually available (a_{in}) and can be expressed as Eq. (2)

$$\text{SPI} = \frac{a_{tot}}{a_{in}} \tag{2}$$

Thus comparison of different algal fuel production pathways amongst themselves or with other fuels is possible by computing the SPI and assessing the relative 'expense' of each product/service (since useful by-products other than just fuel may arise from a particular production strategy). It further allows identifying the bottlenecks of a process that demand high area and will help in optimizations that allow for a more sustainable and economic process.

8.2 Environmental Indicators

The status of environmental, social and economic sustainability associated with a product or process is assessed with the help of indicators. A number of bodies strive to define appropriate indicators for determining the sustainability of biofuels and hundreds of indicators have been proposed. Certain criteria for the selection of these indicators have been proposed by the Roundtable on Sustainable Biofuels, the

National Biodiesel Board and several researchers (Mata et al. 2013; Efroymson and Dale 2015). They are listed below:

- Simple, accessible, widely applicable, practical, sufficient and non-redundant
- Generic to all feedstock
- Adaptable and accommodating to update information
- Efficient and inexpensive to measure
- Predictable and responsive to management.

A generic set of bioenergy indicators suggested by McBride et al. (2011) included GHG emissions, soil and air quality, water quality and quantity, biodiversity, and productivity. Efroymsen and Dale proposed 16 specific environmental indicators spread across the abovementioned six broad categories (Efroymson and Dale 2015). The indicators were selected for algal biofuels and included the bulk density of soil, salinity, phosphate and nitrate concentration in water, consumptive water use, peak storm and minimum base flow of water, presence and habitat of any taxa of concern, released algae abundance, CO_2 and N_2O emissions, tropospheric ozone, carbon monoxide concentration in the air, individual measurements of total particulate matter that were less than 2.5 mm diameter and 10 mm diameter and productivity.

Indicators considered essential for biofuels by Mata et al. (2013) are listed below (Mata et al. 2013):

1. Life Cycle Energy Efficiency (LCEE): It gives the efficiency of the fuel produced and is computed as the ratio of the total energy content of the fuel and co-products produced to the total energy consumed by the process.
2. Fossil Energy Ratio (FER): It is the ratio between the energy content of the final fuel product and that of the fossil energy consumed for the production.
3. Contribution to Global Warming (GW) or Carbon Footprint (CF): The concentration of different GHG emissions such as CO_2 and CH_4, which can lead to global warming, are expressed here as equivalent CO_2 emission per unit energy of fuel product.
4. Land Use Intensity (LUI): It is obtained by measuring the total land area employed for production per unit energy of the fuel.
5. Carbon Stock Change Emissions (CSCE): These account for the annualized emissions from carbon stock changes that arise from changes in land use.

8.3 Cost Benefit Analysis

A cost benefit analysis compares the costs associated with a proposed action with the benefits that are to be derived from it. The comparison is most often carried out in monetary terms. An economic cost benefit analysis of algal fuel systems gives information on the feasibility and profitability of the process. A number of cost benefit analyses have been carried out on the first and second generation biofuels. Fewer studies exist on algae due to its relatively recent application and data

limitations. The costs associated with each stage of the production process, both direct and indirect, are estimated and compared against the net benefits obtained. For example, a comparison of cost benefit analysis of fossil fuels, algal biodiesel and rapeseed biodiesel carried out by Kovacevic and Wesseler 2010 revealed that algal and rapeseed biomass conversion was dominated by the benefits from by-products but the energy recovery cost in algae being very high, nullified some of these benefits (Kovacevic and Wesseler 2010). The benefit of greenhouse gas (GHG) mitigation was found only with algal fuels. The estimated costs for pesticides and fertilizers were low for both biofuels. GHGs and negative impact on food prices amounted to significant costs in rapeseed and fossil fuels. The study concluded that a significant reduction in the cost of algal production processes is required for its commercialization (Kovacevic and Wesseler 2010). Decisions can thus be made based on such analyses. A number of factors exist that cannot be translated into monetary terms. While the cost of algal fuel is found to be higher than fossil fuels in this study, environmental benefits are also expected. Thus depending on the sustainability perspective, which is not always purely monetary, decisions can be made.

8.4 Life Cycle Assessment

A life cycle assessment (LCA) is a concept that involves scrutinizing the entire life cycle of a product/process from procuring of raw materials to packaging of the product and disposal of wastes (Georgianna and Mayfield 2012). It involves breaking down a process to various fragments and assessing it for overall economic feasibility and environmental impacts. LCA studies mostly involve a 'cradle to grave' approach to help identify crucial bottlenecks in a process such as the most energy draining stages or those that have significant impacts on the environment (Boer et al. 2012; Kumar et al. 2010). The approach is expected to help efficiently design processes by tailoring them for optimum and economic productivity while not compromising with the environment. The concept of LCA emanated from environmental concerns in the 1960s (Williams 2009). In the following decades, the concept greatly improved and is now much encouraged by various governments and academicians. Two international standards are currently available for LCA (i) ISO 14040 (2006E) that defines the principles and framework for LCA and (ii) ISO 14044 (2006E) that lays down requirements and guidelines.

A life cycle assessment for an algal fuel production will help isolate the key processes in the production that hamper its commercialization or burden the environment. Improving these identified processes could lead to a more economical and environmentally feasible production. LCA comprises of four steps as briefly described below (Williams 2009):

1. Defining the Goal and 'Scope' of the study: The goal of the study, its intended application, functional unit, system boundaries, data specificity and presentation,

assumptions and allocations are defined. A functional unit is an arbitrary unit that is defined in order to relate input and output processes such as 'x kilograms' of alga biomass produced or 'y mega joules' of biodiesel produced (Udo de Haes et al. 2006). It is extremely essential as it allows comparison between LCA studies of competing systems. A system boundary or perimeter is defined in order to establish what unit processes/operations are included in the LCA such as algal cultivation, harvesting, drying and transportation.

2. Life Cycle Inventory (LCI): The LCI of the biofuel production process being analysed includes an inventory of the biofuel production pathway. Data if not available from first-hand studies and literature is often collected from the USLCI database or the Greenhouse Gases, Regulated Emissions and Energy use in Transportation (GREET) model.
3. The life cycle impact assessment (LCIA): The environmental impacts of the process are assessed here based on the results from the LCI such as management of co-products, energy balance, carbon and water footprint.
4. An assessment report: The data from the assessment along with its evaluation and interpretation are discussed here. The final conclusions and recommendations from the study are stated.

8.4.1 Existing LCA Studies

A number of studies have attempted LCA for algal biofuel. A review by Singh and Olsen (2011) observe that nutrient requirement could be reduced to half by recycling harvest water (Singh and Olsen 2011). Use of seawater or wastewater could decrease the freshwater requirement to 10% of the original used in the particular LCA study. Energy balances became favourable with the recycling of carbon and the use of raceway ponds and flat plate photobioreactors (PBRs) instead of tubular PBRs. Greenhouse gas (GHG) emissions from algae were comparable to those from canola and ULS diesel whereas costs were not. Other studies concluded that biomass drying and solvent extractions were the most energy expensive processes contributing to 90% of the net energy consumed. An examination of LCA studies by Slade and Bauen (2013) suggest that the overall energy input to the production system is higher than desirable indicating that algal biomass production does not look attractive when carried out for energy generation (Slade and Bauen 2013). However, a number of alternatives to the processes used in the existing study are available. For example, it was shown that cultivation of biomass in raceway ponds rather than PBRs resulted in a more favourable energy balance, which has been reported in previous studies. Tweaking the production strategy by altering the unfavourable processes is thus possible and could improve the energy balance. This review also agreed that productions costs could be reduced to half provided low cost nutrients and water were obtained.

Most of these studies are, however, incomplete in many respects and thus their results cannot be deemed conclusive and accurate. Some of the common

inadequacies of existing LCA studies on algal biofuels are listed below (Kumar et al. 2010; Slade and Bauen 2013):

- Despite the general ISO requirements and guidelines available, the approach suffers from non-homogeneity due to lack of appropriate standardizations specific for LCA of algal fuels.
- Since data of large scale production is either unavailable or proprietary, several studies have extrapolated lab scale data to large scale for the analysis.
- Data gaps are often filled by assumptions based on existing literature of different algal species or even plants.
- Data specificity is often ignored. Data specific to a geographical region cannot be extrapolated to another region or country without errors just as data procured over a particular season or time cannot be generalized for the whole year.
- Impacts to the environment that arise indirectly from the biodiesel production process such as land required for waste disposal/management of co-products are often not included within the system boundary rendering the study incomplete and subsequently inaccurate.
- Variations in assumptions, system boundaries, functional units and analysis methodologies used in the different studies make comparison between them difficult.

An attempt to address these issues has been made by Collet et al. (2015) following a detailed investigation of 41 LCA studies and a number of recommendations have been made to this end (Collet et al. 2015).

Despite the non-homogeneities between the various LCA studies, all LCA analyses indicate that production of algal biofuel is currently weighed down by both economic and ecological disadvantages (Boer et al. 2012). However, identification of energy expensive processes via LCA allows room for improvement of these processes (Fields et al. 2014). Thus with appropriate standardizations, LCA can prove a very effective tool in determining impacts and consequences of a particular algal fuel production process which in turn would serve to design a more practical production strategy.

8.5 Ecosystem Service Analysis

Ecosystem services refer to the benefits derived by human beings from their interaction with the ecosystem. The Millennium Ecosystem Assessment (MEA) commissioned by the United Nations in 2000 defined ecosystem services as “the benefits provided by ecosystems to humans, which contribute to making human life both possible and worth living”(National Research Council 2013). These services include both tangible benefits that can be easily translated to monetary values and intangible benefits. Increase in population and growth of the economy demands an increased requirement of the services. In order to allow for a sustainable fulfillment of these requirements without detrimental effects to the ecosystem, trade-offs are

required. Ecosystem service analyses are thus conducted to enable making of informed decisions regarding the exploitation of these services.

While a number of assessments or analysis of algal biofuel life cycle are effective in incorporating the evaluation of net energy ratios, greenhouse gas emissions, water footprint, etc., they do not account for the effects on ecosystem services (Zaimes et al. 2015). Sustainability assessment of algal biofuels would therefore be incomplete without this analysis. Ecosystem services can be distributed into the following categories (Joly et al. 2015):

- Provisioning services such as fuels, food and water. Algal biofuel is a source of fuel. However, its production also demands the consumption of fossil fuel. Food/fodder may be obtained as by-products from the different fuel production pathways.
- Regulating services such as soil and water quality regulation and climate regulation: While biofuel productions may negatively impact the quality of water, its production when coupled with wastewater treatment can be said to improve water quality. Spent biomass has been suggested as a good source of phosphor and could substitute for chemical fertilizers. The net greenhouse gas balance of the concerned pathway would alter the climate.
- Cultural services that may be aesthetic, recreational, spiritual, etc. Non-arable lands can be used and managed for algal cultivation.
- Supporting services such as habitat formation and nutrient cycling.

Since there is considerable debate regarding the beneficial and damaging impacts of algal biofuels on ecosystem services, its thorough analysis would be desirable right at the planning stage to predict and avoid any negative impacts that the production strategy and fuel use may have on the ecosystem services.

9 Conclusion

Designing an efficient, sustainable (both economic and ecological) and safe method of lipid extraction and obtaining maximum FAME recovery is still a challenge in algal fuel industry. As discussed in Sects. 5 and 6, researchers have tried a range of extraction techniques and performed comparative studies. Though few techniques like toxic solvent free SCO_2 and IWT have been said to be yield and energy efficient and suggested to be feasible at pilot scale, the conclusion is based on lab scale performance. This chapter also attempted to compare the feasibility of certain extraction and trans-esterification techniques based on the energy consumption data from the literature. However, there is no rational way to integrate and compare results from various investigations as they are performed under different conditions with different strains and often different methods.

Thus as discussed above in Sect. 8, an elaborate and ‘wholesome’ assessment of the biofuel production pathway is essential. Existing studies on various algal fuels are incomplete and error prone due to the multiple reasons cited throughout the

chapter. A combination of one or more of the available assessment strategies may be employed to analyse the sustainability and feasibility of the pathway. As pointed out earlier, several ecological services and products cannot be easily translated into monetary equivalents. These, however, are often crucial to environmental sustainability and must thus be accounted for. Standard guidelines that would help incorporate such aspects are thus desirable. The identification of process bottlenecks from such analyses, isolate those operations that call for economical alternatives, allowing for the design of better algal fuel production pathways. Therefore, while immediate commercialization of the algal fuel is not possible, refining these production pathways based on their sustainability assessment data could very well allow for its commercialization in the future.

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Catalytic Conversion of Microalgal Lipids to Biodiesel: Overview and Recent Advances

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Contents

1 Introduction

Biodiesel is a renewable and sustainable alternative to the petroleum fossil fuels. Biodiesel can be produced from variety of feedstocks such as edible, non-edible oils, animal fats, and microalgal lipids (Ma and Hanna 1999; Sharma et al. 2008). Fast growth rates and high lipid accumulation have proven microalgae as a promising

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feedstock for biodiesel production (Chisti 2007; Rawat et al. 2013). Catalytic conversion of feedstock oil to fatty acid alkyl esters (FAAE), i.e., biodiesel is the most widely used method (Helwani et al. 2009). The conversion process depends upon number of factors such as quality of feedstock oil, choice of catalyst, acyl acceptor, use of solvent, and reaction parameters. The recent advances such as process intensification by microwave and ultrasonication have improved the yields of catalytic conversion of lipids to biodiesel. This chapter deals with the brief overview, recent advances, and challenges in catalytic conversion of microalgal lipids to biodiesel.

2 Microalgal Lipids as a Feedstock for Biodiesel

Microalgal lipid is considered as a greener and sustainable feedstock for biodiesel synthesis. Microalgae have faster growth rates and higher lipid accumulation capability than the terrestrial plants. Microalgae also offer other environmental benefits such as CO_2 mitigation and wastewater utilization. Microalgae have shown lipid accumulate of up to 20–70% lipid per dry cell weight. Various microalgal strains such as *Chlorella, Dunaliella, Nannochloropsis, Scenedesmus, Neochloris, Nitzschia, Porphyridium, Phaeodactylum*, and *Isochrysis* have been studied for assessing their potential as a biodiesel feedstock (Amaro et al. 2011). Ensuring high lipid accumulation in microalgae is a crucial parameter for making the biodiesel production process economical. Microalgal lipid accumulation can be enhanced by altering the cultivation conditions and nutrients in the media (Singh et al. 2016a). Nitrogen, light, and CO_2 stress are widely used strategies for enhancing lipid accumulation in microalgae (Singh et al. 2016b).

Microalgal oils are composed of neutral lipids, polar lipids with some amount of hydrocarbons, sterols, waxes, and pigments (Singh et al. 2014). The neutral lipids are considered as most suitable for the biodiesel synthesis because of their easy conversion to fatty acid alkyl esters (FAAE). In microalgal cells neutral lipids act as the energy storage components and are mainly composed of triglycerides (TAG) and some amount of free fatty acids (FFA). While the polar lipids serve the structural roles (phospholipids in cell membrane) as well as physiological functions such as cell signaling (sphingolipids) (Sharma et al. 2012). Triglycerides and free fatty acids both can be converted into biodiesel via transesterification and esterification process, respectively. However, free fatty acid could cause saponification during the reaction if alkali catalysts are used. Microalgal lipids are known to contain high free fatty acid content.

Biodiesel fuel properties are influenced by the number of carbon atoms in the chain, degree of unsaturation, percentage composition of saturated and unsaturated fatty acid in microalgal lipids. Thus it is important to choose microalgal strains with suitable fatty acid composition for biodiesel synthesis which complies with the international standards (Guldhe et al. 2015b; Singh et al. 2014). Microalgal lipids are composed of saturated, monounsaturated, and polyunsaturated fatty acids. Microalgal lipids have shown C14:0, C16:0, C18:1, C18:2, and C18:3 as major contributing fatty acids (Song et al. 2013). These fatty acids are considered as most

suitable for quality biodiesel production. The cultivation conditions and nutrients supplied also have an influence on the fatty acid composition of the microalgal lipids. Table 1 depicts the fatty acid compositions and lipid content of different microalgae used for biodiesel production.

3 Transesterification Process

In the process of biodiesel synthesis, triglycerides and methanol are interacted in a reaction called as transesterification or alcoholysis. Methyl esters of fatty acids, i.e., biodiesel and glycerol are the products formed in the transesterification reaction (Cook and Beyea 2000). A catalyst is used to facilitate this reaction via improved rate of reaction and high product yield. Excess alcohol is added to shift the equilibrium of this reversible reaction towards the products side. Alcohols such as methanol, ethanol, propanol, butanol, and pentanol can be used in the transesterification process. Methanol is used more commonly because of its low cost and its physical and chemical advantages (Helwani et al. 2009). NaOH dissolves easily in alcohols and reacts with triglycerides. In a transesterification reaction stoichiometrically, a 3:1 molar ratio of alcohol to triglycerides is needed. Generally the ratio needs to be higher to drive the reaction equilibrium towards maximum ester yield. Biomass-derived fuels share many of the same characteristics as their fossil fuel counterparts. Once formed, they can be substituted in whole or in part for petroleum-derived products. The general reaction mechanism is shown in Fig. 1.

4 Catalysts

The transesterification reaction can be catalyzed by chemical (alkali and acid) or enzyme catalysts. The chemical alkali catalysts include NaOH, KOH, carbonates, and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Sulfuric acid, sulfonic acids, and hydrochloric acid are commonly used as chemical acid catalysts. Enzyme lipases from various sources are used as biocatalyst for biodiesel synthesis. Alkali-catalyzed transesterification is faster than acid-catalyzed transesterification (Benemann 1997). The catalytic conversion has not been meticulously studied for microalgal biodiesel production. Table 2 shows the various catalysts used for conversion of microalgal lipids to biodiesel.

Table 1 Fatty acid profile and lipid content of different microalgae

Microalgal strain	Lipid content (%)	Fatty acid composition (%)									References
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	SFA	PUFA	
Chlorella sp.	30	0.30	0.41	2.41	25.94	3.82	0.47	0.99	–	–	Praveenkumar et al. (2012)
Scenedesmus sp.	16	–	15.62	4.06	2.97	15.23	7.00	22.99	18.59	56.86	Talebi et al. (2013)
Chlorella vulgaris	17.3	–	14.55	1.183	10.51	23.62	13.80	32.10	25.06	70.7	Talebi et al. (2013)
Ankistrodesmus falcatus KJ671624	23.3	–	–	21.15	–	28.18	19.25	14.33	–	–	Singh et al. (2015)
Chlorella vulgaris	20.82	0.78	36.97	5.10	0.5	4.96	4.38	8.42	–	–	Song et al. (2013)
Staurastrum sp.	10	4.97	40	16.50	–	–	–	–	–	–	Song et al. (2013)
Monoraphidium sp. KMN5	20.84	–	41.03	–	17.67	10.16	3.03	1.53	58.7	16.26	Tale et al. (2014)
Scenedesmus sp. KMN4	28.63	–	41.27	–	20.53	9.21	1.91	.57	61.82	12.77	Tale et al. (2014)
Nannochloropsis gaditana.	–	3	16	17.1	–	3.9	8	9	19.4	68.3	Hita Peña et al. (2015)
Neochloris oleoabundans	29	0.43	19.35	1.85	0.98	20.29	12.99	17.43	20.76	64.6	Gouveia et al. (2009)

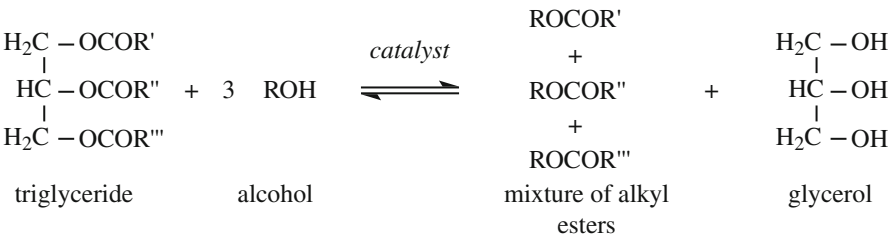


Fig. 1 Transesterification of oils to fatty acid alkyl esters

4.1 Homogeneous Chemical Catalyst

4.1.1 Homogeneous Acid Catalysts

Homogeneous acid catalysts can produce biodiesel from low cost lipid feedstocks, generally associated with high FFA concentrations (cooking oil and greases, FFA>6%). Microalgal lipids are also known to have high FFA concentrations in the lipids. For acid-catalyzed systems, sulfuric acid, HCl, BF_3, H_3PO_4, and organic sulfonic acids have been used by various researchers (Vyas et al. 2010). Mathimani et al. (2015) comparatively studied the transesterification of *Chlorella* sp. Lipids using various types of catalyst. In their study they used homogeneous acid (H_2SO_4), homogeneous alkali (NaOH), heterogeneous acid ($Fe_2(SO_4)_3$), and heterogeneous alkali (CaO) catalysts. The study showed that the maximum biodiesel yield was detected with the transesterification process catalyzed by homogeneous acid catalyst.

The mechanism of the acid-catalyzed transesterification of oils is depicted in Fig. 2. Mechanism represented here is for monoglyceride, it can be applied to di- and triglycerides. The protonation of the carbonyl group of the ester forms the carbocation (II). The carbocation undergoes a nucleophilic attack of the alcohol, which leads to the tetrahedral intermediate (III), followed by elimination of glycerol to form the new ester (IV), and to redevelop the catalyst H^+. According to this mechanism, if water is present in the reaction mixture carboxylic acids can be formed by reacting with carbocation. Thus to avoid the loss of product acid-catalyzed reaction needs to be performed in the absence of water (Schuchardta et al. 1998).

4.1.2 Homogeneous Alkali Catalyst

The most commonly used alkali catalysts for biodiesel synthesis are KOH, NaOH, and CH_3ONa (Gemma et al. 2004). The reaction mechanism for alkali-catalyzed transesterification was determined as a three step process. The alkali-catalyzed transesterification is faster than the acid-catalyzed reaction. The mechanism of

Table 2 Conversion of microalgal lipids to biodiesel by using various types of catalysts

Microalgae	Catalyst	Catalyst loading (% wt/oil wt)	Molar ratio Alcohol: Oil	Reaction conditions [Temperature (°C), Time (Hours), Stirring (RPM)]	Biodiesel yield (Y)/ conversion (C) %	References
Chlorella protothecoides	Sulfuric acid	100	56:1	30, 4160	$Y \approx 60$	Miao and Wu (2006)
Oedogonium sp.	Sodium hydroxide	–	–	–, 3, 300	$Y > 90$	Hossain et al. (2008)
Spirogyra sp.	Sodium hydroxide	–	–	–, 3, 300	$Y > 90$	Hossain et al. (2008)
Nannochloropsis oculata	Al_2O_3 supported CaO	2	30:1	50, 4, 1100	$Y = 97.5$	Umdu et al. (2009)
Chlorella protothecoides	*Candida* sp. 99–125 sp. lipase	30	3:1	38, 12, 180	$C = 98.15$	Xiong et al. (2008)
Scenedesmus obliquus	*Pseudomonas fluorescens* lipase	10	3:1	35, 12, 200	$C = 90.81$	Guldhe et al. (2015b)
Scenedesmus obliquus	*Aspergillus niger* whole cell lipase	6 BSPs[a]	5:1	35, 36, 200	$C = 80.97$ $Y = 90.82$	Guldhe et al. (2016)

[a]Biomass support particles

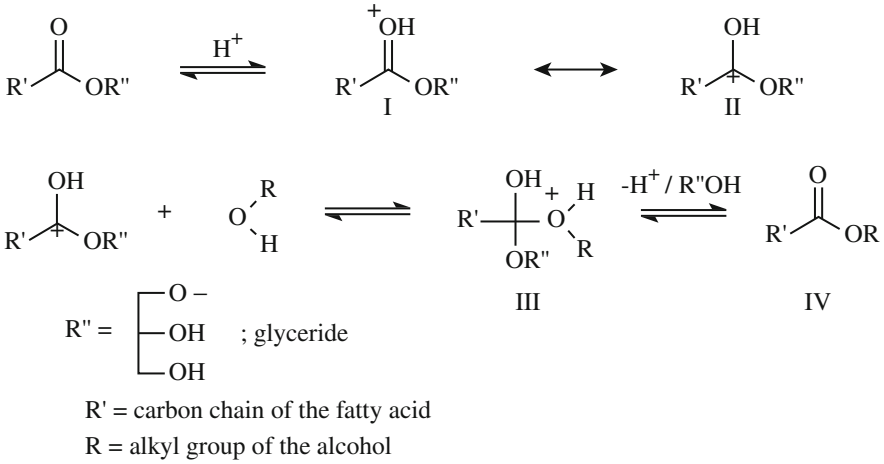


Fig. 2 Mechanism of the acid-catalyzed transesterification of oils

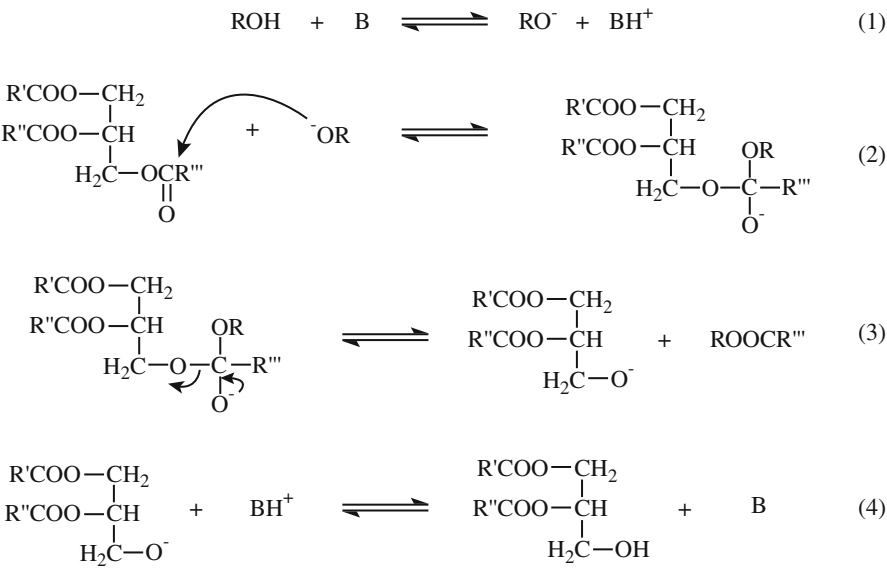


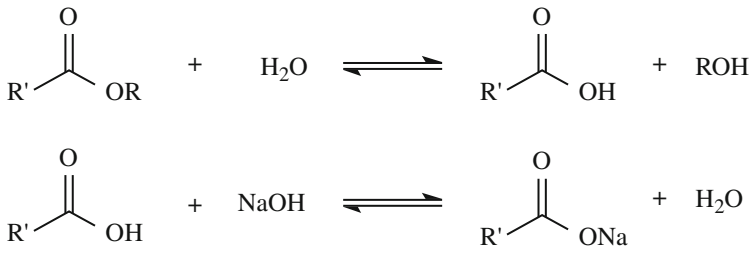
Fig. 3 Mechanism of the base-catalyzed transesterification of vegetable oils

the base-catalyzed transesterification of oils is shown in Fig. 3. In the first step the base catalyst reacts with the alcohol to produce an alkoxide and protonated catalyst. In the second step nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride leads to a tetrahedral intermediate from which the alkyl ester and the corresponding anion of the diglyceride are generated. The anion of diglyceride

deprotonates the catalyst, thus regenerating the active species, which is now available to react with a second molecule of the alcohol, for another catalytic cycle. Diglycerides and monoglycerides follow the same mechanism to form a mixture of alkyl esters and glycerol.

In the alkali-catalyzed transesterification reaction, catalyst is dissolved in methanol by stirring. The mixture of catalyst and alcohol mixture is added to the oil. The reaction mixture is stirred continuously at ambient pressure. After completion of the reaction two liquid phases are produced: ester and crude glycerol. Crude glycerol settles down at the bottom after several hours of settling. After settling is complete, water is added to the reaction mixture followed by stirring, and the glycerine is allowed to settle again. A two step process washing is performed for ester recovery, which needs to be performed with extreme care. First step is water wash and then the acid treatment with stirring. Air is carefully passed through the aqueous layer while gently stirring. This process is continued until the clear ester layer is formed. After settling, the aqueous solution is drained, and water is added again for the final washing (Demirbas 2005; Demirbas 2008). Since base-catalyzed transesterification proceeds faster than the acid-catalyzed reaction and their less corrosive nature than the acidic compounds, industrial processes usually apply base catalysts.

If feedstock oil contains high amount of free fatty acids the alkali catalyst leads to the soap formation which affects the biodiesel yield and also causes problem in the washing steps (Singh et al. 2014). Even when water-free alcohol or oil mixture is used, some water is generated in the reaction mixture by the interaction of the hydroxide with the alcohol. The presence of water leads to the hydrolysis of some amount of ester, with resultant soap formation (Fig. 4). The undesirable saponification reaction hampers the ester yields and makes it difficult to recover the glycerol due to the formation of emulsions (Freedman et al. 1984).



R' = carbon chain of the fatty acid

R = alkyl group of the alcohol

Fig. 4 Saponification reaction of the fatty acid alkyl esters produced

4.2 Heterogeneous Chemical Catalyst

There are numerous heterogeneous catalysts that have been studied for biodiesel synthesis. The heterogeneous catalysts utilized in synthesis of biodiesel are grouped as solid acid and solid base catalyst. Solid acid catalyst includes a wide range of chemicals viz. resins, tungstated and sulfated zirconia, polyaniline sulfate, heteropolyacid, metal complexes, sulfated tin oxide, zeolite, acidic ionic liquids, and others have been used by researchers. Solid base catalysts also include a wide range of catalysts viz. calcium oxide, hydrotalcite (also called layered double hydroxide), alumina, and zeolites (Lam et al. 2010; Sharma et al. 2011). Homogeneous acid and base catalysts used for biodiesel synthesis have several disadvantages, e.g., corrosion of the reactors, metal pipes, storage tanks, and engines. Tedious washing process for their removal is energy intensive and generates wastewater. The heterogeneous catalysts give the advantage of easy separation and thus reuse (Chouhan and Sarma 2011). Reuse of heterogeneous catalyst could improve the economics of the conversion process. Zhang et al. (2012) used KOH/La-Ba-Al_2O_3 as a heterogeneous catalyst for conversion of microalgal lipids. The reaction was carried out at 60 °C for 3 h and they observed the highest conversion of 97.7% in their study with 25% loading of KOH on modified alumina. To reduce the cost of catalyst several low cost heterogeneous catalysts derived from the waste materials, eggshell, bones, mollusks fish waste, etc., are applied for biodiesel production (Singh et al. 2014). Nur Syazwani et al. (2015) synthesized the CaO catalyst from Angel Wing shell and applied for transesterification of *Nannochloropsis oculata* lipids. They observed the 84.11% biodiesel yield with 9% catalysts concentration and 1:150 oil to methanol molar ratio in 1 h reaction time. Leaching of the heterogeneous catalysts into the final product, i.e., biodiesel is the major concern. Leached catalyst into the product could hamper the fuel quality as well as its performance in the engines (Singh et al. 2014). A large number of heterogeneous catalysts have been studied for the synthesis of biodiesel from the edible and non-edible plant based oils; however, application of these catalysts on the microalgal based oil is yet to be thoroughly studied.

4.3 Biocatalysts

Enzyme lipase is employed as the biocatalyst for conversion of oils to biodiesel. Lipase is capable of catalyzing both esterification and transesterification process. Thus lipases can be effectively used for conversion of microalgal lipids with high free fatty acid concentration to biodiesel (Guldhe et al. 2015a). Enzyme catalyst provides several advantages over the chemical catalysts. Enzymes are known to possess high selectivity and specificity which lead to high purity product formation. Thus enzymatic transesterification gives high purity biodiesel and by-product glycerol. Less purification steps and minimal use of chemical minimize the

wastewater generation unlike in chemical catalyzed biodiesel synthesis. Enzyme catalyst does not require high temperature conditions and can function effectively at atmospheric pressure. Thus the energy input for the enzyme catalyzed reactions is lower than the chemical catalysts (Robles-Medina et al. 2009). Major constraint while using lipases as a catalyst is its high price. Lipase if immobilized can be separated from the reaction mixture and reused for several batches of the conversion process. Novel immobilization techniques such as cross-linked protein coated microcrystals, magnetic support particle, and nanofiber are used for lipases to improve their catalytic activity, reuse potential, and stability in solvents (Guldhe et al. 2015a). The reuse potential of biocatalyst can reduce the cost of catalytic conversion. The activity of lipases can be inhibited by the presence of excessive alcohol (<3 moles). Thus several researchers have suggested the stepwise addition of methanol in the reaction mixture (Fukuda et al. 2001; Guldhe et al. 2015b). Stoichiometrically 3 moles of methanol is needed in the transesterification reaction for 1 mole of triglyceride. In lipase catalyzed transesterification 1 mole equivalent of methanol with respect to oil is added thrice after periodic interval during the reaction. Enzyme catalysis can be carried out in two ways, viz., using immobilized extracellular lipases and immobilized whole cells (intracellular) producing lipase. Xiong et al. (2008) used the *Candida* sp. 99–125 sp. lipase for the transesterification of *Chlorella protothecoides* lipids and observed 98.15% biodiesel conversion. The application of biocatalyst for conversion of microalgal lipids to biodiesel can make the process greener.

5 Acyl Acceptors Used in Transesterification of Microalgal Lipids

Transesterification is the common used process to convert triglycerides into biodiesel. This consists of the reaction of triglyceride and an acyl acceptor. Carboxylic acids, alcohols, and other ester can be used as an acyl acceptor. Glycerol is produced in transesterification when alcohol is used as an acyl acceptor while triacylglycerol is produced when ester is used as an acyl acceptor. Several acyl acceptors have been studied by the researchers for transesterification process. Methanol is the most widely used acyl acceptor in transesterification process (Helwani et al. 2009). Methanol is short chain alcohol which leads to faster reaction rate and also its inexpensive nature makes it favorable acyl acceptor at an industrial scale biodiesel production process. The major drawback of methanol is its toxic nature which raises the concerns regarding environmental and accidental risks. Methanol as an acyl acceptor is more toxic for lipase activity compared to ethanol. Use of ethanol as an acyl acceptor can improve the catalytic performance of lipase and also its reuse potential (Raita et al. 2010). In addition, ethanol can be produced from renewable sources via fermentation, which makes the process of biodiesel production greener. Only few studies report use of ethanol as acyl acceptor. The

literature unanimously recommends a stepwise addition of alcohols to reduce their toxic effect towards enzyme catalyst (Guldhe et al. 2015a). Further research is needed for exploring the potential of other compounds as acyl acceptor for transesterification reaction.

6 Solvents Used in Transesterification of Microalgal Lipids

The feedstock oil and alcohol are immiscible and thus slow down the reaction rate. To overcome this problem researchers have suggested the use of solvent in the transesterification reaction. Solvents increase the mass transfer rate which eventually results in better reactant interactions and fast reaction rate (Abbaszaadeh et al. 2012). Various solvents have been applied in the transesterification. Hexane, tetrahydrofuran, and diethyl ether are most popular solvents for transesterification reaction in biodiesel synthesis. Lam and lee (2013) investigated the effect of various solvents (hexane, ethanol, tetrahydrofuran, toluene, methyl acetate, ethyl acetate, and chloroform) on the transesterification of *C. vulgaris* oil using sulfuric acid as a catalyst. Among the various solvents studied by them tetrahydrofuran significantly improves the reaction rate and also reduces the methanol and catalyst amounts needed for reaction.

The solvents need to be removed from the biodiesel as well as glycerol after the completion of the reaction. Solvents are readily available at low cost, thus its use does not drastically affect the economics of the production process. However, high volatility, toxicity, potential hazards, and environmental risk raise concerns regarding their usage. To overcome these concerns recently ionic liquids have been employed as the solvent in transesterification reaction. Ionic liquids are termed as green solvents because of their properties such as negligible vapor pressure, high solubility, and tunable as per reaction requirement (Mohammad Fauzi and Amin 2012). Much more attention needs to be given on the novel solvents such as ionic liquids for their application in microalgal biodiesel production process.

7 In-Situ Transesterification

In-situ transesterification couples the extraction of lipids and transesterification via catalysis together. In-situ transesterification is alternative to the conventional process, which has the potential of reducing the processing steps and the overall conversion cost. The in-situ process aids the conversion of the oil to biodiesel directly from the oil bearing biomass. In-situ transesterification process eliminates the solvent extraction step. This technique thus reduces the requirement of solvents used for extraction step (Ehimen et al. 2010). This process is also gaining recognition as a lipid measurement procedure for algae (Laurens et al. 2012). In-situ transesterification can be done by using dry or wet microalgal biomass. Recently

microwave and sonication assisted in-situ transesterification are studied for increasing the biodiesel yield. Tran et al. (2012) studied the in-situ transesterification of wet *C. vulgaris* biomass catalyzed by *Burkholderia* lipase. They observed 97.3% biodiesel yield when sonication pretreated biomass was subjected to in-situ transesterification in hexane as a co-solvent. This technique has shown potential to reduce the number of processing steps, amount of organic solvent used, and thus the overall production cost of biodiesel synthesis.

8 Process Intensification by Microwave and Ultrasound

Microalgal biodiesel is still far from commercial realization because of the high cost of production. Process intensification to reduce the reaction time and increase the yields could aid in reducing the biodiesel production cost. Catalytic conversion of oils to biodiesel can be intensified by using microwave or ultrasound techniques. The microwave or ultrasound assisted lipid conversion processes offer the advantages of shorter reaction time and higher yields over the conventional heating processes. The transesterification reaction assisted by microwave and sonication has been extensively investigated with vegetable oils in recent years to enhance the biodiesel yield. Microwave irradiation directly delivers energy to the reactants in the transesterification reaction. Thus the microwave assisted reaction completes in shorter time because of the effective heat transfer. The mass transfer of reactants in a sonicator is about 10 times faster than the conventional mode of stirring (Gole and Gogate 2012). Microwave and sonication techniques can be effectively coupled with in-situ transesterification of microalgal biomass (Guldhe et al. 2014). In in-situ transesterification process these intensification techniques serve dual purpose of breaking the cell wall as well as effective mass transfer. These process intensification techniques are, however, associated with high energy consumption. Further investigation is needed to improve the efficiency of using microwave or sonication for transesterification of microalgal lipids.

9 Challenges and Future Prospective of Catalytic Conversion of Microalgal Lipids

Biodiesel from microalgae is identified as promising future transport fuel. However, at present the high production cost is the major bottleneck in its commercial scale production. Conversion of microalgal lipids to biodiesel is a key step in production process. Lot of attention has been provided on the up-stream steps such as strain selection, cultivation, and lipid enhancement in microalgal biodiesel production process. There is need for thorough research on the microalgal lipids conversion process using various catalysts. Varying lipid quality, low yields, long

reaction time, scaling-up, and achieving desired quality product are some of the major challenges in catalytic conversion process of microalgal lipids. High free fatty acid content in microalgal lipids advocates the use of acid or enzyme catalyst for conversion reaction. Numerous heterogeneous catalysts have been investigated for conversion of vegetable and other feedstock oils. This promising group of catalysts needs to be thoroughly studied for its application in microalgal biodiesel production. In-situ transesterification has shown potential to reduce the production cost by avoiding the extraction step. Low product yields in in-situ transesterification process need attention from the researchers to make this technology efficient. Process intensification by microwave and ultrasound techniques has shown potential to improve the yields in conventional as well as in-situ transesterification processes. Scaling-up of the efficient conversion process is also a challenging task. Comprehensive techno-economic and environmental risk assessment studies need to be conducted for competent conversion technologies for microalgal biodiesel production. Efficient and economically viable conversion technologies will lead the microalgal biodiesel production towards sustainability and greener future.

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Biomethanation Potential of Algal Biomass

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1 Introduction

The enhanced worldwide demand of energy necessitated augmenting the alternative source of energy. Globally, the ever increasing populations demands and greater energy consumption is reaching new heights each and every year (Singh et al. 2011a; Vaish et al. 2016a). So to fulfill the escalating demands of energy, it is required to increase the rate of energy generation considering the environmental impacts on climate in terms of green house gases (GHG) (Singh and Gu 2010). Rapid generation of greenhouse gases not only warms the planet but also causes shifting or change in the climate cycle. Climate change had worst effect on the economic growth of agriculture based developing countries (Gahlawat et al. 2009; Srivastava et al. 2015). So to maintain economic growth in lack of conventional resources of energy it is very important to use renewable energy sources based on biomass transformation (Singh et al. 2011b; Ward et al. 2014). Thereby, anaerobic digestion has gained widespread consideration in recent years that degrades number of carbonaceous matter present in the natural environment that can cause oxygen depletion (Appels et al. 2008). Biomethanation process benefits society as well as environment by providing biogas as clean biofuel and bio slurry (end product) is obtained by freely available raw material (Appels et al. 2011).

Several feedstocks are being investigated to be used in the process of biomethanation including aquatic and terrestrial. Researchers have strongly promoted the use of marine biomass because of its high energy potential, i.e., around 100 EJ/year, as compared to terrestrial biomass, i.e., around 22 EJ/year or solid waste, i.e., around 7 EJ/year (Chynoweth et al. 2001). Therefore, algal biomass has paved its way to be used as biomethanation feedstock because of its high productivity rate, resilience to broad range of temperature, nutrient content, salinity, and no competition with food crops (Bruhn et al. 2011; Nielsen and Heiske 2011; Jones and Mayfield 2011). Algal biomass has tremendous potential of becoming a feasible aquatic energy crop (Bruhn et al. 2011; Chynoweth et al. 2001). Still, generation of energy from algal biomass is lacking behind due to economic constraints (Jones and Mayfield 2011). One solution can be cultivation of algae using wastewater that can be co-digested with activated sludge. Consequently, research for production and utilization of algae as feedstock is gaining momentum in the past few decades. This chapter gives an overview of utilizing algal biomass as potential feedstock for biomethanation process.

2 Algal Biomass: A Potential Feedstock

In the current global scenario, energy security, climate change, and carbon dioxide fixation has attracted scientists to search for alternative source of energy. In this context algae have proved itself as a promising source since it not only sequesters significant quantity of carbon but is also coupled with high lipids, carbohydrate, and

nutrient content (Singh and Gu 2010). Therefore, from the last few decades extensive research has been going on to determine the potentiality of algal biomass as a source of alternative to biofuel and for biogas generation. Golueke et al. (1957) were the pioneers to study the feasibility of converting sunlight energy to methane by algal fixation which was followed by fermentation. In his study he achieved 0.5 m^3 of biogas per kg of volatile biomass with 63% of methane. Because of its high capability to be used as biomethanation feedstock a strong re-interest in cultivating algae for anaerobic digestion is gaining importance. It has been anticipated that biomethanation of algae feedstock will be able to achieve higher efficiency and sustainability for production of biomethane.

Algae are simple, autotrophic microorganisms that range from unicellular to multicellular forms. They dwell in diverse environments like saline, coastal, non-agricultural lands, shallow lagoons, raceway ponds, closed ponds, marginal lands, etc., and therefore do not participate in competition with agricultural lands for food production. Chisti (2007) suggested that high rate raceway ponds can generate up to 127 tons/ha/year of biogas with maximum achievable amount up to 263 tons/ha/year. Likewise, Carlsson et al. (2007) proposed production up to 50–60 ton/ha/year and through photo bioreactor he achieved production up to 150 tons/ha/year. Using sunlight and freely accessible raw materials like CO_2 and nutrients obtained from wastewater, they can generate high amount of biomass, lipids, carbohydrate along with other essential co-products (like omega three fatty acids, astaxanthin, etc.) per hectare as when compared to any other kind of terrestrial biomass. A wide number of closed photo bioreactor including horizontal and vertical tubes, open and closed systems are being explored for cost-effective production of algae. High productivity is attained in a controlled environment of photo bioreactor but high capital and operating investments are also required as compared to other open systems. Thus, algae can play a principal role in treatment, management, and utilization of wastewater along with mitigating its environmental impact.

2.1 Macroalgae

Macroalgae commonly called as seaweeds form multicellular thallus like rhodophyta, chlorophyta, and phaeophyta (Andersen 2005; Richmond 2004). Various macroalgae can be looked for production of biogas particularly methane and carbon dioxide (Gupta et al. 2012). The performance of macroalgae depends on type of species, composition, season, location, etc. (Costa et al. 2012). In one of the study, Gunaseelan (1997) compared several land and water based biomass for digestion process and found that macroalgae exhibited 0.31–0.48 m^3 CH_4/kg volatile solid as when compared to land based biomass like grass that exhibited 0.34–0.42 m^3 CH_4/kg and wood exhibited around 0.32–0.42 m^3 CH_4/kg. It should be noted that biomethanation rate can be increased by co-digesting macroalgae with composts or other material like activated sludge (Costa et al. 2012). As biogas

generation from macroalgae is technically feasible option but due to its high operating and installment cost it is not yet economically feasible. The cost of production, installation, and operating expenditure must be curtailed by 75% to achieve economical feasibility (Roesijadi et al. 2010).

2.2 *Microalgae*

Microalgae are basically microscopic algae, i.e., around 5–50 μm and photosynthetic bacteria like cyanobacteria that get cultivated in salt/freshwater (Richmond 2004). The chemical composition of microalgae greatly depends on number of environmental aspects such as light intensity, temperature, and nutrient availability (Becker 1994; Rodolfi et al. 2009). Microalgae have higher fraction of protein in the range of 10–60% DM, lipids round 2–90% DM, carbohydrates (starch, sugar, and other polysaccharides) in the range of 5–50% DM (Chisti 2007; Spolaore et al. 2006). Adding to this, they also contain some highly important materials such as pigments, eicosapentaenoic (EPA), decosahexaenoic (DHA), and essential vitamins like nicotinate, A, B_1, B_2, B_6, B_{12}, C, E, folic and pantothenic acid (Becker 1994; Spolaore et al. 2006). High content of lipids make microalgae as an attractive feedstock for biomethanation process because of high theoretical methane yield, i.e., around 1.0 LCH_4/g VS for lipid content, 0.85 LCH_4/g VS for proteins, and 0.42 LCH_4/g VS for carbohydrates (Li et al. 2002; Sialve et al. 2009). While on practical basis, methane productions are approximately around 0.09–0.34 LCH_4/g VS subject to digestion. Therefore, the positive aspect related to digestibility of microalgae is high methane content, i.e., around 60% (De Schamphelaire and Verstraete 2009; Sialve et al. 2009). Adding to this the biogas generated at the end of the process does not contain sulfur that corrodes the equipment. At the end, for enrichment of biogas by utilizing algae to eliminate carbon dioxide seems plausible (Converti et al. 2009; Sialve et al. 2009). Table 1 shows carbohydrate composition of various micro and macro algae species.

3 Biomethanation: The Process

Biomethanation process involves the digestion of organic fraction of substrate to yield carbon dioxide and methane as end product with digestate as valuable by-product. The process is basically divided into four stages (i) hydrolysis, (ii) acidogenesis, (iii) acetogenesis, and lastly (iv) methanogenesis. Among these four processes any of them can be limiting phase during the process. Generally, when dealing with complex substrate like microalgae, hydrolysis becomes the limiting step (Mussgnug et al. 2010; Vaish et al. 2016b). Figure 1 highlights the steps involved in the process of biomethanation of algal biomass.

Table 1 Carbohydrate composition of different macroalgae and microalgae

Carbohydrate composition of Macroalgae			Carbohydrate composition of Microalgae
Rhodophyta	Chlorophyta	Phaeophyta	
Polysaccharide	*Polysaccharide*	*Polysaccharide*	
Mannan	Carrageenan	Laminarin	Starch
Ulvan	Agar	Mannitol	Total carbohydrate
Starch	Cellulose	Alginate	Arabinose
Cellulose	Lignin	Fucoidan	Glucose
		Cellulose	Fucose
Monosaccharide	*Monosaccharide*	*Monosaccharide*	Glucose
Glucose	Glucose	Glucose	Galactose
Mannose	Galactose	Galactose	Mannose
Rhamnose	Agarose	Fucose	Rhamnose
Xylose		Xylose	Ribose
Uronic acid		Uronic acid	Xylose
Glucuronic acid		Mannuronic acid	
		Guluronic acid	
		Glucuronic acid	

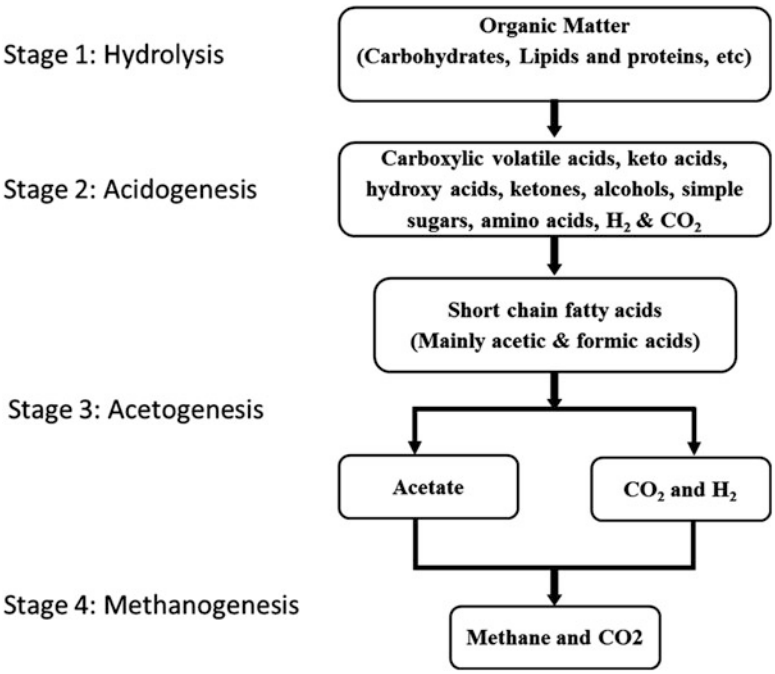

Fig. 1 Stages in Biomethanation Process

3.1 Hydrolysis

Hydrolysis is the first and primary stage in the process of biomethanation of different complex organic feedstocks. Hydrolysis stage is essential as the anaerobic microbes can only utilize soluble organic material that can penetrate through cell wall. The organic material is composed of lipids, carbohydrate, and proteins that get converted into amino acids, monosaccharide, and long chain fatty acids (LCFA) (Batstone et al. 2002). Extracellular enzymes are involved in hydrolysis of such complex organic material. The hydrolysis of carbohydrate is completed in few hours while of proteins and lipids take few days and this makes the hydrolysis a limiting stage (Deublein and Steinhauser 2011). Since, algae do not contain lignin, cellulose, and hemicelluloses (as their breakdown is difficult by anaerobic microorganisms), the algae as biomethanation feedstock is favored.

3.2 Acidogenesis

This is the second stage of the process during which two groups of microorganisms degrade soluble organic molecule like monosaccharide and amino acids through facultative bacteria. Alcohol, hydrogen, acetic acid, formic acid, and carbon dioxide are produced after completion of this reaction. Few of the end products such as formic acid, acetic acid, and hydrogen are directly consumed by the methanogens during methanogenesis. As compared to hydrolysis, the kinetics of this stage is faster.

3.3 Acetogenesis

The third stage of the process converts alcohols, butyric acids, propionic acid, valeric acid, etc., into hydrogen, carbon dioxide, acetic acid via acetogenic bacteria. Very low hydrogen partial pressure is required for acetate formation from propionic acid, valeric acid, or butyric acid (Deublein and Steinhauser 2011). This indicates that the acetogenic bacteria live in close symbiosis with the methanogenic bacteria (Anderson et al. 2003). Table 2 shows acetogenic reaction of various substrates. Acetogenesis and methanogenesis process works simultaneously until and unless over acidification occurs (Van den Poel 2014). The overall reaction is as follows:

$$CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2H_2O$$

The reaction table for different substrate is as follows:

Table 2 Examples of acetogenic reactions

S. No.	Substrate	Reaction
1.	Propionic acid	$CH_3(CH_2)COOH + 2H_2O \rightarrow CH_3COOH + 3H_2$
2.	Butyric acid	$CH_3(CH_2)COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$
3.	Valeric acid	$CH_3(CH_2)_3COOH + 2H_2O \rightarrow CH_3COO^- + CH_3CH_2COOH + H^+ + 2H_2$
4.	Lactic acid	$CH_3CHOHCOO^- + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2H_2$
5.	Ethanol	$CH_3(CH_2)OH + H_2O \rightarrow CH_3COOH + 2H_2$

3.4 Methanogenesis

This phase is the last stage that sums up the process of biomethanation process. In this stage compounds such as hydrogen, carbon dioxide, and acetic acid get transformed into methane and carbon dioxide via methanogens. The two types of bacteria are involved that are strictly anaerobic microbes that degrade acetic acid and are termed as acetoclastic methanogenic bacteria while those degrading hydrogen are called as hydrogenotrophic methanogens. The former is the main route of formation of methane, i.e., around 70% and 30% by the latter. The reaction for formation of methane from acetate is as follows:

$$CH_3COOH \rightarrow CO_2 + CH_4$$

while from hydrogen, the reaction is

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

This chemical reaction has dual function in the process of biomethanation as it not only produces methane but also gaseous hydrogen is eliminated. High ammonia concentration limits the activity of methanogens during the digestion process of algal substrate as well (Hansen et al. 1998; Ramos-Suárez and Carreras 2014). The specific methane yield could be calculated by the modified formula of Symons and Buswell (1933). This is theoretical maximum yield that does not account for cell maintenance and anabolism:

$$C_aH_bO_cN_d + \left(\frac{4a - b - 2c + 3d}{4}\right)H_2O \rightarrow \left(\frac{4a + b - 2c - 3d}{8}\right)CH_4 + \left(\frac{4a - b + 2c + 3d}{8}\right)CO_2 + dNH_3$$

In the above equation, organic matter is stoichiometrically converted to CH_4, CO_2, and ammonia.

4 Operating Parameters

4.1 Temperature and pH

In biomethanation process, temperature and pH are considered as crucial parameters that determine the efficiency of the process. It is reported that low temperature and pH control operating conditions were considered as feasible options that cause reduction in ammonia toxicity present in biomethanation process (Kayhanian 1999; Massé et al. 2003). Both the parameters will be discussed separately.

4.1.1 Temperature

Temperature directly influences the biogas digestion process. At the range of 30–35°C temperature mesophilic methane producing bacteria are vigorously active and at the temperature range of 50–60°C thermophilic methane producing bacteria are active. The most favorable temperature for biogas production is 35°C. However, methane production can occur over a broad range of temperatures. Temperature below 32°C may lead to the increase in volatile acid to alkalinity ratio. When temperatures rise higher than 32°C, a greater destruction rate of volatile solids and the production of methane occurs (Gerardi 2003). For example, methane conversion efficiency and productivity of *Spirulina maxima* are enhanced when the temperature is raised from 15 to 52°C coupled with volatile solid reduction at temperature around 35°C (Samson and LeDuy 1986). Golueke et al. (1957) reported that when temperature is raised from 35 to 50°C, it can enhance the rate of algae biodegradability from 5 to 10% for a multispecific algal biomass. Nonetheless, Chen (1987) found a maximal methane productivity under mesophilic temperatures appear to be optimal situations for algal anaerobic digestion, especially at 40°C (Table 3).

Carrere et al. (2010) applied thermal pretreatment for algal disintegration at temperature ranging from 50 to 270°C. However, generation of recalcitrant and/or inhibitory composites occurs when the temperature is increased above 180°C that reduces biomass digestibility (Wilson and Novak 2009). To improve the energy demand and increase the profitability of the process, pretreatment at low temperature, i.e., <100°C is required (Ferrer et al. 2008). Biomass solubilization is the outcome of high activity of thermophilic and hyperthermophilic bacterial populations (Alzate et al. 2012; Carrere et al. 2010) while low pretreatment works as a thermophilic or hyperthermophilic pre-digestion step (Lu et al. 2008). However, compared to pretreatment temperature, the exposure time plays a major role for algal biomass solubilization in addition to methane generation at this array of temperature (Appels et al. 2010). Chen and Oswald (1998) found that the maximum methane generation increase is around 33% after pretreatment at 100°C for 8 h and it revealed that temperature is the major parameter that influences

Table 3 Effect of low temperature pretreatment of microalgae

References	Microalgae	Pretreatment conditions	Anaerobic digestion conditions	Result
Chen and Oswald (1998)	Microalgal biomass grown in wastewater	100°C, 8 h	Batch	CH_4 production increased by 33%[a]
Gonzalez-Fernández et al. (2012a)	*Scenedesmus* biomass	90°C, 3 h	Batch 35°C	CH_4 production increased by 220%[a]
Gonzalez-Fernández et al. (2012b)	*Scenedesmus* biomass	80°C, 25 min	Batch 35°C	CH_4 production increased by 57%[a]
Alzate et al. (2012)	*Scenedesmus* and *Chlamydomonas* biomass	55°C, 12 and 24 h	Batch 35°C	CH_4 production decreased by 4–8%[a]
Alzate et al. (2012)	*Acutodesmus obliquus* and *Oocystis* sp. Biomass	55°C, 12 and 24 h	Batch 35°C	CH_4 production decreased by 3–13%[a]
Alzate et al. (2012)	*Microspora* biomass	55°C, 12 and 24 h	Batch 35°C	CH_4 production increased by 4–5%[a]

[a]Compared to control

microalgae anaerobic digestion. Gonzalez-Fernández et al. (2012a) in their experiment revealed that the final methane production was found highest with the pretreatment of *Scenedesmus* at 90°C compared to 70°C and the methane yield with 90°C was 2.2-fold, i.e., around 170 mL/g COD as compared to the control with untreated biomass. Alzate et al. (2012) in their study revealed that the different cultures of microalgae have the resistance to different levels of temperature and time of exposure.

4.1.2 pH

Anaerobic microorganisms can be divided into acidogens and methanogens. For acidogens and methanogens, the optimum pH range is 5.5–6.5 and 7.8–8.2, respectively. The optimum pH range for both the cultures ranges from 6.8 to 7.4. Since methanogenesis is the most important step, pH should be kept close to neutral. Compared to acidogens, methanogens are more sensitive to small changes in pH (Khanal 2008). Ammonium toxicity of the microorganism may get reduce by the proper controlling of pH during the biomethanation process (Fernandes et al. 2012). The growth of microorganisms and the composition of total ammonia nitrogen (TAN) will be affected by pH during the digestion of substrate containing high concentrations of TAN. The increase in pH results in increased toxicity as there is increase in fatty acid (FA) form of ammonia which is considered as the actual toxic agent. The instability caused by ammonia results in production of volatile fatty acids (VFAs) accumulation, which again advances towards sudden

drop in pH and thus declines the concentration of FA. The lesser methane yield is the consequence of interaction amidst FA, VFAs, and pH. Shanmugam and Horan (2009) observed that unionized ammonia was highly correlated with poor biogas production at pH higher than 8.5, which provide evidences for ammonia toxicity, in particular for leather flesh waste. They also accounted that the ammonia nitrogen was minimum at pH 4.5, but high VFA inhibited the methanogens. The ammonia toxicity can be minimized by controlling pH within the growth optimum range of microorganisms (Chen et al. 2008). Reducing the volumetric organic loading rate to the point where VFAs are consumed faster than produced can solve the problem.

4.2 Hydraulic Retention Time

HRT refers the time that wastewater or sludge spends in the digester. It is a key parameter in biomethanation processes. HRT must be high in sufficient amount to encourage the dynamic populations of microbes that executes in reactor, particularly methanogens. Moreover, interference with hydrolysis must be prevented as hydrolysis is usually the limiting step of the overall conversion process. HRT plays an important role in slowly degradable complex organic pollutants (Speece 1996). The constant and higher methane yield will be attained when the process is operated at low loading rate and high HRT. Contradictory to this, when the highest loading rate or else least hydraulic retention time is reached, a decrease in the yield of methane occurs. Thus, hydraulic retention time controls the transformation of volatile solids to gaseous product (Gerardi 2003). Figure 2 demonstrates temperature-dependent sCOD removal efficiencies of different anaerobic methane digesters by continuous reactions.

4.3 Loading Rate

Optimal loading rate depends on the type and biological composition of the algal feedstock that enables efficient conversion of organic matter. When the cells are directly subjected to the biomethanation, resistance of cell wall in hydrolysis stage limits the availability of intracellular content for anaerobic microorganisms. Thus, distinctive characteristics of the algal species formulate the variation for given loading rate or else for hydraulic retention time as was reported by Asinari Di San Marzano et al. (1982); Chen (1987).

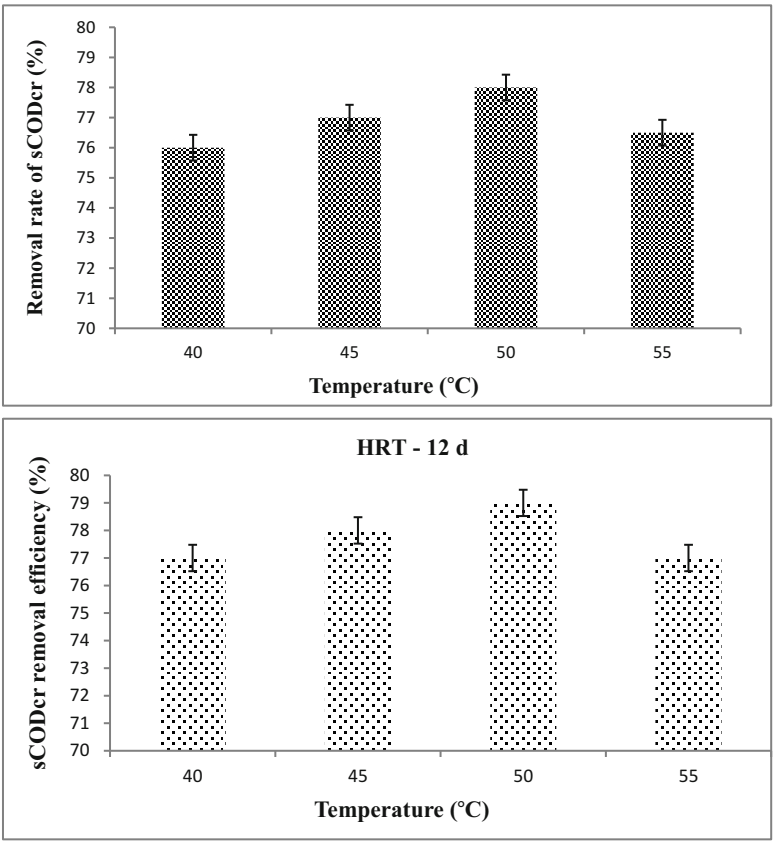

Fig. 2 Temperature-dependent sCOD removal efficiencies of different anaerobic methane digesters by continuous reaction (**a**) HRT—10 d (**b**) HRT—12d (Source: Kim et al. 2006)

5 Biomethane Production from Algal Biomass: A Sustainable Approach

The intense increase in academic and industrial research in algae is the resultant of technological solution for CO_2 fixation and as a source of biofuels. The strong interest for biomethanation is mainly due to its capability to operate, transform, and treat broad range of organic fraction of wastes into renewable form of energy (Vaish et al. 2016b). The management of bulk amount of remaining biomass and the high quantity of fertilizers play pivotal role in the perspective of setting up massive cultures. Biomethanation is the major process that can resolve the waste disposal problem as well as maintains the energetic and economic balance for such promising technology. Undeniably, the digestion of algal biomass into biomethane after lipid extraction can recover additional amount of energy as compared to

energy recovery from cell lipids (Sialve et al. 2009). The efficiency of algae as a substitute to biofuel crops with CO_2 fixation is a matter of strong concern (Chisti 2008; Li et al. 2008).

Minowa et al. (1995) reported that microalgae as compared to other terrestrial biomasses usually have a higher photosynthetic efficiency. The proper recovery of fuel from algae helps in utilizing it instead of fossil fuel. The algae produce fuel with the renewable process using solar energy and it makes it highly promising source (Tsukahara et al. 2001). The biomethanation of organic wastes helps in minimizing the effect of global warming and therefore considered as sustainable option as treatment technology (Pantaleo et al. 2013). Biomethanation is the process where microorganisms degrades and stabilizes the organic wastes in lack of oxygen. It directs towards the generation of biogas (combination of methane, carbon dioxide, and few impurities), microbial biomass, and fertilizer which will be applied as nutrient source in agricultural fields (Chen et al. 2008; Rajagopal et al. 2011). The significant benefits of biomethanation process are low energy demand, low sludge production, and green renewable energy recovery (Massé et al. 2010; Xia et al. 2012).

Therefore, the technology has net positive energy production and the biomethane generated could moreover replace fossil fuel which eventually helps in greenhouse gas reduction (Shanmugam and Horan 2009). Biomethanation provides the route for recycling of nutrients which is essential for sustainability and economic feasibility of production of commercial scale algal biofuel (Keymer et al. 2013). The digestate generated from biomethanation of algal biomass is loaded with nitrogen and phosphorus nutrients. Digestate nutrient values of 2940 mg/L ammonia-nitrogen, 320 mg/L of potassium, and 390 mg/L of total phosphorous have been reported by Collet et al. (2010). The clear liquid digestate will produce higher concentrations of TAN and phosphorus from anaerobically digestion of algae. These results significantly reveal that the high strength nutrient-rich digestate could be generated from the biomethanation of microalgae biomass (Zamalloa et al. 2012).

6 Future Prospects

Current researches have enabled better understanding of the complexity of different algal species that are to be used as biomethanation feedstock. In future, this present knowledge will be very beneficial for the biomethanation of algae and will optimize and eventually increase the biomethane production rate. Each individual algal species should be treated and processed specifically to optimize the yield of the gas. Biomethanation, very efficiently, integrates algae based biofuel production and algae based wastewater treatment. However, several technical issues are related to digestion of algal biomass that includes low concentration of substrate and ammonia inhibition that can be overcome by pretreatment methods. The digestate formed at the end of the process can be again utilized for regrowth of algal species that will

help to close the nutrient loop which is associated with mass production of algal biomass and thus environmental sustainability could be achieved. Therefore, with the greater understanding about biological characteristics of different algal species in the process of biomethanation plays a promising role in providing clean and sustainable form of energy obtained from algae.

7 Conclusions

Biomethanation of algal biomass has an excellent future potential of production of biomethane as a renewable feedstock that could foster large amount of biogas. Algae when used as feedstock can foster high amount of methane content and thus can be economically feasible. The economic consideration of algal feedstock could be recommended at industrial scale only when extensive research to demonstrate the operational feasibility of the process is executed. Moreover, exploration of more diverse microbial community and other innovative substrate needs to be examined thoroughly. Therefore, biomethanation is required to generate biomethane from algal biomass. The residue left after lipid extraction for biodiesel production can be subjected to anaerobic digestion for biomethane production. For production of commercially viable biomethane, the process must be significantly improved in terms of efficiency, algal biomass growth, lipid extraction, and biomethane production. To achieve this, technological and research breakthroughs are required for production of commercially feasible feedstock. If this happens, algal biomass will play a promising role in production of biomethane as a sustainable feedstock. Finally, guiding technology and preferred routes for biofuel production are needed to be explored so that it can be incorporated for policy making processes.

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Technological Advances in Biohydrogen Production from Microalgae

Sheena Kumari, Mahmoud Nasr, and Santhosh Kumar

Contents

1 Hydrogen: A Future Energy Carrier

The global demand for energy is on an exponential rise over the years, while the fossil fuels reserves are diminishing at a faster pace (Nasr et al. 2014a). Additionally, fossil fuel combustion significantly affects the environment due to CO_2 release (Nasr et al. 2013a). Accordingly, scientists are searching for exploring new and

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alternate energy sources that are sustainable and possibly could replace fossil fuels (Nasr et al. 2014b).

Hydrogen is emerging as a potential energy carrier of the future owing to its renewable nature, zero carbon dioxide emission during combustion (Nasr et al. 2013b), has more energy per unit weight and could be used in fuel cells (Nasr et al.2013c).

2 General Characteristics of Microalgae

Microalgae are primitive tiny plants found in aquatic habitats, however, lack various structures found in tracheophytes (vascular plants) such as leaves and roots (Amos 2004). The main cell composition of green algae includes nucleus, cell wall, chlorophyll and other pigments, pyrenoid, stigma and flagella. Cyanobacteria (blue-green algae) and green algae have the ability to carry out plant-type photosynthesis (Schnackenberg et al. 1996). Cyanobacteria are now categorized as prokaryotes due to their anatomical similarities to bacteria. Microalgae and Cyanobacteria perform oxygenic photosynthesis (Eq. 1), where water is split by sunlight into O_2 and a strong reductant, typically ferredoxin, typically used to reduce CO_2 to carbohydrates (sugars) (Tiwari and Pandey 2012).

$$CO_2 + H_2O + \text{sunlight} \rightarrow [CH_2O] + O_2 \quad (1)$$

(CH_2O—represents carbohydrate general formula).

3 General Mechanisms of Hydrogen Production

Biohydrogen production is the biological conversion of water, sunlight and/or organic substrates into hydrogen by the action of nitrogenase or hydrogenase enzymes. Hydrogen, a by-product of nitrogen-fixation by the enzyme nitrogenase, is produced by reducing molecular nitrogen into ammonia. Hydrogenase is another key enzyme in biohydrogen production that catalyses the formation and decomposition of hydrogen (Tiwari and Pandey 2012).

Biologically, hydrogen can be produced through photobiological process (green algae, photosynthetic bacteria or cyanobacteria), or through dark fermentation (heterotrophic bacteria) (Hena et al. 2016). Microbial hydrogen production mainly involves three distinct mechanisms, based on the abundance of carbon and other energy sources (Nasr et al. 2015): (1) Dark fermentation, where the organic matter is converted to hydrogen, carbon dioxide and soluble metabolites (mainly volatile fatty acids) by a group of heterotrophic obligate or facultative anaerobic bacteria, in the absence of light (Nasr et al. 2013d), (2) Photofermentation, where organic acids (e.g. VFAs) are converted to hydrogen by the action of photosynthetic bacteria

(e.g. *Rhodobacter*) in the presence of light and (3) Biophotolysis, where carbon dioxide and sunlight are used as energy sources for the dissociation of water into molecular hydrogen and oxygen by photoautotrophic organisms (cyanobacteria and green algae) (Gaffron and Rubin 1942).

Biological process of hydrogen generation has multiple benefits which include less energy demand and minimum capital investments (Nasr et al. 2014a). However, biological conversion efficiencies of substrates to hydrogen gas are influenced by various external factors including pH, temperature, substrate concentration, type of inoculum, etc. (Nasr et al. 2014b). The biohydrogen production efficiency of microalgae depends heavily on the species involved and their growth requirements.

4 Photoautotrophic Hydrogen Production

Photosystems (PS) are the complexes of pigments, such as chlorophylls, carotenoids and phycobiliproteins, in addition to several dozen proteins that are the functional units of photosynthesis (Singh et al. 2015). PS are able to capture photons by light harvesting pigments (also known as antenna) and then alter light (photon) into chemical energy via photosynthetic reaction centre (Miura et al. 1997). The initial form of chemical energy is further transformed as reductant to metabolic energy (reduced ferredoxin which then generates nicotinamide adenine dinucleotide phosphate (NADPH)) and a membrane potential proton-motive force which is then transformed into adenosine triphosphate (ATP) (Florin et al. 2001). ATP and NADPH are used to fix CO_2 into glucose, further used along with nitrogen (as ammonia or nitrate), phosphorous (as phosphate) and other inorganic nutrients as the primary building material for all other algal cell components (carbohydrates, proteins, nucleic acids, fats, etc.) (Schnackenberg et al. 1996).

Eukaryotic microalgae are photoautotrophic (possess chlorophyll A and other pigments) organisms, and perform oxygenic photosynthesis using photosynthetic systems (PSII and PSI). Photosynthesis is a two-stage process (Fig. 1), which includes light reaction (the photo part) and Calvin cycle (the synthesis part) (Florin et al. 2001). During the light reaction of photosynthesis, the pigments in PSII (P680) absorb light (photons with wavelengths $<$680 nm), creating a powerful oxidant which can split water into protons (H^+), electrons (e^-) and O_2 (Miura et al. 1997). A series of electron carriers and cytochrome complex transfer the released electrons to PSI (Schnackenberg et al. 1996). The photons (wavelength $<$700 nm) are absorbed by the pigments in PSI (P700) where NADPH is formed by reducing nicotinamide adenine dinucleotide phosphate (NADP+) by adding a pair of e^- and H^+. Additionally, the oxidized ferredoxin (Fd_{ox}) is reduced to reduced ferredoxin (Fd_{red}), which is directed to the enzyme hydrogenase (Hase) for hydrogen liberation (Tiwari and Pandey 2012).

Adenosine triphosphate (ATP) is also produced during light reactions by the addition of a phosphate group to ADP by chemiosmosis, through a process known as photophosphorylation (Bishop and Bishop 1987). The formation of ATP and

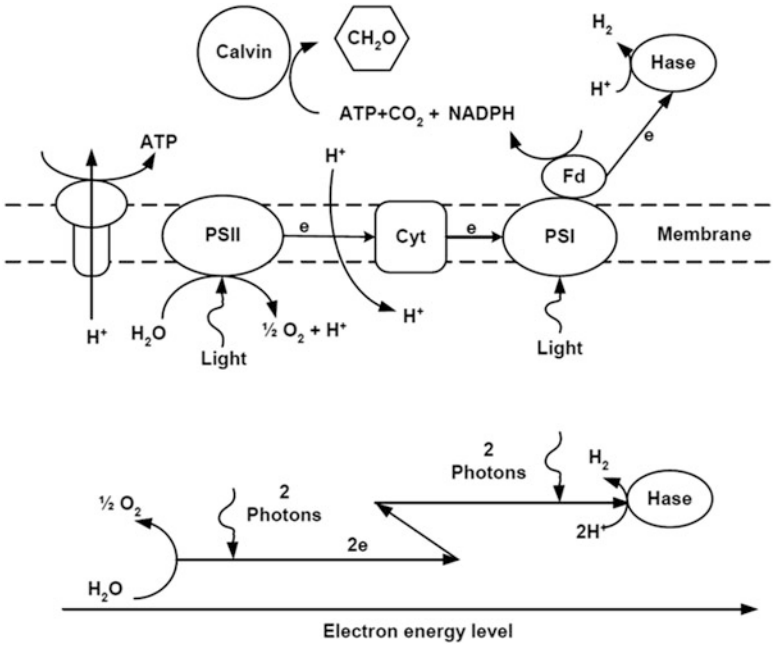


Fig. 1 Schematic representation of photosynthesis and biophotolysis process (Amos et al., 2004)

NADPH thus marks the first step in the photosynthesis (conversion of light to chemical energy). Subsequently, the ATP and NADPH formed during light reactions are reduced with CO_2 from the air (carbon fixation) via reductive pentose phosphate pathway or Calvin cycle for cell division (Weissman and Benemann 1977).

During Calvin cycle, the fixed carbon is reduced to carbohydrate by the addition of electrons (Miura et al. 1986). The excess reduced carbon thus formed is accumulated as carbohydrates (CH_2O) and/or lipids inside the cells. The metabolic steps involved in Calvin cycle are termed as dark reactions, or light-independent reactions, as it does not require direct light (McCully and McKinlay 2016).

5 Hydrogenase-Dependent Hydrogen Production

The hydrogen ions liberated during microalgal photosynthesis by splitting the water molecules to hydrogen ion and oxygen are transformed to hydrogen gas by the action of hydrogenase enzyme (Hena 2016). Hydrogenase oxidizes reduced ferredoxin to liberate molecular hydrogen in anaerobes, (Eq. 2, where the electron carrier "X" is assumed to be ferredoxin) (Winkler et al. 2002). Thus, addition of external iron source may be required for hydrogen production (Fig. 2). However,

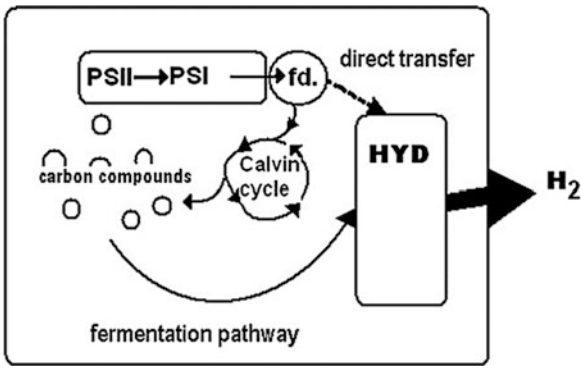


Fig. 2 Hydrogenase-mediated hydrogen production [8]

the oxygen-labile nature of hydrogenase is a bottleneck for sustainable hydrogen production using microalgae.

$$2H^{+}+2X_{reduced} \rightarrow 6\ H_2+2X_{oxidized} \quad (2)$$

Hydrogenase enzyme has been found in green algae, *Scenedesmus obliquus* (Florin et al. 2001), in marine green algae *Chlorococcum littorale* (Schnackenberg et al. 1996), *Playtmonas subcordiformis* (Cao et al. 2001) and in *Chlorella fusca* (Winkler et al. 2002), though, hydrogenase activity was not reported from *C. vulgaris* and *Dunaliella salina* (Cao et al. 2001). Gaffron and Rubin (1942) have indicated that the electron donation (reducing) capacity of hydrogenase does not arise from water constantly, but may evolve intracellularly from starch like organic compounds. Greenbaum et al. (1995) have shown good conversion rate of light to hydrogen (10–20%), with light at 400–700 nm wavelength. Furthermore, they found that a mutant strain of *Chlamydomonas* could achieve CO_2 fixation and hydrogen liberation using one photosystem only (photosystem II). Miura et al. (1986) proposed hydrogen production by a photo/dark cycle, and found that starch was reduced from CO_2 during photosynthesis (in the presence of light), after which the starch was converted to hydrogen gas, organic acids and/or alcohols under anaerobic and dark conditions. They indicated that oxygen sensitivity of hydrogenase is overcome by green algae during anaerobic phase, and under light conditions, photosynthetic bacteria convert organic acids and alcohols to hydrogen gas. Asada and Kawamura (1986) have indicated that hydrogen gas could be produced by cyanobacteria through auto-fermentation in dark and anaerobic conditions, where the highest activity among the investigated cyanobacteria was witnessed for *Spirulina* species. Gaffron and Rubin (1942) found that *Scenedesmus* spp could produce hydrogen molecules under light conditions after exposure to dark and anaerobic conditions.

6 Nitrogenase-Dependent Hydrogen Production

In photosynthetic bacteria, nitrogenase enzyme plays a major role in catalysing hydrogen gas production (Kapdan and Kargi 2006). Nitrogen-fixation in prokaryotic organisms like cyanobacteria is catalysed by nitrogenase, whereas it is absent among eukaryotes, such as microalgae (Oldroyd and Dixon 2014). In photosynthetic bacteria, nitrogenase facilitates hydrogen production, though hydrogenases may involve both in hydrogen production and uptake under specific conditions. Nitrogenase catalysed hydrogen liberation arises as a side reaction during nitrogen-fixation process where photosynthetic bacteria, in the presence of light, could convert organic acids and other organic substrates into H_2 and CO_2 (Fig. 3). During nitrogen-fixation in cyanobacteria, molecular nitrogen is reduced to ammonia by the utilization of a reducing agent (ferredoxin) and ATP through an irreversible reaction (Flores et al. 2005).

$$N_2 + 6H1^+ + 6e^- \leftrightarrow 2HN_3$$

$$12ATP \leftrightarrow 12(ADP + Pi)$$

Nitrogenase mediates reduction of proton in the absence of nitrogen gas.

$$2H^+ + 2e^- \leftrightarrow H_2$$

$$4ATP \leftrightarrow 4(ADP + Pi)$$

In the presence of oxygen, ammonia and at high N/C ratio, nitrogenase activity is found inhibited, though hydrogen production can be resumed after ammonia depletion (Koku et al. 2003). Therefore, the process needs an atmosphere with limited ammonium and free oxygen availability (Yokoi et al. 1998). For example, in

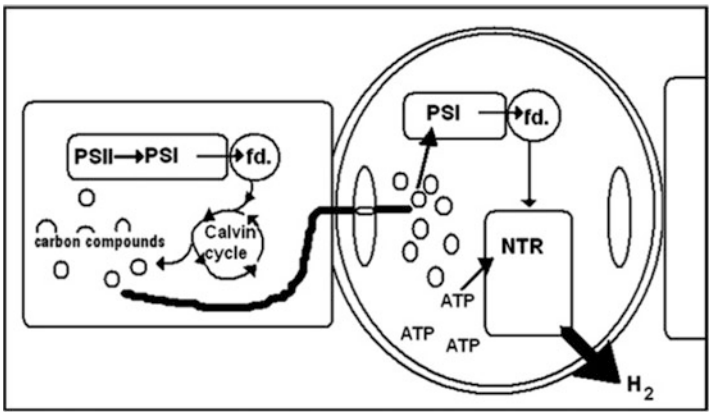


Fig. 3 Hydrogenase-mediated hydrogen production [8]

R. sphaeroides, a complete inhibition of hydrogen production was noticed at ammonia concentrations higher than 2 mM (Yokoi et al. 1998). Hydrogen production was also found to be limited in medium containing ammonia salts, whereas proteins (e.g. albumin, glutamate and yeast extract) enhanced hydrogen production when used as a nitrogen source (Oh et al. 2004). At higher nitrogen levels, the metabolism is directed more towards utilizing organic substrates for cell synthesis and growth than hydrogen production (Fascetti and Todini 1995). Removal efficiency of ammonia along with stimulation of hydrogen production could be enhanced by the supplementation of carbonate (Antal Lindblad 2005).

Localizing nitrogenase enzyme in the heterocysts of filamentous cyanobacteria is the most effective mechanism for depriving nitrogenase from oxygen and to provide it with energy ATP and reducing power (Llama et al. 1979). In filamentous cyanobacteria, vegetative cells perform oxygenic photosynthesis, whereas organic compounds are broken down to supply nitrogenase with reducing power. ATP is supplemented by PSI-dependent and anoxygenic photosynthesis within the heterocysts (Weissman and Benemann 1977). Additionally, previous studies on the enhancement of hydrogen production have indicated that the hydrogen-generation ability of cyanobacteria can be improved through nitrogen deprival (Weissman and Benemann 1977; Miyamoto et al. 1979)

7 Biophotolysis

Hydrogen gas is produced by microalgae under certain growth conditions, resulting in an overall net dissociation of water, known as "biophotolysis" (Miura 1995). Biophotolysis is a very interesting biological process, where water is converted to hydrogen and oxygen using solar energy. Microalgae have all the required genetic, enzymatic, metabolic and electron transport systems to convert water into hydrogen gas using light (Kapdan and Kargi 2006).

The biophotolysis process can be presented as:

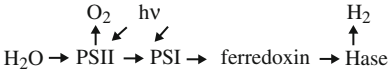
$$2H_2O + \text{light} = 2H_2 + O_2$$

Biophotolysis can be classified as "direct" and "indirect photolysis". In direct biophotolysis, the reduced ferredoxin generated by the splitting of water during photosynthesis is directly used to reduce the hydrogen-producing hydrogenase or nitrogenase, without intermediate CO_2 fixation (Miura 1995). However, if the hydrogen is generated from carbohydrates produced by microalgae during normal photosynthesis, then the process is known as "indirect biophotolysis" (Gaffron and Rubin 1942).

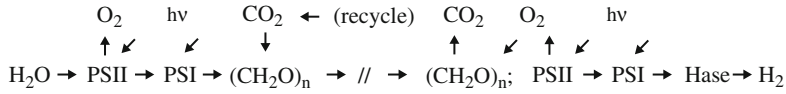
7.1 Direct Biophotolysis

In direct biophotolysis, the electrons generated by the absorption of light energy by PSII are transported to ferredoxin using light energy captured by PSI. The above process is catalysed by the enzyme hydrogenase (Benemann 1996) present in the stroma of algal chloroplast. Hydrogenase accepts electrons liberated from reduced ferredoxin and donates them to two protons to generate one H_2 molecule. Equation (3) presents direct biophotolysis reaction (green algae and in vitro systems; potentially in cyanobacteria), whereas Eq. (4) demonstrates direct biophotolysis reaction with respiratory O_2 uptake (green algae, possibly cyanobacteria) (Hena 2016).

Though direct biophotolysis is an attractive method for sustainable hydrogen production, in practice, the process is strongly limited by the powerful inhibition of hydrogenase activity by concomitantly released oxygen (Miura 1995). To combat the above challenge, alternate methods have been proposed such as spatial separation of hydrogen and oxygen, chloroplast immobilization, oxygen scavenging and gas purging.



(3)



(4)

7.2 Indirect Biophotolysis

Reduced carbon from the photosynthetic process is generally deposited as endogenous carbohydrates, in the form of starch in microalgae and glycogen in cyanobacteria (Dauvillée et al. 2006). These stored intracellular energy reserves such as carbohydrates may act as electron donors or reducing equivalents for hydrogenase and nitrogenase to function (Antal and Lindblad 2005). The energy from carbohydrates is released via fermentation in dark conditions, and the surplus reducing power may be shifted to protons (H^+) by hydrogenase generating hydrogen (Gfeller and Gibbs 1984). Thus, indirect biophotolysis encompasses two stages, stage-1: photosynthesis for carbohydrate production, and stage-2: hydrogen production through dark fermentation of the stored carbohydrate. Through the above two-stage process, the release of oxygen and hydrogen can be spatially separated from each other (Benemann 1996). This separation makes the hydrogen purification process relatively simpler as CO_2 can be easily separated from the gas (H_2/CO_2) mixture (Bélafi-Bakó et al. 2006).

Miura et al. (1997) studied hydrogen production using natural light/dark cycles via indirect biophotolysis. In their study, they have found that during photosynthesis, the CO_2 is reduced to starch which is then fermented to hydrogen gas and organic acids under anaerobic and dark conditions. Another mechanism for indirect biophotolysis is by heterocystous cyanobacteria; filamentous species that could achieve water-splitting and CO_2-fixing photosynthesis, as well as exclude O_2 and reduce N_2 (Prince and Kheshgi 2005). In heterocysts, localization of nitrogenase provides an oxygen free environment for cyanobacteria to fix nitrogen from air (Prince and Kheshgi 2005). Another approach of indirect biophotolysis is to carry out two reactions in separate stages, first O_2 production (with CO_2 fixation) followed by H_2 production (with CO_2 release) (Miura 1995).

8 Challenges and Technological Advancements in Biohydrogen Production from Microalgae

Although hydrogen production from microalgae is a much exploited area, there are few major drawbacks that limit its application at its commercial level. Some of these well-known challenges include (1) O_2 sensitivity of hydrogenase enzyme, (2) non-dissipated proton gradient and state transitions, (3) small antenna size, (4) competition for photosynthetic reductant and (5) requirement of specific photobioreactor (Dubini and Ghiradi 2014). Nevertheless, the past decade has shown good progress in overcoming some of these shortcomings mainly through genetic engineering approaches. Several attempts have been made at pilot and industrial scale for the production of hydrogen by microalgae. However, most of these successful studies involved either genetically engineered microalgal strains or sulphur-deprived conditions (Dubini and Ghirardi 2014; Gimpel et al. 2015). Some of the recent advancements in this area are discussed in brief below.

Oxygen sequestration to mitigate the O_2 sensitivity of hydrogenase enzymes is been investigated as an alternative approach for enhancing hydrogen biosynthesis in microalgae. Wu et al. (2011) have reported that introduction of leghaemoglobin (LbA) proteins (oxygen sequester protein from the root nodules of legumes) increased the hydrogen production in *Chlamydomonas* sp to fourfold compared to its wild type. With further modifications to the above strain, Wu et al. (2011) could increase the gene expression of HemH (ferrochelatase gene) and LbA (leghemoglobin gene) to 6.8 fold in the transgenic strain of *Chlamydomonas*. Similarly, two genetically modified *Chlorella vulgaris* strains (YSL01 and YSL16) with upregulated hydrogenase gene (*HYDA*) expression could liberate hydrogen through photosynthesis in the presence of oxygen (Hwang et al. 2014). Other alternative approaches were tested to remove O_2 which included the establishment of new pathways in *Chlamydomonas*. It is known that pyruvate oxidase (PoX) enzyme is involved in the decarboxylation of pyruvate to CO_2 and acetyl phosphate. The reaction is O_2 dependent and was assumed that the intracellular O_2

levels in *Chlamydomonas* could be reduced by introducing this gene (Dubini and Ghirardi 2014). The engineered algae strain could produce 2.5 fold higher hydrogen compared to its wild strain under very low light (30 μE m^{-2} s^{-1}) and sulphur-deplete growth conditions (Gimpel et al. 2015).

Incomplete inactivation of O_2 formation was also achieved in the transgenic strain of *Chlorella* sp. by knocking down the *PSBO* gene, a nuclear gene that encodes the plastid manganese-stabilizing protein of photosystem II (Lin et al. 2013). This was achieved by antisense RNA technology, i.e. by introducing a short interference RNA antisense-*PSBO* fragments to knock down the *PSBO* gene expression in the transgenic *Chlorella* sp. This was resulted in a tenfold improvement in hydrogen evolution in transgenic strain of *Chlorella* sp. (Lin et al. 2013).

Biohydrogen production by microalgae is also limited by the competition of hydrogenases enzymes for photosynthetic reductant from ferredoxin with other key enzymes viz., Ferredoxin-$NADP^{++}$ reductase (FNR), Ferredoxin/thioredoxin reductase (FTR), nitrite reductase, sulphite reductase and glutamate synthase that are involved in major metabolic pathways (Dubini and Ghirardi 2014). To improve the electron flow and to reduce the competitions, engineering of FNR and the hydrogenase genes have all been exploited under in vitro conditions (Oey et al. 2016). Studies have also focused on engineering electron competitors genes such as RuBisCo, cyclic electron flow, starch degradation and respiration (Ruehle et al. 2008; Pinto et al. 2013) with a reportedly increase in hydrogen yield (Oey et al. 2016). Obtaining additional reducing power through genetic engineering is therefore expected to increase hydrogen yields in microalgal cells due to the reduced competitions by different enzymes. A study by Doebbe et al. (2007) has shown that the expression of HUP1 (hexose transporter gene) from *Chlorella kessleri* in *C. reinhardtii* has resulted the production of an improved strain that are able to use glucose as both carbon and electron source. They have also noticed a 1.5-fold improvement in hydrogen production rate in the modified strain. In another study, Pinto et al. (2013) have shown that expression of a genetically modified small sub-unit of Rubisco (RBCS-Y67A) in *C. reinhardtii* has resulted in the elimination of photosystem II activity in the modified strain leading to a 15-fold rise in hydrogen production rate under sulphur-deplete conditions (Dubini and Ghirardi 2014).

A truncated antenna mutant of *Chlamydomonas* sp. showed an eightfold increase in biohydrogen production under sulphur deprivation due to the increased light capture efficiency and decrease photo inhibition in the mutant strain (Kosourov et al. 2005). It is expected that the reduction of antenna size increases light harvesting efficiency of the microalgal cells as it enhances the light absorption and distribution leading to overall increase in photon conversion efficiency of the microalgal cells (Beckmann et al. 2009; Oey et al. 2013, 2016). A twofold increase in hydrogen production is also reported in *C. reinhardtii* by downregulation of three major proteins of light harvesting complex II (LHC II), i.e. LHC MB1, 2 and 3 with the use of RNAi constructs (Oey et al. 2013). Similarly, D1 mutant of *Chlamydomonas* sp. with non-functional photosystem II has exhibited a substantial improvement in hydrogen production rate in the transgenic strain (Scoma et al. 2012; Oey et al. 2016). It was also reported that the hydrogen production in microalgae can

also be enhanced by modifying (locking) the electron transport chain and by lowering the cyclic electron transport thereby reducing the competition for electron with the hydrogenases (Kruse et al. 2005a; Tolleter et al. 2011; Oey et al. 2016).

Further, the use of latest gene editing technologies such as transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFN) and CRISPER/Cas systems could offer specific and lasting gene editing (Cho et al. 2013; Sizova et al. 2013; Gao et al. 2015; Oey et al. 2016). In the recent past, immobilization of algal cells has shown excellent potential in biotechnological industry including biohydrogen production from microalgae. An immobilized wild type and *tla1* sulphur-deprived mutant strain (truncated antenna) of *C. reinhardtii* on alginate films could produce higher hydrogen gas for a longer period (over 250 h) (Kosourov et al. (2011). More recently, several studies have also underlined the possibility of using microalgae to perform photoheterotrophic degradation of organic acid-rich dark fermentation effluent (Zhang et al. 2014).

9 Conclusions

Microalgae are regarded as a cheaper and viable source of biohydrogen production compared to other biomass based fuels. Biohydrogen production from microalgae can be achieved by various means such as biophotolysis and photofermentation. Though much progress has been made at the in vitro level, commercialization of this technology is far from real. This may require the integration of advanced engineering (next generation reactor configurations) and biotechnological approaches (genetic engineering). Though researchers were successful in integrating or modifying a specific challenge or character, scale up of the process may require inclusion of multiple characters into a single microalgal strain. Inclusion of all the required traits into a single microalgal strain for continuous biohydrogen production is still one of the biggest challenges that limit its commercialization. Advances in metabolic engineering may play a major role in development of sustainable substitutes for the long-term biohydrogen production such as simultaneous wastewater treatment and biohydrogen production using microalgae in the near future.

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Hydrothermal Liquefaction (HTL): A Promising Pathway for Biorefinery of Algae

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1 Introduction of Hydrothermal Liquefaction and Its Role for Algae Biorefinery

Algal biofuel has been proposed as the next (commonly called "third") generation biofuel (Gouvea 2011). Among processes of algae conversion technology, three main routes to produce liquid biofuels from algae are biodiesel via the extraction or transesterification, bio-oil via pyrolysis, and biocrude via hydrothermal liquefaction (HTL) (Gupta and Demirbas 2010). Both pyrolysis and HTL are technology of thermochemical liquefaction. The direct liquefaction of algal biomass into biocrude oil is defined as the HTL of algae, which is in a closed oxygen-free reactor by pressurizing inert gases (e.g., N_2 or He) or reducing gases (e.g., H_2 or CO), at a certain temperature (250–380°C) and pressure (5–28 MPa) (Déniel et al. 2016; Guo et al. 2015; Ramirez et al. 2015; Changi et al. 2015; Tian et al. 2014; López Barreiro et al. 2013a). During HTL, the hot compressed water (i.e., near-critical water) is used as both solvent and reaction medium (Akizuki et al. 2014). A key challenge for HTL with organic solvent was its high cost. HTL using hot compressed water as the solvent has the advantages of being abundant, non-toxic and non-flammable, inexpensive, and naturally stored in biomass (Huang and Yuan 2015).

A pattern process of continuous HTL in detail is shown in Fig. 1. Compared to other technologies of liquid biofuel production such as oil extraction or pyrolysis

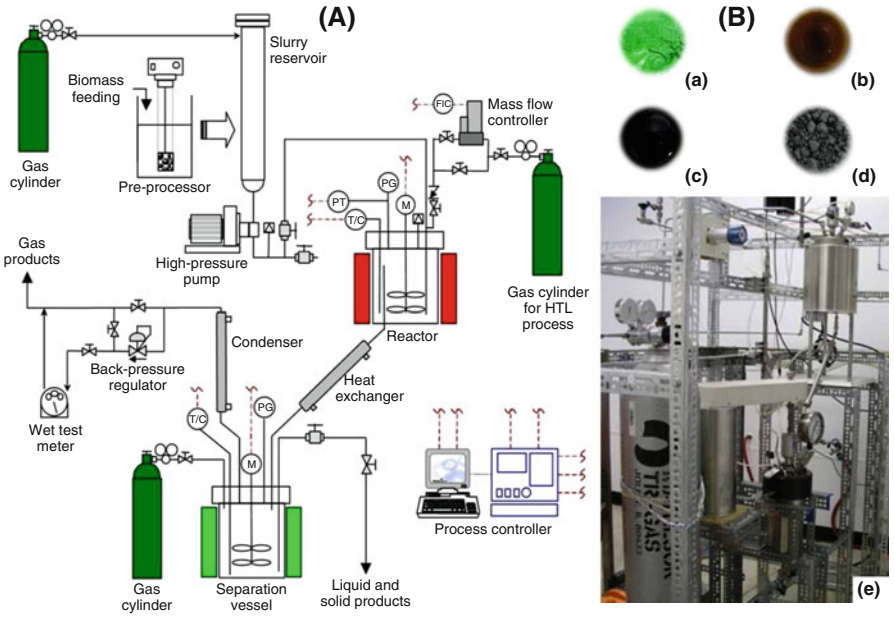

Fig. 1 Schematic diagram of a continuous-flow HTL process (Tian et al. 2017). (**A**) A flow chart of HTL; (**B**) Pictures of algae HTL: (**a**) algae slurry, (**b**) aqueous products, (**c**) biocrude oil, (**d**) solid residue, (**e**) HTL reactor. Figure was reprinted with the permission of Elsevier

(Gupta and Demirbas 2010; Crocker 2010), HTL of algae has some advantage (Peterson et al. 2008). Firstly, the drying process of feedstock is eliminated due to HTL can treat wet algal feedstock, because the total solids (TS) of feeding feedstock are usually 10–25%; Secondly, whole algae components, not only lipids but also proteins and carbohydrates, can be converted, leading to a higher biocrude yield. In other words, high lipids content is not a decisive factor of feedstock screening anymore; thirdly, mass transfer is enhanced due to sub-/super-critical water that can play the role both of the reaction medium and solvent; in the same time, enthalpy of phase change can reduce latent loss and enhance energy efficiency of HTL since hot and high-pressure water is not in the form of vapor.

The concept of algae HTL was derived from the 1970s (Tian et al. 2014). Elliott group firstly summarized the advances from 1983 to 1990 on direct thermochemical conversion of biomass to hydrocarbon fuels (Elliott et al. 1991). Hydrothermal conversion attracted growing interests in recent two decades. Researchers mainly focused on direct liquefaction, especially using organic solvents during process and feedstock is mainly lignocelluloses. After that time, the importance of algae as a promising feedstock for HTL has been addressed (Tian et al. 2014). Japan was one of the pioneering countries focused on this field, but ceased after 2005. More studies have been reported since 2010 due to the high price of petroleum (Toor et al. 2011). Different conditions were then discussed, such as holding temperature and retention time at the holding temperature (Akhtar and Amin 2011). Besides academic activities, the US Department of Energy (2012) began to add HTL as one of the major pathways for biomass conversion technologies (US-DOE 2012). Then, US National Renewable Energy Laboratory (NREL) and Pacific Northwest National Laboratory (PNNL) took part in composing technical reports focused on "Whole Algae Hydrothermal Liquefaction" (NREL & PNNL 2013; US-DOE & PNNL 2014).

HTL is a process that can convert algal biomass into four phases: biocrude, aqueous products, solid residue, and gaseous products (Tian et al. 2014). The most important target product through HTL is biocrude, and biocrude has the potential to co-refine in an existing fossil refinery to produce energy and chemicals (Ramirez et al. 2015; Zhu et al. 2013). Other products, i.e., aqueous, gaseous, and solid phases can be seen as intermediates. Biorefinery is an integrated concept which reuses these intermediates through other technologies. To full use of these products, a synergistic algal biorefinery is described in Fig. 2. There are four operational units, including algal biomass preparation, HTL reaction, HTL products recovery, and products upgrading/refining. Actually, one critical problem is how to reuse the process wastewater to resolve this issue, two strategies (Orfield et al. 2014) were proposed (Fig. 2): one pathway with featuring cultivation of algae on the aqueous products and recycling of the algal biomass back through another HTL process for boosted oil yields (marked ① in Fig. 2); the other pathway with onsite gasification of the aqueous products to fuel gas for energy recovery (marked ② in Fig. 2).

For one thing, major limitations for algal biofuel production are the consumption of water, nutrients, energy, and environmental pollution (Rampton and Zabarenko 2012). One promising strategy of the algal biorefinery is to reuse and recycle

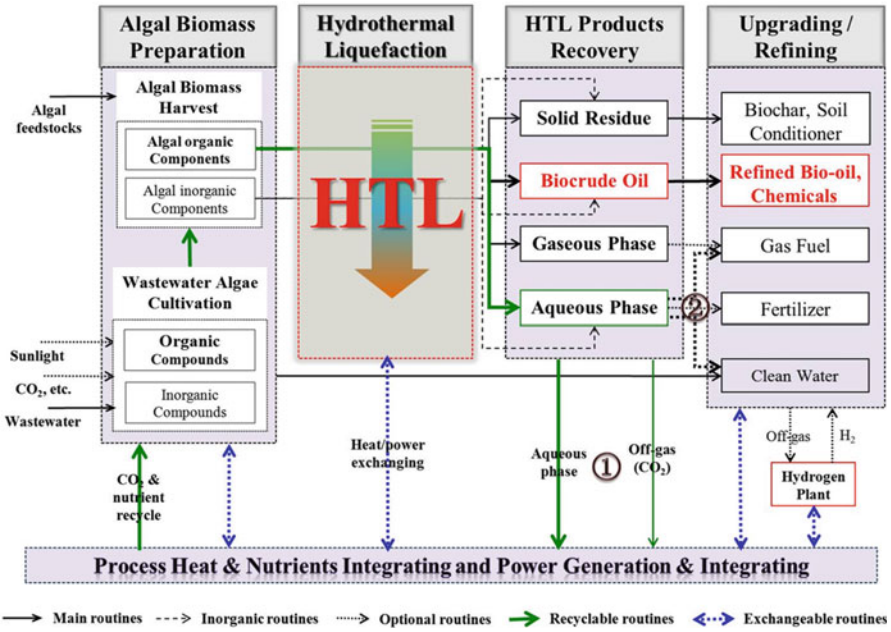

Fig. 2 The algae biorefinery concept through HTL. Figure was drawn derived from Tian et al. (2014) and NREL & PNNL (2013)

nutrients, wastewater stored in post-HTL aqueous (Fig. 2-①) via the algae cultivation. This concept was later formally proposed as "Environmental-Enhancing Energy" (Zhou et al. 2013) (E2E). E2E is established based on the full use of feedstock components by maximizing the recovery of hydrocarbon and reusing nutrients. Not only nutrients (nitrogen, phosphorus, etc.) stored in post-HTL wastewater but also the whole processes besides HTL collected could be fed back for algae cultivation. CO_2 produced through HTL and/or released from other sources such as the thermal power plant can be used for algae growth. In E2E, biocrude oil is converted from algae via HTL, the post-HTL wastewater was cleaned and carbon dioxide is captured via algae cultivation, the augmented algal biomass will be further converted into biocrude oil via HTL. Thus, the E2E paradigm realizes multiple stages of algae production and biofuel conversion, and at the same time cleans wastewater and captures CO_2. In addition, the inorganic compounds also play an important role in the process; some metal elements in aqueous products can be used for algae growth (Richmond 2004). On the other hand, massive water (Elliott et al. 2015) and hydrogen/fuel gas (Hoffmann et al. 2013) were need during the whole process, especially in "products upgrading/refining" unit (Fig. 2). Under this view, aqueous phase can be treated via hydrothermal gasification (HTG), which can produce gas fuel (mainly CH_4) and clean water (Fig. 2-②). As results, the generated hydrogen can be supplemented for biocrude upgrading, and aqueous phase was cleaned to prevent the environmental pollution and supply clean water for the process (Elliott et al. 2015). Of course, assorted related technology must be prepared, for instance, the facilities of separation, purification, and storage for hydrogen. This strategy was

mainly advocated by Elliott group in recent years. Orfield et al. (2014) incorporate recent experimental results into an analysis of these two strategies (Orfield et al. 2014). For the Strategy One (Fig. 2-①), the land footprint could be further reduced by 10%, and the optimal cost of algal oil could be reduced to $1.59 L/oil. For the Strategy Two (Fig. 2-②), the cost was $1.64 L/oil. Actually, in the Strategy One, the recyclable routine can be multi-recycled. Through this multi-recycled process, research indicated that the algae biomass amplification ratio can reach nearly ten times (Zhou et al. 2013).

Considering the technology advantage of HTL among the liquid biofuel conversion technologies and the flexibility in the biorefinery, HTL might be the most suitable technology in an algae biorefinery. However, several bottlenecks still have to be resolved before the application of HTL for an algae biorefinery.

2 Key Factors Affecting on Algae HTL

A simplified scheme for the hydrothermal processes (Tian et al. 2014; Kruse et al. 2013) is presented in Fig. 3. The hydrothermal processes can be classified into three types according to different operational conditions (mainly referred to temperature) and target products (Kruse et al. 2013): (1) hydrothermal carbonization (HTC) for the production of solid biochar; (2) HTL for the production of biocrude oil; and (3) HTG for the production of fuel gas. HTG dominates at high temperatures in near/super-critical conditions whereas HTC takes place at mild temperatures.

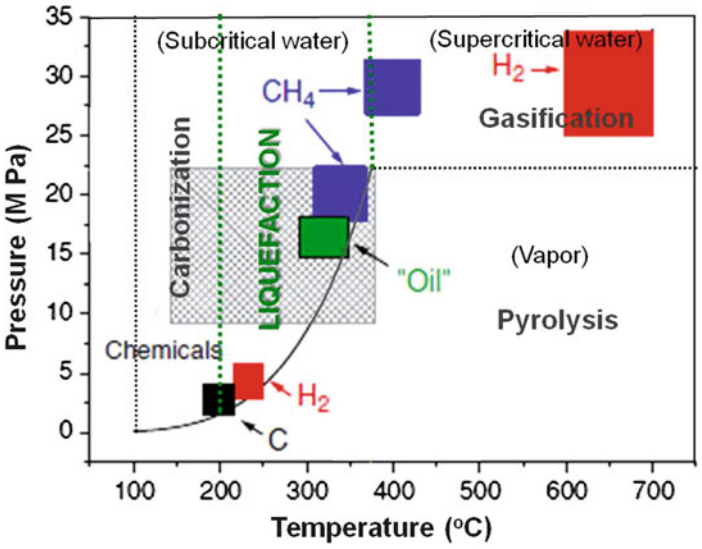

Fig. 3 The simple principle of hydrothermal process. Figure was revised from Kruse et al. (2013)

Under HTL conditions, increasing temperature always means an increasing pressure due to the conditions set by the vapor–pressure curve. Hydrothermal conversion of biomass is established by mimicking the formation of natural fossil fuels including petroleum, natural gas, and coals (Tian et al. 2014). According to biogenic hypothesis of oil and gas, all fossil fuels found in nature are formed through hydrothermal conversion of biomass buried beneath the ground under the conditions of millions of years of high-temperature and pressure (Hunt 1996). Hydrothermal conversion is a process with physical and chemical changes of biomass in a heated, pressurized, and oxygen-free closed reactor, where algal biomass with long-chain organic compounds is firstly broken into short-chain components (Peterson et al. 2008).

Hydrothermal processes take place in liquid water at elevated temperatures. The pressure in the system must be at or above saturated pressure. They can be classified by other different regions above the vapor–pressure curve and the critical point in the phase diagram of water. In contrast to hydrothermal processes, steam assisted pyrolysis is represented by different conditions below the vapor–pressure curve. The state of water aggregation is dry steam, i.e., pyrolysis (Fig. 3). The biocrude produced by HTL is similar to its counterpart in dry thermochemical conversion, flash pyrolysis. It should be noted that no significant char/coke formation is observed during HTL in contrast to flash pyrolysis (Kruse et al. 2013).

HTL of biomass is a well-known process for the production of a liquid biocrude. Products of this process are two liquid phases (biocrude and aqueous phase) and gas phase (Valdez et al. 2012). As HTL starts, the yields of gases, aqueous products, and biocrude increase. The pathway from algae to aqueous products represents the release of intracellular proteins and carbohydrates, and their subsequent decomposition during HTL. The pathway to biocrude represents the decomposition of the cell wall, hydrolysis of big molecules, and reforming of produced small. Part of biocrude oil is converted into gaseous products and might also be converted to the aqueous products, such as triglycerides and phosphorous (Valdez et al. 2012, 2014; Valdez and Savage 2013). Reactions as such could account for the slight increase of yields of aqueous products with the increase of time. A similar characteristic for three processes is that aqueous products are formed since water is involved for all routes.

Based on the illustration of principle of hydrothermal reaction, some of the key factors have emerged, like the process temperature and pressure. However, these conditions are just the important process factor, while, the feedstock properties are the deceive factors that can decide the reaction “ceiling” no matter the process condition changing. In addition, the process operational parameters, i.e., retention time, heating rates, total solids catalysts, and even products separation, should be considered, because they are all the key factors either. In this section, we will discuss the key factors from upstream to downstream for HTL.

2.1 Feedstock Selection

Obviously, feedstock selection is the upstream factor for HTL. Algae used for HTL include microalgae and macroalgae (Tian et al. 2014). Microalgae were the dominant algae species, including *Spirulina*, *Dunaliella*, *Chlorella*, *Nannochloropsis*, *Scenedesmus*, *Desmodesmus*, and even natural mixed microalgae. They were originally used for food/feed or some special chemicals. The contents of lipids, proteins, and carbohydrates are main organic components of algae. Most algae were chosen for HTL have low lipids content, usually lipids <20% (basis of dry ash free, daf). Algae with high lipids content were commonly associated with slow growth rates and low biomass productivities (Tian et al. 2014; Williams and Laurens 2010). This was because high lipids content was usually caused by nutrient limitation, which negatively impacted algae growth and biomass accumulation (Williams and Laurens 2010). Natural algae are almost all fast-growing with high proteins and low lipids. In other words, algae having low lipids content, mean high proteins or carbohydrates relatively, and have high growth rates. One key difference of biocrude via HTL conversion and biodiesel from algae extraction is the limitation of lipids content (Mata et al. 2010). For biodiesel production, lipids content was the main criterion for screening suitable algae species and demanded growing of pure algae species which could tremendously increase the operational cost (Demirbas and Demirbas 2010). One of the advantages of HTL is that all organic components of algal biomass not limited to lipids can be converted into biocrude (Tian et al. 2014). HTL is suitable for converting different wet biomass, including low lipids algae, into biocrude. However, high lipid algae may require simpler processes for post-HTL oil upgrading and refining than other algae strains.

Lipids in algae are mainly nonpolar aliphatic compounds, which are principally referred to triacylglycerides. Lipids are water insoluble under ambient conditions. Proteins are major components of algae, which consist of at least one peptide chain. Nitrogen is the key element for proteins (González López et al. 2010). Proteins also contain sulfur, and there are some sulfur-containing amino acids, such as methionine and cysteine (Richmond 2004). Carbohydrates include polysaccharides, starch, cellulose, and hemicellulose. Lignin together with cellulose and hemicellulose is named lignocelluloses. Lignocelluloses are major components of some plants and macroalgae. Furthermore, they are the main components of cell wall (Gupta and Demirbas 2010). Compared with lignocellulosic biomass, many microalgae with low carbohydrates can be easily converted into biocrude with higher oil HHV due to their low content of oxygen (Tian et al. 2014). The distribution of the main components in algae is highly dependent on growth conditions and might be different even for the same species (Richmond 2004).

In general, the major organic components refer to lipids, proteins, and carbohydrates; the inorganics usually called ash. In terms of the relationships between the biocrude yield with algae components, Biller and Ross (2011) firstly gave the following equation to estimate this (Biller and Ross 2011):

$$\text{Biocrude yield } (\%\text{basis of dry, d}) = x \times L + y \times P + z \times C.$$

L: Lipid content, P: Protein content, C: Carbohydrates content; x: generated part yield by Lipid, y: generated part yield by Protein, z: generated part yield by carbohydrates.

Then they calibrated the equation by measuring the HTL yields of independent compounds (Biller and Ross 2011):

$$\text{Biocrude yield } (\%\text{d}) = 0.80 \times L + 0.18 \times P + 0.06 \times C;$$

Using the same approach and identical model compounds, Teri et al. (2014) referred the results was that (Teri et al. 2014): Biocrude yield (%d) = 0.95 × L + 0.33 × P + 0.06 × C;

Through the results from the regression analysis, Leow et al. (2015) have given the more precious results as (Leow et al. 2015): Biocrude yield (%d) = 0.97 (±0.10) × L + 0.42(±0.07) × P + 0.17(±0.35) × C.

From these researches, there is no doubt that the potential yields for converted algae components into biocrude via HTL were in the order: lipids > proteins > carbohydrates (Biller and Ross 2011). Figure 4 has revealed further relationships of three algal components response to biocrude oil yield. However, they didn't take ash effect into account. Algae ash has negative effects on HTL biocrude production

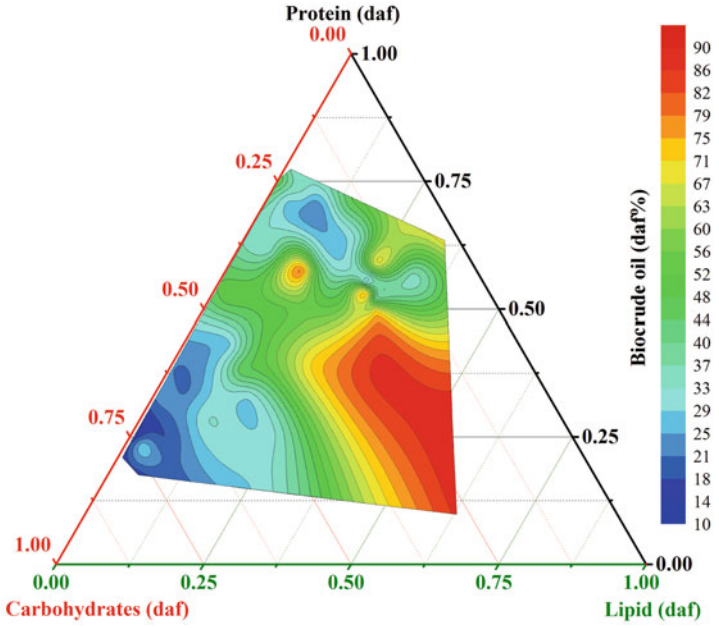

Fig. 4 The relationships of three components response to biocrude yield. Figure was drawn based on the data from literature (Tian et al., 2014)

(Tian et al. 2015). First, a high ash content means less organic materials converted into biocrude oil and more solid residue left. Second, more ash could cover the surface of organic matter and further hamper mass and heat transfer of hydrothermal reaction (Tian et al. 2014, 2015). Natural mixed algae were usually found containing more ash, less proteins and lipids, resulting in less biocrude oil production (Chen et al. 2014a; Tian et al. 2015). In particular, macroalgae have higher ash than microalgae, resulting in lower yields of biocrude oil (Jin et al. 2013). Note that there is no quantitate relation between ash content and biocrude. Biocrude yield is affected by many factors and highly dependent on algae species and their organic components. For instance, Chen et al. (2014a, b) recently reported a biocrude oil yield of 49.9% (daf) from mixed-culture algae with a high ash content of 47.5% (d) (Chen et al. 2014a). The growth media used for cultivating algae can contain many inorganic compounds, which are incorporated on/into the algal cells via biosorption/absorption (Richmond 2004). Algae contain inorganics in range about 5–40% (d). Inorganic material such as alkali salts can act as catalysts and inhibitors for biomacromolecule decomposition in hydrothermal systems (Roberts et al. 2015; Feng et al. 2014). Thus, the effects of feedstock on HTL still need further study.

The HTL can be compatible with current algae conversion technologies. Algae residue (Audo et al. 2015) after extraction of biodiesel, nutrients, such as carotenoid or other high-value products, can be further converted into biocrude via HTL (Savage 2012), thus constituting an example of biorefinery of whole algae (NREL 2013; PNNL 2014). Algae have the ability to contribute to resolving the issues of energy and the environment through the E2E paradigm (Zhou et al. 2013). Regarding the feedstock for the HTL, algae can be co-converted with other biomass or organics. In some cases, algae biocrude might be benefited by mixing with other biomass. This process is called "co-liquefaction."

2.1.1 Co-Liquefaction

Algae were once attempted to co-liquefy with coal for better conversion efficiency. For instance, Ikenaga et al. (2001) examined the co-liquefaction of microalgae such as *Chlorella*, *Spirulina*, and *Littorale Yallournor*, with Illinois No.6 coal in 1-methylnaphthalene under hydrogen at 300–400°C (Ikenaga et al. 2001). Besides the industrial single microalgae, natural mixed algae are also considered as a HTL feedstock (Chen et al. 2014a; Tian et al. 2015). In the view of composition of algae components affecting on HTL biocrude, as discussed above, the potential contributions of different algae components to biocrude were different. More than these, the interaction between two algae components or even among all three components could happen in single algae species (Changi et al. 2015). So, how it may happen if the two or even more different algae species are mixed together for HTL?

Co-liquefaction did not affect the molecular composition but affect the relative amount of each component into biocrude. Jin et al. (2013) have investigated co-liquefaction of microalgae (*Spirulina platensis*) and macroalgae (*Entermorpha prolifera*) (Jin et al. 2013). As a result, a positive synergetic effect existed during

the co-liquefaction, and this synergetic effect was dependent on reaction conditions. Co-liquefaction alleviated the severe reaction conditions compared to the separate liquefaction and also promoted the in situ deoxygenation of the biocrude. The energy recovery from the co-liquefaction is larger than the average value from the separate liquefaction. After that, the synergistic interactions of co-liquefaction were also observed between *Chlorella pyrenoidosa* and Rice husk (Gai et al. 2015a), and mixed-culture algae and swine manure (Chen et al. 2014b).

2.2 Process Parameters

Besides feedstock selection, operational parameters for HTL of algae in recent studies include (Akhtar and Amin 2011) total solids (TS) of algae, holding temperature (HT), retention time (RT) of holding temperature, and catalyst selection. Figure 4 presents the effects of three typical operational parameters (i.e., TS, HT, and RT) on biocrude characterization via HTL of algae (Jena et al. 2011). The biocrude characterizations in Fig. 4 include oil yield (%, d), higher heating value (HHV, MJ/kg), and the atom ratio (including O/C, N/C and H/C). In general, like illustrated in Fig. 5, HT was the most effective factor and it could significantly affect the oil yield, HHV, and deoxygenation. There were right values of TS and RT for maximum oil yields.

Actually, the role of processing conditions including TS of algae, HT, pressure and gases, heating rates, RT, and catalysts are all very important for algae HTL. The

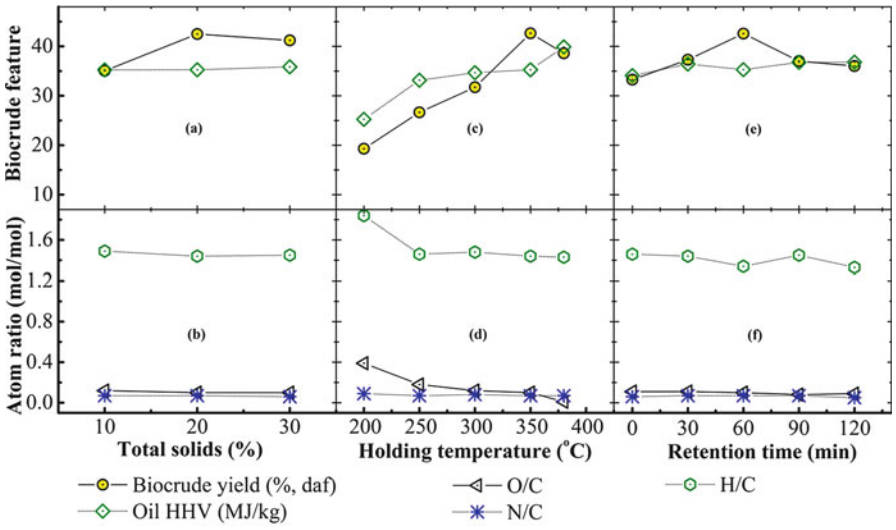

Fig. 5 Temperature effect of elements recovery of HTL biocrude and aqueous. Figure was drawn derived from Yu et al. (2011b)

effects of these parameters on the optimum biocrude yields have been discussed specially (Akhtar and Amin 2011). The process condition during HTL is the inevitable topic.

2.2.1 Total Solids (TS) of Algae

TS of algae refers to the algae concentration for HTL. To a certain extent, this parameter still highly relates to feedstock, so we discussed it first. The selection of TS used in HTL depends on many factors, such as algae species, the scale of the reactor, and economics. Increasing the algae/water ratio to extremes may not have a positive influence on the yield and quality of biocrude, where a slow algae feedstock concentrations lead to poor economics. From engineering experience, the target biomass concentrations should be 15–20% in order to achieve practical economies. For instance, TS of 20% is suggested for *Spirulina platensis* (Fig. 4) (Jena et al. 2011). TS $< 10\%$ was suggested using marine algae as feedstock (Tian et al. 2014), such as *Dunaliella tertiolecta* and *Laminaria Saccharina*. However, there is no clear relationship between TS and oil yield. More issues related to mass transfer, thermochemical conversion, and energy consumption may occur if the TS is too high, whereas the volume efficiency and productivity of the HTL reactor will be reduced if the TS is too low (Tian et al. 2014). Compared with microalgae *Spirulina platensis*, solid residue was increased with the increase of TS for macroalgae *Sargassum patens*, indicating that microalgae could be more easily converted into biocrude oil than macroalgae. In general, TS of algae/algae concentration just only affected on the biocrude yield but not the oil characterizations (including HHV and elements distribution), like shown in Fig. 4.

2.2.2 Holding Temperature (HT)

HT has a remarkable influence on the performance of HTL, as discussed in Fig. 4. In general, suitable holding temperature varies depending on algae species (Tian et al. 2014). Many studies used HT were at 300–350°C. However, high biocrude yields were also reported in 250–300°C using *Enteromorpha prolifera* (Zhou et al. 2010), *Chlorella pyrenoidosa* (Yu et al. 2011b) as feedstocks.

Influence of temperature on the yield of liquefaction products seems sequential. Initially the rise in temperature triggers biocrude yield. After reaching a maximum for the biocrude yield, further increase in temperature actually inhibits biomass liquefaction. Very high temperature is not usually suitable for production of liquid oils both in terms of operational cost and liquid oil yield. HT was selected depending on the competition of hydrolysis, fragmentation, re-polymerization, and other reactions (Toor et al. 2011). De-polymerization of algal biomass is a dominant reaction during the initial stage, re-polymerization becomes active at later stages which lead to the formation of char (Möller et al. 2011). In general, there are two reasons for this behavior (Tian et al. 2014; Toor et al. 2011): (1) The secondary

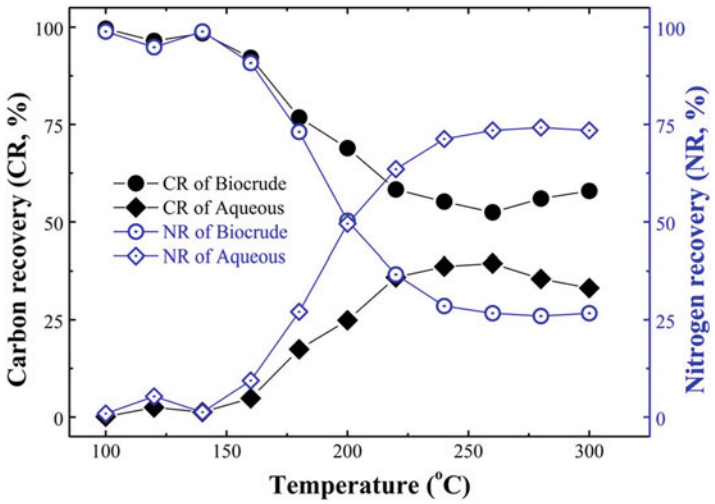

Fig. 6 Temperature effect of elements recovery of HTL biocrude and aqueous. Figure was drawn derived from Yu et al. (2011b)

decompositions and boundary gas reactions become active at high temperatures which lead to the formation of gases; (2) there combination of free radical reactions leads to the char formation due to their high concentrations. These two mechanisms become dominant at high temperatures, which reduce the production of oil from biomass. Moreover, at temperatures <280°C, in complete decomposition of individual biomass components suppresses the biocrude yield. Under hydrothermal conditions, lignin and cellulose fragments rapidly decomposed at temperatures>250°C. Thus, it can be presumed that 300–350°C would be an effective temperature range for decomposition of biomass for subcritical water conditions (Kruse et al. 2013).

Carbon recovery and nitrogen recovery of HTL products (biocrude and aqueous, etc.) were also significantly affected by HT (Fig. 6) (Yu et al. 2011b). The biocrude yield using *Chlorella* as feedstock tended to increase very slowly when HT increased up to 350°C, and then decline as more gases are formed at higher HT. As HT increased, carbon recovery and nitrogen recovery of biocrude decreased but aqueous products increased. After HTL, parts of carbon and nitrogen in the algae feedstock remained in aqueous phase. They also indicated that the sum carbon recovery of biocrude oil and aqueous products reached about 91%, while the sum of nitrogen recovery was nearly 100% at 300°C. This result indicated that mass transfer of elements of carbon and nitrogen at about 300°C were mainly through the phases of biocrude and aqueous. This result is very important, since this mass balance feature would give some ideas to support the algae biorefinery in Fig. 2.

2.2.3 Pressure and Gases

The main purposes of applying initial pressure are to maintain water in liquid phase, actually water is under subcritical condition, and if not so, water will be under vapor (Fig. 3). Subcritical water can reduce the enthalpy of phase change of water, enhance the solubility of biomass, and improve the energy efficiency (Peterson et al. 2008; Toor et al. 2011). The reaction pressure directly reflects water density and water in the liquid state will be characterized with the thermal vapor pressure. Higher water density accelerates the release of more H^+ from high temperature and compressed water, thereby increasing the effect to acid-catalyzed reactions. A common method is using inert nitrogen to replace the air inside the HTL reactor and keep the reactor in an oxygen-free atmosphere. HTL of algae was hardly affected by the initial pressure of inert gas (Yu et al. 2011a), whereas HTL of cattle manure was negatively impacted by initial pressure (Yin et al. 2010). In HTL of microalgae, there are still no obvious conclusions about the effect of water density on biocrude yields. More solid residue was produced at higher initial pressure, which could be resulted from the re-polymerization of biocrude due to reduced activation energies (Peterson et al. 2008; Toor et al. 2011).

Reducing gases, such as CO and H_2, were also investigated in the early study of HTL in order to further reduce the oxygen content of biocrude. However, reducing gases have shown that the effect of biocrude yield was not very significant (Toor et al. 2011; Akhtar and Amin 2011). In addition, when using reducing gases, operation and instruments of dynamic adding pressure may be needed to get the suitable pressure.

2.2.4 Retention Time (RT) of HT

RT is another important operational parameter affecting the HTL product. Most studies have demonstrated that a range of 30–60 min is commonly suggested for HTL, like reported in Fig. 5, the highest oil yield obtained at 60 min. The mechanism of retention time on the degradation of algal components has not been well understood. For instance, cyclic oxygenates formation in the aqueous products (Chen et al. 2014a) and the role of RT on their production are not clear. Some intermediates, such as amino acids, produced from protein via HTL were not stable. One example is the Millard reaction, which takes place between amino acids from protein and reducing sugar from carbohydrates (Changi et al. 2015). The best condition of RT depended by the critical point before forming biochar.

In current researches, the RT value started from the gradually rising temperature reaching to critical point of HT. In other words, they didn't count the time for temperature rising. Actually, during this time, the reactions have happened.

2.2.5 Heating Rate

Limited by the current technology/experimental condition for temperature rising, the real heating rates of most researches were very low. The role of heating rates on biocrude production has not been well understood. It is well known that fast pyrolysis can maximized bio-oil production through a combination of fast heating rate to a mid-range temperature during a very short residence time (Akhtar and Amin 2011). For practical purposes, empirical correlation may be suitable to estimate the yield of liquid oil, as a function of heating rates shown as the following equation (Zhang et al. 2009):

$$\text{Liquefaction } (\%) = [0.0042 \times \ln(\text{heating rate}) + 0.5514] \times 100$$

A higher heating rate usually leads to higher amounts of biocrude yield (Zhang et al. 2009). Higher heating rates are supportive for degrading of biomass while slow heating rates usually lead to the formation of char residue due to secondary reactions (Brand et al. 2014). This is true for both pyrolysis and algae HTL. Similar to fast pyrolysis, HTL consists of beneficial primary reactions (pyrolytic and hydrolytic degradation) and non-beneficial secondary reactions, i.e., recombination and secondary cracking. However, heating rates impart low effect on the product distributions in HTL than in pyrolysis. The reason is that the better dissolution and stabilization of fragmented species in hot compressed water medium. Secondary reactions become also dominant at very high heating rates which result in high gas yields as in case of supercritical gasification (Zhang et al. 2009). Moreover, biocrude yield is not very sensitive to large variations in high heating rates. Suitable heating rates can lead extensive fragmentation and minimal secondary reactions. On such bases, moderate heating rates may be enough to overcome heat transfer limitations and to produce high mass of biocrude (Akhtar and Amin 2011).

HTL is typically performed with slow heating and/or reaction times of tens of minutes or longer, but recent results suggest that shorter reaction times may be sufficient (Bach et al. 2014). Comparing with conventional HTL for 60 min, the fast HTL of *Nannochloropsis sp*. at reaction times of 1–5 min has been investigated to performed that the biocrude yield and corresponding energy recovery are the highest reported for liquefaction of algae (Faeth et al. 2013). For a reaction time of 1 min, as the set-point temperature increases, light biocrude (e.g., hexane solubles) makes up less of the total biocrude. The biocrudes produced by fast HTL have carbon contents and higher heating values similar to biocrudes from the traditional isothermal liquefaction process, which involves treatment for tens of minutes. These results indicate that biocrudes of similar quality maybe produced in higher yields and in a fraction of the time previously thought necessary. Such a decrease in the reaction time would greatly reduce the reactor volume required for continuous biocrude production, subsequently reducing the capital costs of such a process. The reaction ordinate is a useful parameter for interpreting results from algae liquefaction performed at different temperatures and RT (Brand et al. 2014; Bach et al. 2014; Faeth et al. 2013).

2.2.6 Catalysts

Catalysts are undoubtedly important for HTL, which could affect the reaction rates, the composition of HTL products. Both homogeneous (alkali, alkali salts, etc.) and heterogeneous catalysts (metallic oxide, etc.) were investigated for the catalysis of algae HTL (Yeh et al. 2013; Savage 2009).

So far, the majority of the work has focused on homogeneous catalysis by acid, alkali, or metal salts partly because homogeneous catalysts are cheap. Compared with heterogeneous catalysts, the main characteristics of homogeneous catalysts are aqueous products without suffering from coking (Yeh et al. 2013; Savage 2009). Homogenous catalysts employed for HTL are (Tian et al. 2014): acids (H_2SO_4, HCl, and acetic acid), metal ions (Zn^{2+}, Ni^{2+},Co^{2+}, and Cr^{3+}), and salts and alkalis ($CaCO_3$, $Ca(OH)_2$, HCOONa, and HCOOK; Na_2CO_3, NiO, $Ca_3(PO_4)_2$). Alkalis are often used to break up carbon–carbon bond, thus beneficial for the formation of gases. Acids and alkalis enhance the hydrolysis of algal biomass, while metal ions favor the dehydration during HTL (Yeh et al. 2013; Savage 2009). Based on the conversion efficiency the catalytic activity is in the following order: K_2CO_3>KOH>Na_2CO_3>NaOH (Karagöz et al. 2005); the yields of biocrude were in the descending order of the used homogeneous catalysts: Na_2CO_3>CH_3COOH>KOH>HCOOH (Ross et al. 2010).

But homogeneous catalysts have the drawbacks of special requirement on HTL reactor material and being difficult to recycle. In comparison, heterogeneous catalysts have the advantages of reaction selectivity and recovery after HTL (Yeh et al. 2013; Savage 2009). Recently heterogeneous catalysts have received increasing attention, in particular the development of non-noble metal based catalysts. However, there is a long distance before its application to HTL. Biller et al. (2011) reported that the use of heterogeneous catalysts enhanced the deoxygenation of biocrude. Co/Mo/Al_2O_3 and Pt/Al_2O_3 seemed to selectively deoxygenate carbohydrates and proteins, whereas Ni/Al_2O_3 preferred deoxygenating lipids, supported by more alkanes formed in the biocrude. Usage of Ni/Al_2O_3 catalysts also appeared to enhance gasification (Biller et al. 2011).

The results of HTL using catalysts were not always positive mainly due to the problem of inactivation. For instance, one study unexpectedly reported higher oil yield and HHV was achieved without catalysts than using catalysts (Biller et al. 2011). This situation may attribute to carbon deposition on the surface of catalysts after HTL (Yeh et al. 2013; Savage 2009), which might reduce the contact area between feedstock and noble metal atoms, and further decrease the activity of catalysts.

2.3 Separation Procedure of HTL Products

Separation procedure is a key factor of downstream for HTL. The standard procedure for HTL products separation has not yet been well established. So far, there is few articles focus on this topic. Figure 6 has summarized three model figurations of

post-HTL separation schedule. Three major HTL products separation have been found among the literature. This classification was made according to the different treating methods for recovery of aqueous phase.

The mode A of separation procedure (Fig. 6-A, marked as M-a) has performed its flexibility, and relatively paid more attention (Yu et al. 2011a, b; Jena et al. 2011; Li et al. 2014; Chen et al. 2014a; Tian et al. 2015; Gai et al., 2014, 2015b). Using right solvents, both heavy and light biocrude can be separately obtained. Heavy biocrude can be recovered by vacuum filtration (Li et al. 2014; Tian et al. 2015; Jena and Das 2011) or soxhlet extraction (Yu et al. 2011a; Chen et al. 2014a; Gai et al. 2015b). The procedures mostly were used for recovering of biocrude by vacuum filtration after extracted by some solvents. Biocrude derived from HTL was usually including water and this situation might not be adequately for extraction (Li et al. 2014). For better products recovering, soxhlet extraction was applied for this post-HTL procedure. The raw oil (residue oil) should be dried before using soxhlet extraction for recovery of biocrude. According to the ASTM stand for testing of petroleum, Yu et al. (2011a, b) proposed a procedure for the HTL products recovery. In their research, the products are firstly filtrated under vacuum and separated into light and heavy fractions, and the heavy fractions retain on the filter; heavy fractions (residue oil) are dried and then separated into heavy biocrude and solid residue by using toluene extraction. Biocrude presented here was mainly referred as heavy biocrude, since in many cases, light biocrude were not separated and mixed with aqueous products. Actually, light biocrude was water insoluble and above the aqueous phase, and it can be recovered by extraction using some hydrophobic solvent (Li et al. 2014), like diethyl ether or n-hexane, like “Solvent 2” in Fig. 7.

The mode B of separation procedure (Fig. 6b, marked as M-b) (Valdez et al. 2012, 2014; Valdez and Savage 2013; Valdez et al. 2011) was carried out through direct addition organic solvents for extraction and washing reactor (e.g., dichloromethane and acetone). Noting that used solvents also must be some hydrophobic. The mixture products excluding gases after HTL can be also naturally stratified into three layers by using a separating funnel: The top layer consists of light fraction of biocrude oil, the middle is aqueous product, and the bottom includes heavy biocrude oil and solid residue. The aqueous product was recovered by phase separation of gravity and biocrude was further recovered by the vacuum filtration, respectively. The solid residue retains on the filter. Biocrude in M-b refers to total biocrude, containing both heavy and light biocrude. M-c was carried out under room temperature by gravity, so this procedure was low-cost but the work efficiency is the problem.

The mode C of separation procedure (Fig. 6c, marked as M-c) was carried out through first removing water for drying the reaction mixtures via vacuum evaporation (Peng et al. 2014a, b; Wu et al. 2013). M-c was not very popular for products separation of algae HTL. The advantage of this method is that the dried mixture is easily for further extraction using kinds of solvent, and the moisture of biocrude would be very low which is useful for next upgrading.

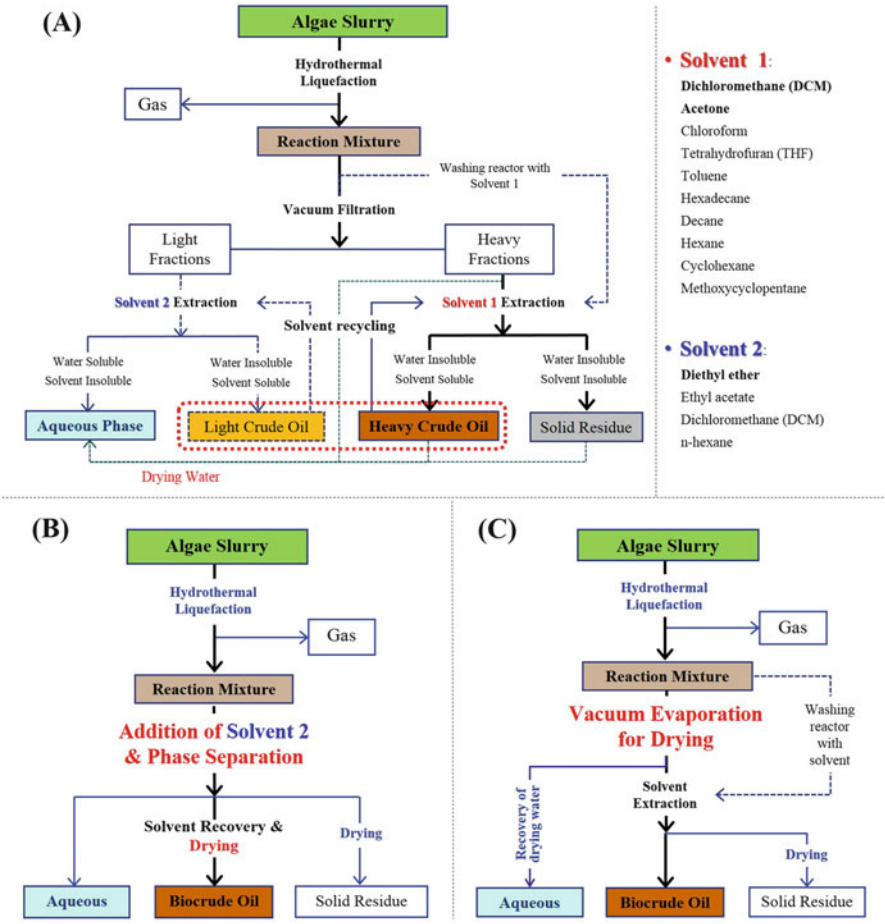

Fig. 7 Summary for three separation schedule models of HTL products in the batch scale. (**a**) Vacuum filtration for aqueous removal, (**b**) Static extraction and separation using hydrophobic solvent, and (**c**) Vacuum evaporation of water

Using different solvents also effects on biocrude recovery. Nonpolar solvents (hexadecane, decane, hexane, and cyclohexane) and polar solvents (methoxycyclopentane, dichloromethane, and chloroform) were tested for separation of biocrude, and the results indicated that hexadecane and decane (nonpolar solvents) were found to result in the highest yields of biocrude, however, had lower carbon content than those with polar solvents (chloroform and dichloromethane).

Besides HTL products recovery biocrude oil, aqueous products and water involved in the procedure could be collected and referred as "post-HTL wastewater"(Zhou et al. 2013; Gai et al. 2015c; Zhang et al. 2015; Pham et al. 2013). Different modes of separation procedure have very different characterization

of "post-HTL wastewater." For instance, the most "clean" "post-HTL wastewater" would be generated from M-c, the next order would be M-b or extracted aqueous phase of M-a, the last is non-extracted aqueous phase of M-a. These generated "post-HTL wastewater" especially are important in the algae biorefinery of E2E (Zhang et al. 2015).

3 Research Focuses and Current Status

To realize the algal biorefinery via HTL, besides getting the basic knowledge of algae HTL and its key effect factor, research must understand how it works and develops the process towards to industrial production. These are actually the research focuses.

3.1 Reaction Mechanism

The mechanism of HTL of algae has not yet been clearly elucidated. Quantitative reaction models based on the governing reaction network are needed for reactor design and process optimization (Changi et al. 2015; Kruse et al. 2013). To closely track all products streams, rather than only pursue biocrude oil on the energy purpose, a systematic analysis was given in this section.

3.1.1 Reaction Network

As discussed above, the operational conditions of HTL such as feedstock selection, TS, HT, and RT significantly affect the distribution of products. Based on the literature data (Jena et al. 2011; Li et al. 2012; Anastasakis and Ross 2011), Fig. 8 has given a diagrammatic analysis of relationship on HTL phases' conversion. Fig. 8a is a ternary contour based on the distribution of four HTL products from literature data. From Fig. 8a, we can clearly see that feedstock is the deceive factor, the process parameters can significantly affect the HTL products distribution in some range. Fig. 8b presented a potential reaction network, showing the dominant reaction directions and paths for the algae HTL. Comprehensive analysis of these three algae species is helpful for understanding of the HTL reaction network.

The initial algae materials referred as solids in Fig. 8. As the reaction starts, the yields of gases, aqueous products, and biocrude increase as solids decrease, suggesting direct reaction paths from the solids to all of the other phases. The reaction of solids to aqueous products includes the release of intracellular proteins and carbohydrates and their subsequent decomposition under subcritical water. The pathway from solids to biocrude presumably represents the decomposition of the cell wall, like the phospholipids are hydrolyzed. Intracellular lipids are also

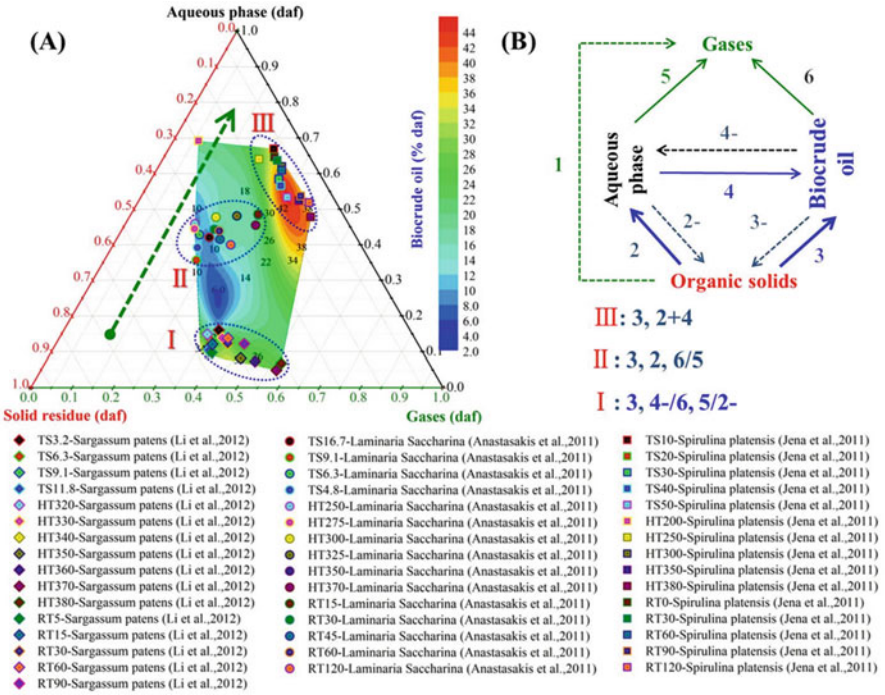

Fig. 8 The diagrammatic analysis of relationship on HTL phases conversion. Figure was drawn derived from Jena et al. (2011), Li et al. (2012) and Anastasakis and Ross (2011). (**a**) Analysis of relations between biocrude and other three phases. Note that the values of triangular coordinates were not their real yields but the respective proportions of aqueous phase, gases, and solid residue. (**b**) A reaction network analysis between biocrude and other three phases according to Fig. 8-A. Fig. 8 reveals that the conversion efficiency of algae HTL highly related with the chemical components of feedstock; in terms of reaction network, the conversion from solids to aqueous phase can significantly affect on the biocrude yield

released and hydrolyzed as the reaction progresses. It is likely that the aqueous products and biocrude continue formation of gases as the reaction ongoing. The biocrude contributes some gaseous compounds that are formed during cracking reactions. The path from biocrude/aqueous to solids may be reversible if secondary reactions occur as has been assumed. Biocrude also probably contribute to the aqueous products, For instance, triglycerides and phospholipids are hydrolyzed to form water-soluble glycerol and phosphates. Such these secondary reactions may account for the slight increasing RT (Valdez et al. 2012, 2014; Valdez and Savage 2013).

HTL reaction network has further meanings of reaction kinetics, which is highly dependent on the algae species and composition of algae components (Valdez et al. 2014; Valdez and Savage 2013). Macroalgae (e.g., *Sargassum patens* and *Laminaria Saccharina* in Fig. 8) usually have lower proteins but higher carbohydrates than microalgae (e.g., *Spirulina platensis* in Fig. 8), leading to higher solids residue

but lower converted aqueous and biocrude. This reaction kinetics has been further investigated by Valdez et al. (2014) and Valdez and Savage (2013).

Some instructive significance for promoting HTL operation process can also be obtained. For instance, a unique two-step sequential algae HTL technology for the simultaneous production of value-added polysaccharides and biocrude has been developed (Miao et al. 2012; Chakraborty et al. 2012); two-stage HTL of high-protein microalgae for reducing the nitrogen concentration in the biocrude to satisfy the oil use in conventional refining processes (Jazrawi et al. 2015). Investigation of HTL reaction network/ kinetics would also be helpful to understand the reaction mechanism in molecular level.

3.1.2 Mechanism of Reaction/Interaction of Model Compounds

Model organic compounds including protein, starch and glucose, triglyceride, and amino acids are very useful to investigate HTL mechanism (Changi et al. 2015) since the real biomass quiet complex for research. A potential reaction scheme for algae HTL was given in Fig. 9. The HTL of organic components in algae includes three steps (Changi et al. 2015; Peterson et al. 2008; Toor et al. 2011; Chen et al. 2014a):

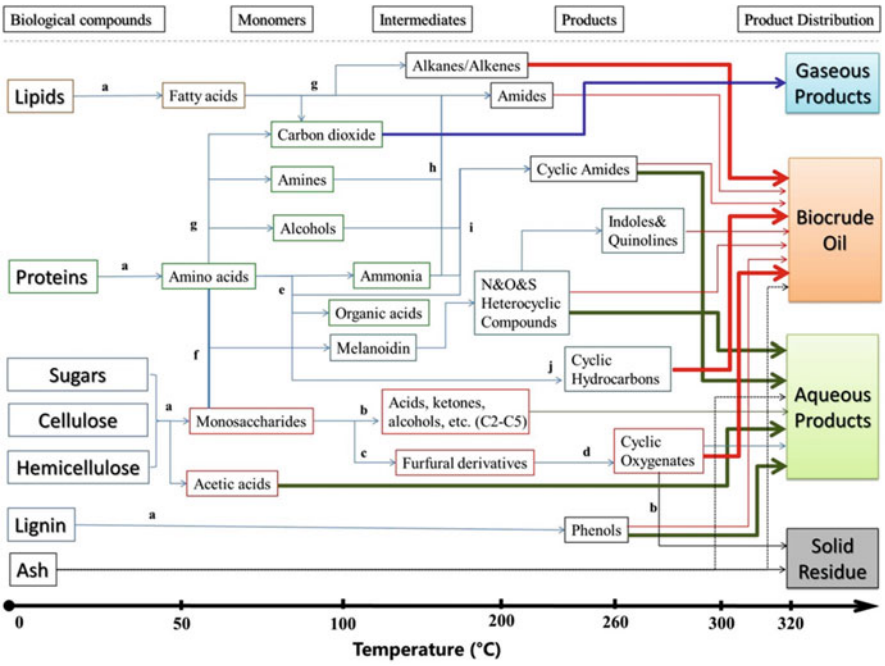

Fig. 9 Potential reaction pathways for HTL of algae: (**a**) hydrolysis; (**b**) decomposition; (**c**) dehydration; (**d**) polymerization; (**e**) deamination; (**f**) Maillard reaction; (**g**) decarboxylation; (**h**) aminolysis; (**i**) cyclization; (**j**) condensation + pyrolysis. The thickness of arrows represents the relative amount of products distributed to different phases. Figure was drawn derived from Chen et al. (2014a)

1. Hydrolysis of proteins, carbohydrates, and lipids into their corresponding monomers such as fatty acids, amino acids, and sugars at lower temperatures;
2. Decomposition of amino acids, fatty acids, and sugars as the reaction temperatures increased. Nitrogen and oxygen were removed from the carboxyl and amine groups via decarboxylation and deamination, respectively. Aminolysis can occur when the HT $> 220°C$. Cyclization involving alcohols, ammonia, and amino acids can occur and cyclicamine derivatives can be generated under HT $> 250°C$; and,
3. Decomposition of intermediates, recombination, and condensation may happen when HT continue to rise. Dehydrohalogenation of alkanehalides subsequently occurred at elevated reaction temperatures and alkynes were generated at about 300°C. Alkanehalides and cyclic hydrocarbons were formed if condensation and halogenation took place.

The pathways for HTL of mixture were clearly different from that of individual model compounds. Binary or even ternary mixture systems are a step closer to understanding the actual processing challenges. Interactions that could increase the nitrogen content in the biocrude include the Maillard reaction, oligomer formation with amino acids and glycerol, and esters forming amides with amino acids. An exception was binary mixtures of polysaccharide and protein, which produced higher than respective biocrude yields (Changi et al. 2015). These enhanced biocrude yields via Maillard reaction also provide a theoretical fundament for co-liquefaction, which has been discussed in Sect. 2.1.1. However, very few studies have been carried out with binary mixtures. The reactions of ternary, quaternary, and more complicated systems under subcritical water have received even less attention. Future work need to come closer to representing reactions of highly complex mixtures until the real algal biomass.

3.2 Operation Mode and Reactor Configuration

Although HTL is a promising technology, algae HTL is still in the early stage. HTL has been already tested for various types of biomass, and began algae HTL testing in continuous-flow and pilot plant recently. The studies presented in Fig. 3 were all batch studies; Table 1 has summarized the current continuous algae HTL. Continuous-flow tests can provide a more reasonable basis for process design and scale-up for commercialization (Elliott et al. 2015).

Figure 1 has presented a continuous HTL process (Tian et al. 2014), in which we can figure out the related operation and instrumentation. The critical units mainly contain feeding, heating, reaction, and separation. Each unit has some problems need to resolve.

1. The high-pressure/high-temperature feeding systems are needed in the continuous HTL process. It is a barrier to implementation through pumping of wet biomass slurries at normal condition is well known (Larsen 2005; Feng et al. 2004).

Table 1 Overview of the continuous algae HTL processes

Process	Time	Developer	Feedstock	Testing scale	References
PNNL-process*	2013	Pacific Northwest National Laboratory (USA)	Macroalgae	1.5 L/h	Elliott et al. (2013)
USyd-process*	2013	University of Sydney (Australia)	Microalgae	Pilot	Jazrawi et al. (2013)
UVir-process*	2013	University of Virginia (USA)	Algae	Pilot	Liu et al. (2013)
ULee-process*	2015	University of Leeds (UK)	Microalgae	Pilot	Biller et al. (2015)
ICL-process*	2015	Imperial College London (UK)	Algae	#N/A	Patel and Hellgardt (2015)

#N/A: not available
*Named by this article according to the developer name

2. Pre-heater, heat-exchanging system must be considered for higher utilization efficiency of heat in the heating unit (Feng et al. 2004).
3. Heating mode and reactor configuration must be carefully considered for the reactor design (Elliott et al. 2015).
4. Separation mode should be developed to work continuous, stable, and high efficiency.

The last but not least, the whole system should make all units work in concert and harmony.

4 Challenges and Prospective of HTL Pathway for Algal Biorefinery

HTL of wet biomass provides a viable route to liquid fuels from biomass, though, subsequent upgrading of the HTL biocrude is necessary since biocrude produced via HTL has high contents of O and N elements, and needs further deoxygenation and denitrogenation before its application as the transport fuel. In addition, treatment of the by-product aqueous is a key component for algal biorefinery via HTL. Thus, in the biorefinery HTL efficiency and utilization of biocrude and co-products especially aqueous are hot topics.

4.1 HTL Efficiency

Most of elements in biomass, i.e., carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, potassium, sodium, etc., exist as either heteroatoms with the carbon or ions

(Gouvea 2011; Demirbas and Demirbas 2010). Only carbon and hydrogen can be used for hydrocarbon liquid fuels. Oxygen and nitrogen are undesirable for oil purpose. Oxygen itself has no heating value, and nitrogen will cause environment pollution if combusted. Thus, biocrude formation via HTL is accompanied with deoxygenation and denitrogenation (Ramirez et al. 2015; Zhu et al. 2013). Figure 9 is a advised van Krevelen diagram of algae and their biocrude (Tian et al. 2014). In general, most algal biocrude has lower H/C than pyrolysis oil, whereas most algae and their biocrude have lower O/C than pyrolysis oil, indicating the unique features of algae HTL biocrude. As shown in Fig. 10, O/C and N/C are significantly reduced

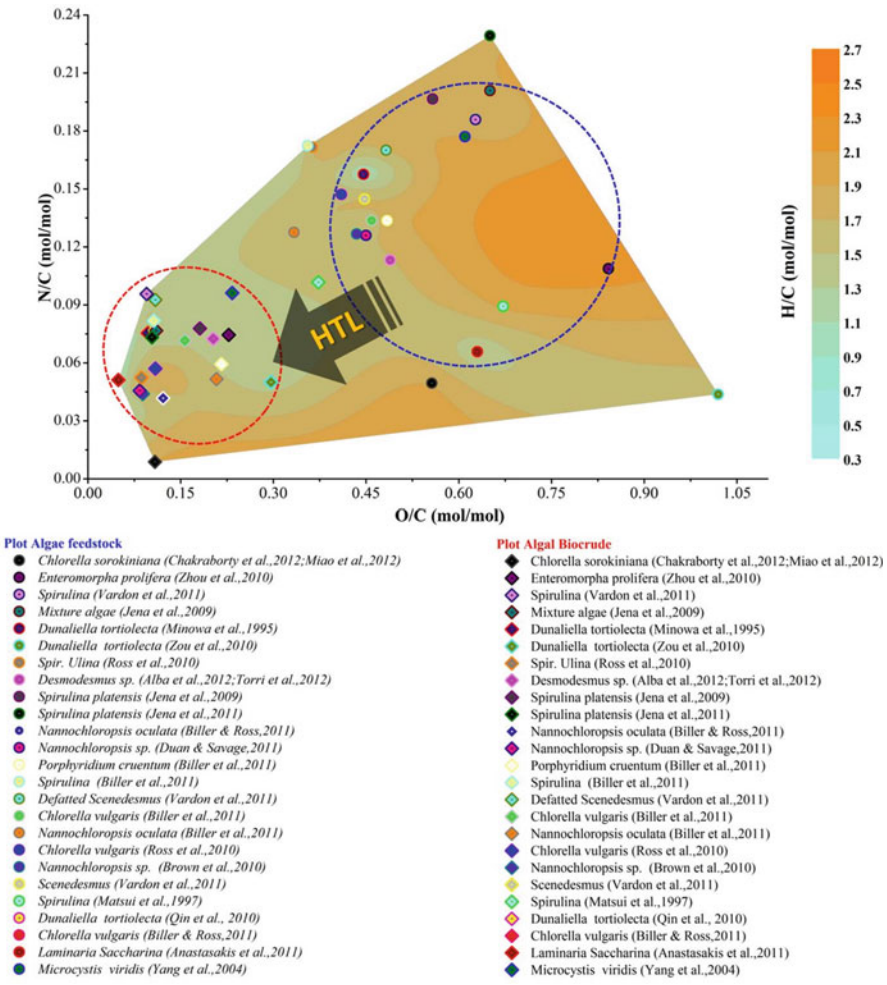

Fig. 10 The relationship of N/C and O/C in the biocrude oil during HTL. Figure was drawn derived from Tian et al. (2014)

during algae HTL, which demonstrates that HTL is an effective process to recover carbon and hydrogen and remove oxygen and nitrogen. However, compared with conventional crude oil oxygen and nitrogen contents in the biocrude are still much high for application as transportation fuel (Ramirez et al. 2015; Zhu et al. 2013). H/C atomic ratio was reduced from about 2.5 to 1.0 during HTL, which is close to crude oil, since petroleum has an average H/C of 1.84 according to Hunt (1996). Thus, biocrude still mainly needs upgrading to reduce O/C and N/C and slightly increase H/C.

The oxygen content of hydrocarbon liquid fuels is less than 1%, whereas algal biomass has around 40–60% oxygen (Gupta and Demirbas 2010). Thus deoxygenation is a main task for converting biomass into hydrocarbon fuels. O/C of most algal biocrude was similar to that of plant oil and biodiesel, and lower than pyrolysis oil (Tian et al. 2014), suggesting HTL results in higher energy density of biofuels than pyrolysis. Oxygen is preferentially removed as H_2O, CO_2, and CO through deoxygenation. Decarboxylation is more attractive form of deoxygenation than dehydration due to more oxygen removal, which could be enhanced by using alkali like KOH (Penninger 1988).

Nitrogen content in biocrude can impact the properties (Gupta and Demirbas 2010), like smell and combustion. Nitrogen in proteins is main contributor for that in biocrude, which will be decomposed and reformed via HTL (Yu et al. 2011b). The high nitrogen content of the biocrude is therefore considered as one of bottlenecks hindering the development of algae HTL to produce transportation fuel (Gupta and Demirbas 2010; Ramirez et al. 2015; Zhu et al. 2013). In a study using cellulose and ammonia as feedstock, the increase of N/C of feedstock resulted in the increase of biocrude yield and nitrogen content, and the decrease of solid residue (Inoue et al. 1999). Another study also demonstrated that nitrogen remarkably affected oil yields and nitrogen contents by using nitrogen-rich albumin and nitrogen-poor sewage sludge (Dote et al. 1996). Studies on nitrogen distribution of HTL products showed that nitrogen was mainly released to aqueous products (Fig. 5), whereas the rest was transferred into either biocrude as nitrogen heterocycles or the gases as NH_3/HCN, depending on operational conditions (Ross et al. 2010). Homogeneous catalysts such as Na_2CO_3 may reduce the nitrogen of biocrude (Inoue et al. 1999; Dote et al. 1996; Dote et al. 1998). Nitrogen distribution receives little attention recently, but it is crucial for sustainable algal biofuels.

4.2 Biocrude Quality and Utilization

In general, major compounds of biocrude identified by GC-MS consist of cyclic nitrogenates (e.g., pyrrole, indole, pyrazine, and pyrimidine compounds), cyclic oxygenates (e.g., phenols and phenol derivatives with aliphatic side-chains), and cyclic nitrogen and oxygen compounds (e.g., pyrrolidinedione, piperidinedione, and pyrrolizinedione compounds) (Ramirez et al. 2015; Zhu et al. 2013). The HHV of most biocrude is in the range of 30–38 MJ/kg, which is very close to petroleum

(42 MJ/kg) (Tian et al. 2014). The important role of HTL is in sustainable energy/chemicals production, meanwhile protection environment. Biocrude oil has high contents of oxygen and nitrogen, and high molecular weight/viscosity (Ramirez et al. 2015; Zhu et al. 2013). Biocrude usually was upgraded through separation of solvent extraction/distillation, hydrogenation/hydrodeoxygenation and followed catalytic cracking, esterification, and hybrid process (Ramirez et al. 2015). As results, oxygen is partly removed as CO_2 or H_2O and nitrogen is partly converted into ammonium.

Besides biofuel production, some groups have paid attention to the potential of HTL for valuable chemicals production. For instance, concomitant extraction of biocrude and polysaccharides (e.g., α-glucan) from *Chlorella sorokiniana* was carried out by a unique two-step sequential HTL technology (Miao et al. 2012; Chakraborty et al. 2012). Another example was the oriented production of organic acids (e.g., acetic acid, formic acid, etc.) using acid- or base-catalyzed HTL (Jin and Enomoto 2011).

So far, the "only biofuel" option is unlikely to be economically viable for algae biomass (Williams and Laurens 2010). Other chemicals can also be produced either from HTL or other process. It might also be possible to extract value-added chemicals from biocrude since it is complex and contains different cyclic nitrogenates and oxygenates besides hydrocarbon (Ramirez et al. 2015).

4.3 Co-Products

As mentioned above, treatment of the by-product aqueous is a key component for algal biorefinery via HTL. There are mainly two pathways for treatment of aqueous (Pathway① and ② in Fig. 2) (Orfield et al. 2014).

Pathway①, which is also called "E2E" (Zhou et al. 2013; Tian et al. 2013) or "algae regrowth pathway"(Orfield et al. 2014), has the potential to resolve the bottlenecks of current algae feedstock including the consumption of water, fertilizer, and energy input (Zhou et al. 2013). Unlike the traditional process through which energy generation (i.e., combustion) could easily bring out environmental problem, the E2E paradigm integrates biocrude production through HTL, and post-HTL water treatment and CO_2 capture through algae growth. A systematic study (Zhou et al. 2013) indicated that the integration of algae HTL and algae growth could lead to the increase of biomass up to ten times (Fig. 11). However, aqueous products generated through HTL contain oxidative and toxic compounds (e.g., phenols, pyridines), which may inhibit algae regrowth while being recycled as nutrients.

Pathway② in Fig. 2 is "catalytic hydrothermal gasification (CHG) pathway" (Elliott et al. 2014), which is used to recover thermal and electrical energy from the aqueous products through the production of gas fuels (CH_4 and H_2), at the same time to clean water. This pathway can provide water for recycle and reuse that it's a major consideration in the design. In addition, the generated gases can use as fuel gas (CH_4) and carbon resource (CO_2).

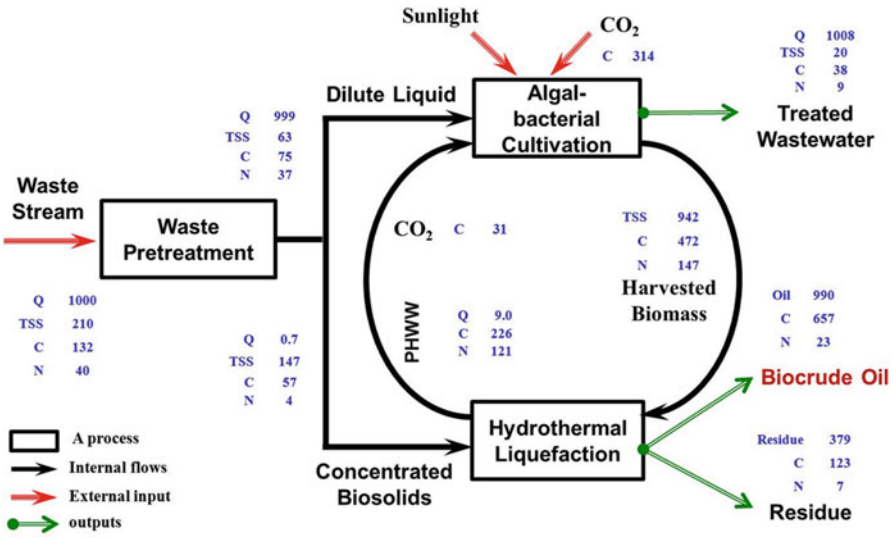

Fig. 11 The mass balance for algal biorefinery of E2E. Figure was drawn derived from Zhou et al. (2013)

In gaseous products, both CO_2 and NH_3 can be fed back for autotrophic algae cultivation, while other gases can be used as fuel gas. However, to really realize this way the separation and purification technology of gaseous products still should be developed.

Gaseous products present primarily CO_2 and other small amount of CH_4, H_2, C_2H_4, C_2H_6, and NH_3 (Elliott et al. 2014). Both CO_2 and ammonia gas can be fed back for autotrophic algae cultivation, while other gases can be used as fuel gas. However, to really realize this using way the separation technology should still develop.

Solid residue is normally ash that highly depends on ash content in the algae. The remained inorganics and salts in aqueous might be recycled for algae cultivation, meanwhile solid in organics can exploit new catalysts since many metal salts have catalysis effects (Roberts et al. 2015).

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Recent Advances in Production of Biofuel and Commodity Chemicals from Algal Biomass

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Contents

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1 Introduction

Rapid development of technocratic society and sustainability of conventional fossil fuel sources are antipodal to each other. During past few decades, we have witnessed dramatic increase in the consumption of fossil fuels, causing several socio-economic changes across the world. Interestingly, conventional fossil fuel reserves are limited, thereby leading to the possibility of energy crisis in near future. Thus, recent focus of research has been shifted towards development of sustainable and renewable sources of energy. Albeit, solar, wind, hydrothermal, and tidal energy sources may be an alternative to upcoming energy crisis, biomass as a source of renewable, and sustainable energy is more interesting due to its ability to produce both chemicals and fuel (Alam et al. 2015; Ahmad et al. 2016). On the contrary, not all the biomass sources can be considered as a sustainable source. For example, first generation biofuel biomass sources such as sugarcane, sunflower, corn, and soybean have conflicts with the food usage which limits their applicability for further consideration (Bardhan et al. 2015), whereas second generation biofuel sources have shown promising results and are used to produce a wide range of biofuel such as bio oil, biochar, biofuel, and hydrogen (Tinwala et al. 2015; Kumar and Pant 2015; Saikia et al. 2015; Pant and Mohanty 2014). However, majority of the products obtained either oxygenates which require further upgradation or other form of fuel which may require modification in existing engine system. For example, bio-hydrogen can be directly produced from biomass derived ethanol (Mondal et al. 2016). On the contrary, application of hydrogen based vehicle is yet to be adopted at large scale. In addition, promotion of lignocellulosic biomass may promote deforestation and depletion of resources as it takes several years for a tree to grow. Noteworthy that energy density of bio oil (10.6 GJ/m^3) obtained from conversion of lignocellulosic biomass is unsubstantial as compared to energy content of the regular gasoline and diesel (35.7 and 39.7 GJ/m^3, respectively). Furthermore, most of the biofuel contains oxygen, therefore used as a fuel additive instead of being directly used as a fuel. Consequently, it necessitates the production of biofuel having comparable properties with gasoline and diesel.

Interestingly, energy density of biodiesel (35.6 GJ/m^3) obtained from algal biomass is found to be comparable with conventional diesel. Moreover, application of algal biomass for biofuel production can be helpful in curbing environmental and aquatic pollution (Chisti 2007). For example, Eduardo et al. have reported an integrated process for the production of biofuel from algal biomass that can be attached to power plants to consume flue gases (Santillan-Jimenez et al. 2016). Although further integration of this technology is possible which we believe that yet to be explored. To be noted that power plants generate huge amount of wastewater and flue gases. Therefore, we hypothesize that algal biomass cultivation may serve as an efficient method to tackle wastewater treatment and flue gas emission simultaneously. However, this approach is yet to be established. Interestingly, unlike lignocellulosic biomass, algal biomass is divided into several categories on the basis of their properties and pigmentation. Filamentous and phytoplankton are

two most abundant varieties of algae which are further classified into four distinct categories, namely golden algae, blue-green algae, green algae, and diatoms. However, two broad major categories of algal biomass are macroalgae and microalgae. Macroalgae based on their pigmentation are further classified into three major categories, namely green seaweed, red seaweed, and brown seaweed. Similarly, microalgae is classified by a variety of methods. Some of them are based on the microalgae cellular structure, pigmentation, and life cycle (Demirbas 2010). Albeit there are several categories of algae, all are capable of utilizing greenhouse gases and sunlight to produce energy and chemicals. However, since oil content of microalgae is relatively higher as compared to macroalgae, thus recent research interest is more inclined towards microalgae. Microalgae is a unicellular microorganism that can survive under both the freshwater and saline water. Diatom algae belongs to the family of phytoplakton and represents largest producers of world's total biomass.

Consequently, a paradigm shift in the biofuel research has been witnessed after consideration of algae as sustainable feedstock for biofuel production (Ullah et al. 2014). The possible reason could be attributed high oil yield with respect to per acre of cultivation land (1200–10,000 gallons per acre of land cultivation land, Table 1, entry 1). It is evident from Table 1 that none of the feedstock are even near to algae in terms of overall oil yield from equal area of land of cultivation. To be noted that free fatty acid content of cottonseed oil, rapeseed oil, and jatropha oil makes them an interesting feedstock for production of oxygen linear chain fuel range hydrocarbons via various routes. However, low oil yield per acre of land remains a challenge.

On the contrary, it is possible to further enhance oil yield by cultivating genetically modified microalgae. In general, algal biomass is extracted using a suitable solvent followed by further processing to yield free fatty acids. Obtained fatty acids are generally converted into FAME via similar process implied for vegetable oil transesterification. The second most common route is hydrodeoxygenation, where free fatty acids (FFA) obtained from algal biomass are hydrogenated to yield oxygen free fuel range hydrocarbons. In particular, long chain hydrocarbons such as diesel and aviation fuel hold great economic and environmental value. Albeit the differences in per acre oil yield for different substrates, the chemical composition of algal biomass based feedstock does not vary significantly. However, the final yield and conversion may vary. This is due to the

Table 1 Oil yield of different crops per acre of cultivated land

Entry no.	Crop	Oil yield (gallons/acre)
1.	Algae	1200–10,000
2.	Cotton	35
3.	Soybean	48
4.	Sunflower	102
5.	Rapeseed/Canola	127
6.	Jatropha	202

fact that FFA are mainly saturated, hence the possibility of C─C cleavage at carbonyl carbon is expected to be higher as compared to unsaturated feedstock where C─C cleavage is also expected in between carbon chain. Moreover, difference in degree of saturation and unsaturation may lead to completely different reaction pathway under varying process conditions. For example, degree of unsaturation of fatty acids may lead to formation of oligomers and aromatics, thus resulting into low selectivity of the linear chain products. Alternatively, it may lead to strong C─C cleavage to produce more coke, thereby a high catalyst deactivation. However, noteworthy that carbon chain length of the feedstock does not alter the overall reaction rate even when experimental conditions such as temperature and solvents are changed. For further study on effect of feed type over conversion and selectivity, review by Hermida et al. can be referred (Hermida et al. 2015).

Nevertheless, several reports on fuel range hydrocarbons production (mainly diesel and aviation grade) from algae and their techno-economic feasibility are available, indicating a new window of possibilities in this area (Ford et al. 2013; Hengst et al. 2015; Bala and Chidambaram 2016; Zhao et al. 2015; Wang 2016; Singh et al. 2016). With respect to techno-economic feasibility analysis between different methods for FFA conversion, study by Netlson et al. is worth reading (Natelson et al. 2015). It is estimated that jet fuel break-even cost could be as low as $0.80/kg which is significantly lower than DOE reported price for jet fuel ($1.0/kg). It is also predicted that under worst case scenario, the maximum cost could go up to $1.04/kg which is not much higher than the DOE projected price. However author's study was based on fatty acids obtained from camelina oil which has similar composition of free fatty acids obtained from algal biomass.

In contrast, high production cost of algae could be a challenge for implementation of FFA derived fuel range hydrocarbons production technologies. However, it has a promising environmental sustainability due to fact that algae cultivation requires carbon dioxide, thus reducing environmental load in an efficient way (Kiran et al. 2014). Moreover, since algae are non-edible, therefore such processes will not make any effect food supply chain. Furthermore, it is possible to FFA yield from algae by cultivating genetically modified algae that in result will lead to higher oil yield per acre. In addition, industrial and domestic waste could be utilized for production of algae. Furthermore, algal biomass production can also serve as an efficient method for consumption of carbon dioxide from flue gases of power plants. These perspectives make algae an interesting and promising feedstock that needs to be explored for their conversion into fuel range hydrocarbons. A general comparison of different generation's feedstock for biofuel production is given (Table 2) followed by various conversion technologies for algal biomass in subsequent section. However, emerging routes such as decarbonylation and decarboxylation have been focused thereafter.

In general, several processes have been developed for production of biofuel from algal biomass. However, for ease of discussion we have divided all the processes

Table 2 1st, 2nd, and 3rd Generation feedstock for biofuel production, advantages, and disadvantages (Pant and Mohanty 2014)

	1st Generation	2nd Generation	3rd Generation
Feedstock	Sugar, starch crops, vegetable oils, soybean, animal fat, straw, etc.	Wood, agri waste, MSW, animal manure, pulp, sludge, grass	Algal biomass
Product	Biodiesel, sugar alcohol, ethanol	Hydro-treating oil, bio oil, FT oil, etc.	Algae oil, fatty acids, esters, lubricants
Advantages	Economic and environment friendly	Non-competing with food, better conversion techniques	High oil content and lipid content
Disadvantages	Limited feedstock, low blending	Lignin content, high oxygenates	Slow growth, difficult extraction

into three major categories, namely thermal, catalytic, and biological routes. All of these processes are well known and reported in literature. The operating conditions and other parameters within these three routes itself vary, thereby resulting into different products and by-products. In Fig. 1, we have summarized the major processes, products, and their advantages as well as disadvantages for algal biomass conversion. Thereafter, a detailed discussion is followed in subsequent sections for explaining further sub-categorization of each route.

2 Thermal Routes for Biofuel Production from Algal Biomass

Albeit various technologies have been developed for production of biofuel from algal biomass, thermal (or thermochemical) route is most preferred and established process for biofuel production at large scale. Possibly, this process is preferred due to the fact that no major pretreatment (except drying) of feedstock is required. The major thermal routes reported in this regard are combustion, gasification, pyrolysis, and liquefaction. In general, all these processes require elevated temperature, thus requires advanced instrumentation. Interestingly, both macroalgae (such as Enteromorpha prolifera, Ulva lactuca L, and others) and microalgae (such as *Chlorella vulgaris*, Spirulina sp., and others) can be processed via thermal routes, thus making it an important process for further considerations (Raheem et al. 2015). A typical diagram showing major thermal routes for algal biomass conversion and products is given below (Fig. 2).

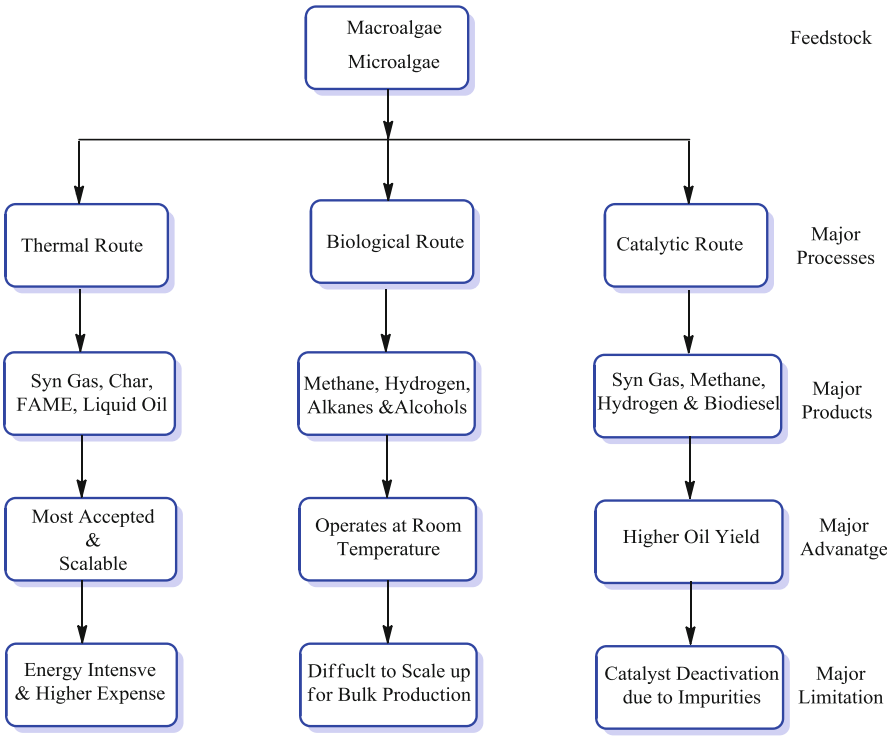

Fig. 1 Major processes for algal biomass conversion, products, advantages, and limitations

2.1 Combustion of Algal Biomass

Direct combustion is a well-known widely accepted process employed for the conversion of second generation biofuel feedstock, e.g., lignocellulosic biomass. Indeed, it is among the most sought-after processes for industrial scale up. Interestingly, the direct combustion process has found crucial applications in the production of energy from algal biomass (Brennan and Owende 2010). In a typical direct combustion process, the algal biomass is charged into combustion chamber with continuous supply of air for complete. The combustion chamber is usually a steam turbine, boiler, or furnace operating at very high temperature range (>800°C) that converts stored chemical energy of algal biomass into heat energy. This heat energy in the form of gases is utilized for preheating, steam generation, or power generation. However, it is crucial to recover the produced heat instantly after generation to avoid the heat loss. Noteworthy that the direct combustion process can be applied at any scale, i.e., for production of energy from domestic application to large and commercial applications (100–300 MW).

However, water content of algal biomass is a major constraint for the successful implementation of the direct combustion process. Thus, it necessitates

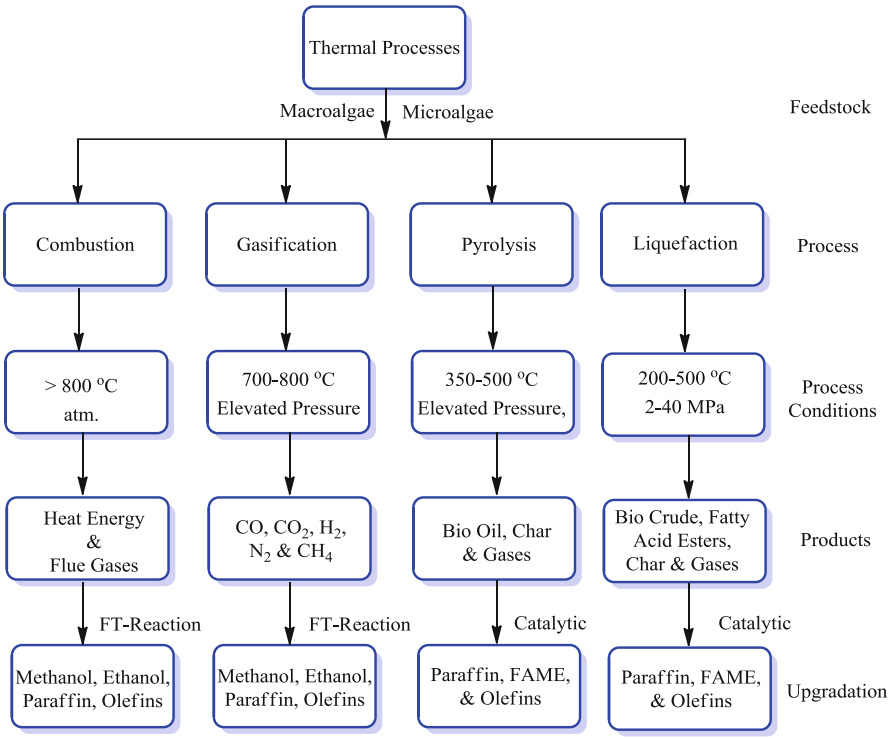

Fig. 2 Thermal routes for biofuel production from algal biomass

the pretreatment of feedstock which essentially includes several steps such as drying, chopping, and grinding, thereby leading to increased energy demand. Consequently, increased energy and equipment costs imparts a negative impact on the overall economics of the process. On the contrary, dry algal biomass enhances the efficiency of CHP power plants up to 40%, thus resulting into overall reduction of power cost. Moreover, application of algal biomass in coal based power plants helps to curb greenhouse gases emission and air pollution (Lane et al. 2014). Therefore, a more detailed and elaborative techno-economic feasibility study is required in this area to calculate the optimum scenario for the application of algal biomass into power plant. Alternatively, a blend of algal biomass and coal can be considered for further studies. Moreover, the gaseous products obtained from such processes may be recovered to produce value added chemicals or feedstock for the growth of algae.

2.2 *Gasification of Algal Biomass*

Gasification is similar process to combustion where algal biomass is partially oxidized via controlled combustion. In contrast, major focus of gasification process is to produce a mixture of combustible gases that can be stored for a period of time which is, so far, unlikely with direct combustion process. In a typical process, algal

biomass is charged into combustion chamber and controlled quantity of oxygen. Simultaneously, water in the form of steam is also supplied. The overall process operates at a temperature range of 700–900°C and produces a mixture of gases consisting of CO, CO_2, H_2, and methane gas (Azadi et al. 2014). It is reported that gasification process proceeds via Fischer–Tropsch reaction which is well-developed and commercially applied technology. Interestingly, Fischer–Tropsch reaction can be used to upgrade the obtained gases into methanol, long chain paraffin, olefins, and other fuel range hydrocarbons. Indeed, syngas obtained from gasification of algal biomass has significant potential to produce an array of chemicals when used a feedstock.

Recent interest in development of technologies for algal biomass gasification is primarily focused towards the production of value added chemicals. For example, methanol is one such valuable chemical that can be produced from the algal biomass derived syngas. Alternatively, a more direct process could be to use the algae itself instead of using syngas because both the processes essentially undergo FT synthesis reaction. However, the major limitation of this process is formation of tar which may suppress the overall yield of the desired product, thus leading to reduced economic advantage. In addition, the presence of tar or undesired impurities may result into suppressing the overall activity of the catalyst during catalytic conversion or upgradation of the algal biomass derived intermediates into useful products. This limitation may be eliminated via designing a suitable reactor configuration (Nikoo and Mahinpey 2008). For example, a combination of FT reactor and reformer can be suggested by NREL in this regard (National Renewable Energy Laboratory 2010).

2.3 Pyrolysis of Algal Biomass

Although, direct combustion and gasification holds a promising future, intensive energy requirement and high cost of reactor setup may be a constraint for such processes for biofuel production from algal biomass. Consequently, low temperature processes have been developed and widely implied for the algal biomass into biofuel. One such interesting and promising process is pyrolysis of algal biomass (Zhang et al. 2007). Pyrolysis of algal biomass is carried out a relatively lower temperature as compared to the gasification and direct combustion process. In addition, products of this process are obtained in all the three phases, i.e., solid, liquid, and gas. The liquid product obtained is essentially bio oil whereas, gaseous products are mostly combustible gases that can be used to meet energy demands of the process and to produce value added chemicals. On the contrary, the solid product properties are comparable to char, thereby finding application as solid fuel source.

Interestingly, pyrolysis of algal biomass is further divided into three sub-categories, i.e., (1) flash pyrolysis, (2) intermediate pyrolysis, and (3) slow pyrolysis. To be noted that all pyrolysis processes applied for the conversion of

lignocellulosic biomass can be applied for the algal biomass as well (Maddi et al. 2011). However, flash pyrolysis has advantage in terms that it is extremely fast, thereby leading to a reduced time for processing of the feedstock. The flash pyrolysis is usually carried out at a temperature range of 350–500°C where algal biomass is brought in contact with heat for a fraction of minutes usually for 2–3 s. In general, feedstock for flash pyrolysis is required to be fine and chopped into smaller particles. Thus, algal biomass has better prospects as compared to lignocellulosic biomass for utilization in flash pyrolysis. The major products obtained through this process are liquid fuel, usually bio oil that can further be upgraded into fuel range hydrocarbons via various catalytic routes. In addition, solid and gaseous products are also obtained that have equal fuel value. However, bio oil yield obtained from flash pyrolysis of algal biomass (e.g., seaweed) is relatively lower as compared to the bio oil yield obtained from flash pyrolysis of lignocellulosic biomass (Yanik et al. 2013). On the contrary, char yield is found to be higher than that of the lignocellulosic biomass. The bio oil yield may improve by utilizing suitable species of algae. To be noted that bio oil yield from pyrolysis of algal biomass depends on several factors such as pyrolysis temperature, flash time, heating rate, and feed composition. Therefore, it is deduced that selecting a suitable algal feedstock and optimizing process parameters may help to improve the bio oil yield further. For example, Budarin et al. have reported the conversion of algal biomass at a very low temperature (130°C) when experiments were performed under microwave irradiation (Budarin et al. 2011). Nevertheless, intermediate and slow pyrolysis processes can be applied for a higher gaseous and solid products yield.

2.4 Liquefaction of Algal Biomass

Thermochemical liquefaction of algal biomass is another interesting process which holds a major advantage. It does not require drying of feedstock which is unlikely the case with pyrolysis (Guo et al. 2015). Indeed, thermochemical liquefaction process can directly be applied for conversion of wet algal biomass into value added products and chemicals. Moreover, it is low temperature process usually operates at a temperature range less than 350°C, thereby leading reduced energy consumptions. On the contrary, this process operates at elevated pressure (10–25 MPa) that may incur additional energy cost (Yang et al. 2016). Furthermore, setting up a reactor may be expensive for liquefaction process due to its complex instrumentation requirements. In a typical liquefaction process, algal biomass is broken down into small molecular fragments using water in bulk quantity under subcritical conditions. Interestingly, this technique is driven from the natural process of formation of fossil fuels which essentially works on the principle of subcritical methods. Consequently, liquefaction yields a viscous crude oil like product called “bio crude.” At present, liquefaction technologies have been applied to recover up to 70% bio crude yield based on dry weight of the algal biomass. The average heating value of bio crude obtained from this processes is in the range of 30–60 MJ/kg which is

comparable to the calorific value of conventional petroleum oil. However, this area is yet to be explored in detail.

Interestingly, algal biomass can be processed by a more recent and robust process called supercritical processing (Bi et al. 2015). The supercritical processing of algal biomass is considered as most efficient of process for the production of biofuel. Unlike, other conventional processes, bio oil produced through supercritical process is simultaneously upgraded into fuel range hydrocarbons. Moreover, product obtained through this process meets the criteria of conventional biodiesel. The major advantage of this process relatively lowers operating temperature as compared to thermochemical liquefaction process. Furthermore, no solvent residues are left over in this process. In a typical supercritical processing unit, algal biomass is charged with a suitable solvent, possibly an alcohol, for extraction of bio oil at elevated pressures. Post this, the produced bio oil undergoes transesterification reaction to produce esters of fatty acids. These fatty acid esters can be utilized as fuel additive to various diesel engines. Noteworthy that bio crude obtained from normal liquefaction process is very complex in process and consists of a wide range of products of different functional groups (Homsy 2012). In contrast, the products obtained from supercritical processing majorly consist of esters as functional group, thus making it easy to determine suitability with the existing fuel (Patel and Hellgardt 2016).

Similarly, supercritical processing of algal biomass when carried out in the presence of water as a solvent produces hydrogen, syngas, and natural gas. Possibly due to its gaseous product composition, supercritical processing is often confused with gasification. However, supercritical processing of algal biomass to produce gaseous products is entirely different from the gasification process. Based on operating conditions and type of feed used, the composition of resulting gas can be optimized. For example, Duman et al. have reported the production of hydrogen rich gas via steam reforming of algal biomass (Duman et al. 2014). Interestingly, up to 100% tar reduction was observed during this process which gives it a cutting edge over other technologies.

3 Catalytic Routes for Biofuel Production from Algal Biomass

Albeit conventional thermal processes are widely accepted and most techniques for production of biofuel from algal biomass, energy consumption needs to be minimized to improve the sustainability of the process. Thus, efforts have been made to develop alternative processes that consumes less energy. In this regard, catalyst supported techniques have emerged as promising method capable of reducing energy consumption significantly, thereby leading to a reduction in overall cost of the process. Worth noticing that more than 90% of industrial chemical processing units utilizes catalysts for production of a wide range of fuel and chemicals.

Interestingly, based on final product obtained, catalytic routes for production of biofuel from algal biomass can be dived into two major sub-categories, i.e., (1) catalytic processes producing gaseous products and (2) the catalytic processes producing liquid fuels. However, the overall process remains similar except the catalyst which is changed to obtain enhanced selectively of desired product.

3.1 Catalytic Process for Production of Gaseous Production from Algal Biomass

Supercritical processing is the primary process applied for the production of gaseous biofuel from algal biomass. As stated earlier, the supercritical process is often referred as gasification. However, gasification is an entirely different process. Interestingly, supercritical processing is also referred as thermal liquefaction which is true to some extent. However, catalytic supercritical processing process for algal biomass conversion is a separate process. Unlike, thermal liquefaction supercritical processing which does not require any catalysts and operates at slightly elevated temperatures, catalytic supercritical processing operates at a relatively lower temperature and thus, consumes less energy. A wide range of catalysts has been reported in this regard that helps to lower the requirement of higher temperature and acts as reforming catalysts simultaneously. For example, Stucki et al. have reported 60–70% heat energy from algal biomass in the form of methane using a ruthenium based catalyst (Stucki et al. 2009). On the contrary, heat energy can be recovered in the form of a relatively cleaner form, i.e., hydrogen by replacing ruthenium catalyst with a nickel based catalyst (Onwudili et al. 2013). Majority of the catalyst employed in this process belongs to the family of transition metals. However, other operating conditions remain similar to that of uncatalyzed supercritical processing.

3.2 Catalytic Process for Production of Liquid Products (Biodiesel) from Algal Biomass

For sustainability of biofuel producing technologies, it is of utmost importance to have their compatibility with existing engines and infrastructure. In this regard, biodiesel produced through transesterification of algal biomass holds an important portfolio. Worthy to note that biodiesel can directly be applied into existing engines and fuel supply chain management system which is unlikely the case with other fuels. This is probably due to the fact that the biodiesel has similar properties and molecular structure with respect to regular diesel. Indeed, biodiesel can replace a significant portion of conventional fuel used in transportation industry (Chisti 2008). This process becomes further important due to similarity in carbon chain

length of the algal biomass esterified biodiesel and regular diesel. Consequently, majority of the algal biomass oil extraction processes are provided with a second stage catalytic reactor for the conversion of bio oil and fatty acid esters. Important to note that transesterification of algal biomass may be carried out either with an acid catalyst or a basic catalyst. Moreover, it is possible to process the wet biomass via this process, which makes it an interesting choice.

In general, the transesterification of algal biomass is done in two steps. Firstly, bio oil from the algal biomass is extracted using various technologies such as supercritical processing. Thereafter, catalytic esterification of obtained bio oil is followed. However, one pot processes for in-situ transesterification of algal biomass have emerged and almost replaced the typical two-step process. Interestingly, this process converts both the free fatty acids and triglycerides into biodiesel. Moreover, it enhances the biodiesel yield by suppressing process losses which are frequent in two-step process, especially during transportation of the fuel. More importantly, this process can be carried out using a variety of homogeneous, heterogeneous catalyst, ionic liquids, and enzymes, thus making it a popular choice in interdisciplinary areas of research (Park et al. 2015). For example, Haas et al. have reported 90% fatty acid methyl ester when experiments were performed at 65°C for 2 h in the presence of sulfuric acid catalyst and methanol (Haas and Wagner 2011). Worth noticing that methanol used here has dual role, i.e., it extracts the oil from algal biomass and esterify it simultaneously (Park et al. 2015). However, the foresaid reaction is reversible in nature; therefore, excess methanol is required to prevent conversion of formed product into reactants. In addition, sulfuric acid is also required in excess due to the fact that huge amount of water present in algal biomass may compete with the protons formed. Thus, further research in this area is required to minimize the methanol and acid requirement. In addition, there is possibility to apply microwave and ultrasound heating methods which may help in improving the overall yield of the process.

4 Biological Routes for Biofuel Production from Algal Biomass

In general, biological routes for production of any chemical and fuel are most preferred and environment friendly route. One possible reason could be the elimination of high temperature requirement. Most of the biological processes operate at room temperature and do not require complex instrumentation system. Moreover, scale up of biological processes is easy as compared to thermal and catalytic routes. To be noted that biological processes consume most of the algal biomass, thereby resulting into maximum utilization of the feed. Interestingly, biological routes do not essentially require pretreatment and cultivated algal biomass can be used as feedstock without any further processing (drying, etc.). Moreover, products

obtained such as methane, hydrogen, alcohols, and alkanes through biological routes are ready to use without further upgradation.

4.1 Production of Methane via Anaerobic Digestion of Algal Biomass

Anaerobic digestion process is primarily applied for production of methane from macroalgae. Interestingly, it is considered to be one of the most efficient processes which eliminates several intermediate steps required during conventional processing of the algal biomass. Moreover, application of anaerobic digestion based technologies may escalate the biofuel production rate. Furthermore, the anaerobic digestion process occurs naturally in digester and does require any special arrangement. In contrast, methane obtained in the form of biogas is a potential greenhouse gas, thereby necessitates the special arrangement to trap the gases. The gaseous product obtained through anaerobic digestion consists of methane and CO_2 in major quantity (Bruhn et al. 2011). CO_2 from methane can be separated using suitable techniques such as gas scrubber, thus resulting into natural gas like composition. Albeit several reactions take place during the anaerobic digestion process, the major reactions which can be considered as limiting factor are hydrolysis, acetogenesis, and methanogenesis (Alaswad et al. 2015).

Interestingly, hydrolysis itself proceeds via two steps, i.e., hydrolysis and acidogenesis. Firstly, algal biomass breaks down into smaller fragment of molecules. These smaller and defragmented molecules are primarily amino acids, fatty acids, and sugars. Thereafter, defragmented molecules are partially consumed by methanogenesis microorganisms and partially convert into intermediate products via acetogenesis step. Primarily, acetates and acetic acids are obtained by the later process. Thereafter, acids are consumed by methanogenic microorganisms to yield methane and energy. To be noted that acetic acids can directly undergo decarboxylation reaction to yield CO_2 and methane whereas hydrogen is generated by the microorganisms. CO_2 and hydrogen further react together to yield methane, thus eliminating the possible danger of global warming. Interestingly, the hydrogen is generated in-situ because of the production of several reducing agent type intermediates during acetogenetic step. There is no clear agreement on the reaction mechanism so far. However, the overall process for the production of methane from algal biomass is given in Fig. 3.

4.2 Hydrogen Production via Biological Routes

Hydrogen is the cleanest fuel that has potential to solve all environmental problems. Primarily hydrogen is produced from conventional sources of fossil fuels via energy

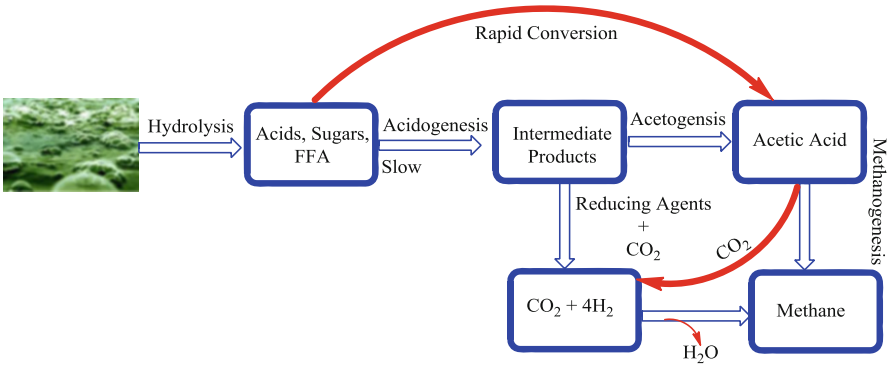

Fig. 3 Anaerobic digestion of algal biomass to produce methane

intensive processes such as reforming of natural gas, gasification of coal, and water electrolysis. Therefore, biological methods for hydrogen production may be an alternative option for sustainability of biorenewable fuel. Indeed, biological routes for hydrogen production may be an ideal process for hydrogen production in terms of energy consumption when compared with conventional processes. Biological processes, in general, do not require elevated temperatures and pressures which give it a winning edge over coal gasification, steam reforming, and water electrolysis. It is interesting to note that algae initially was used a source to produce oxygen. However, Professor Melis and his colleagues from National Renewable Energy (NREL), in 1999, observed that algae could be a promising resource for hydrogen production (Ullah et al. 2014). In general, biological routes for hydrogen production from algal biomass can be broadly classified into two major categories, i.e., (1) fermentation and (2) photosynthetic (Burgess et al. 2011). Interestingly, fermentation is further divided into two categories, namely (a) photo-fermentation and (b) dark fermentation whereas photosynthetic method is categorized as (c) direct biophotolysis and (d) indirect biophotolysis.

However, the yield of hydrogen is primarily dependent upon the type of enzyme used. The three major enzymes which are widely applied for the production of hydrogen from algal biomass are Fe-hydrogenase, NiFe-hydrogenase, and nitrogenase. The nitrogenase is mainly used in fermentation, especially in photo-fermentation. The fermentation route for hydrogen production essentially requires hydrolysis of the algal biomass as first step which is very similar to the methane production process (Xia et al. 2015). Absence of the sunlight, water, and oxygen is essential for dark fermentation process. Once the polysaccharides are broken down into monomeric form, e.g., acid and alcohols, the acidogenic bacteria such as anaerobes cause glucose to undergo glycolysis reaction that finally yields pyruvate and NADH. Thereafter, these intermediates via series of oxidation and reduction steps produce hydrogen. The major advantage of this process is that it does not require sunlight or oxygen to proceed. On the contrary, photo-fermentation essentially requires sunlight in order to produce hydrogen. The entire photo-fermentation

process is almost similar to anaerobic digestion of algal biomass for methane production. This process holds merit over dark fermentation in terms of removal of pollutants and industrial waste. On the contrary, pretreatment of industrial effluents may lead to the requirement of additional processing units.

Interestingly, photosynthetic processes for hydrogen production have attracted the attention of research community for its photosynthesis properties. In this process, hydrogenase is used as major microorganism responsible for the hydrogen production (Rumpel et al. 2014). The direct biophotolysis dissociates the molecular water into hydrogen and oxygen. The chlorophyll content of cyanobacteria or green algae helps in photosynthesis. The photons from the sunlight absorbed by the pigments result into the generation of strong anti-oxidants that split water molecule into oxygen, free proton, and free electron. The electrons generated via this process reduce intermediates products which are utilized by hydrogenase or nitrogenase to yield hydrogen. However, major disadvantage of this process is that hydrogenase activity may reduce if generated oxygen is not removed immediately. On possible reason could be high sensitivity of hydrogenase towards oxygen. On the contrary, the major advantage of indirect biophotocatalysis is the immediate separation of hydrogen and oxygen. The whole process takes place in two steps. Firstly, water molecules split into electron, proton, and oxygen which is a similar process like direct biophotolysis. However, splitting of water is followed by CO_2 fixation to produce more carbohydrates. Thereafter, the formed carbohydrate undergoes a series of reactions to finally yield hydrogen as a source of clean fuel. Interestingly, recent reports suggest that hydrogen can be produced under aerobic conditions from algal biomass (Hwang et al. 2014). However, this area is still under research and would be interesting to further progress.

4.3 Alcohols Production via Biological Routes

Next to hydrogen, alcohols especially bioethanol and biobutanol have potential to play a crucial role in the biofuel industry. Indeed, bioethanol production has been considered among top 12 chemicals suggested by United States Department of Energy for future considerations. Moreover, bioethanol held the major portfolio in the list of first generation fuel. Sugar cane is widely used for the production of bioethanol via different routes. However, possible conflict with food industry ruled out the possibility of commercialization of such technologies. Post this, second generation biofuel production technologies emerged as an alternative to the first generation biofuel production technologies. Thus, bioethanol production from lignocellulosic biomass was emphasized (Balat 2011). However, to best of our knowledge no commercial process is reported for the production of bioethanol from lignocellulosic biomass. One of the possible reasons could be the lignin content of the lignocellulosic biomass which acts as a binder and prevents cellulose and hemicellulose from taking part in further reactions. Moreover, it is difficult to convert lignin into useful products at mild process conditions, thereby resulting

into huge amount of residual waste. Therefore, it is of utmost importance to develop alternative feedstock for the production of bioethanol with low lignin content. In this regard, algal biomass is found to having minimum lignin content, thus making it a suitable feedstock for the production of bioethanol and alcohols via various biological routes (Dawoud et al. 2007).

Like hydrogen production, fermentation (heterotrophic) is the major route for production of bioethanol and other alcohols from the algal biomass such as seaweed, *Chlorella vulgaris*, and *Chlamydomonas perigranulata*. To be noted that these species of algal biomass contains higher amount of starch as compared to other species, thus fits into the criteria to qualify as a suitable feedstock for production bioethanol and biobutanol. However, sugars obtained from starch may breakdown into other products, thereby resulting into low alcohol yield and higher by-products formation. For example, some of the sugars obtained from seaweed are difficult to digest by certain enzymes such as *Saccharomyces cerevisiae*, thus limiting the further prospects of the process (Milledge et al. 2014). Interestingly, initial steps in overall process for production of alcohols from algal biomass are similar to biological routes for methane production. Firstly, algal biomass feed is prepared and charged into a digester or fermenter in the presence of suitable enzymes (acid catalyst for catalytic hydrolysis) to breakdown polysaccharides into reducing sugars (Dawoud et al. 2007). Albeit up to 20% of algal biomass can be hydrolyzed into reducing sugars or monosaccharides, acid hydrolysis is preferred due to its capability to enhance hydrolysis yield up to 50% of the dry total dry weight. Thereafter, hydrolyzed feedstock undergoes fermentation step which is a well-known and commercially available process. However, traces of acid remaining in the feedstock may lead to more problematic situation and result into inhibition of microorganisms responsible for hydrolysis. In general, all kinds of algae (green, red, and brown) can be used to produce alcohols, however, brown algae is preferred due to its higher carbohydrate content.

Recently, application of photobioreactors has been reported for the simultaneous production of algal biomass and alcohols altogether. These reactors contain cyanobacteria capable of producing alcohols while protecting itself from harsh conditions such as salinity of water and temperature. Thus, this process makes an integrated technology for alcohol production from algal biomass by linking photosynthesis and sugar production at one place. Noteworthy that these processes are not a way to produce alcohols but primarily to reduce greenhouse gases such as carbon dioxide. For example, it is reported that photobioreactor technology utilizes 90% of the system carbon dioxide to produce algae, sugars, and bioethanol (National Renewable Energy Laboratory 2010). To be noted that 60–70 L of bioethanol is produced from one ton of CO_2, making it a suitable choice for future considerations. Moreover, the bioethanol yield per acre of land can be up to 10,000 gallons. Thus it would be interesting to see, photobioreactor based technologies for alcohol production from algal biomass.

In contrast, ethanol as fuel faces several difficulties, thus limited to blending purposes. In this regard, biobutanol is an emerging fuel which has potential to replace bioethanol in near future. Worth noticing that biobutanol has relatively

lower vapor pressure and high energy density as compared to bioethanol. In addition, some bacterial species used for biobutanol production digest cellulose along with starch and sugars, thereby leading to possibility of enhanced alcohol production. The production of biobutanol from algal biomass follows a similar process to that bioethanol production. However, biobutanol yield is significantly lower than the bioethanol yield, thereby limiting the commercial prospects. The possible reason could be inability of the bacterial species to convert sugars and starch into long chain alcohols. However, we anticipate that biobutanol production will emerge as a potential replacement for bioethanol production in near future.

4.4 Production of Alkanes via Biological Routes

Albeit algal biomass derived alcohols holds a promising future, majority of fuel produced either needs modifications in existing engines design before application or cannot be used as a standalone fuel. One reason could be their compositional difference when compared with fuel obtained from conventional fossil reserves. Noteworthy that conventional fossil fuels are mainly linear chain alkanes whereas biofuel obtained from algal biomass are composed of oxygenates. Therefore, recent interest in the area of algal biomass derived biofuel is directed towards the development of alkane like hydrocarbons (Klähn et al. 2014; Peramuna et al. 2015). Interestingly, algal biomass for alkane production can be cultivated in closed reactor which may not necessarily require sunlight. In these reactors, sugars obtained from various biorenewable sources such as lignocellulosic biomass are fed to the algae. Depending upon the strain of the algae, the composition and range of alkane produced may vary (Chang et al. 2013). However, further upgradation techniques can be implied to enhance the properties of the obtained alkanes.

Unlike alcohols production, photosynthesis reaction needs to be suppressed, thereby leading to enhanced metabolic activities responsible for production of alkanes. Therefore, alkane production fermentation is generally favored in the dark condition, resulting into a higher yield as compared to photobioreactors. Additionally, the sugar fed to algae enhances its growth rate enabling higher algae cultivation in less area. One reason could be the readily available feedstock in concentrated form. This is in contrast to the photosynthesis procedure which takes longer time to generate food from sunlight, thereby slowing growth of the algae. Moreover, conventional photobioreactors require large infrastructure to produce the same amount of algae that can be produced in dark room for alkane production. This is due to the fact that energy required for algae growth is given in concentrated form and faster growth rate, thus higher production in reduced time.

Interestingly, sugar obtained from lignocellulosic biomass can be utilized for the production of biofuel in the presence of a suitable microorganism. However, majority of the fuel obtained from lignocellulosic biomass are cyclic oxygenates such as furans, lactones, pyrones, and esters that may not qualify the criteria of oxygen free alkane obtained from the biomass. On the contrary, it is possible to

produce linear chain alkanes from the lignocellulosic biomass via a similar process applied for algal biomass conversion. But the cyclic oxygenates produced as a by-product in this case may create operational issues, thus limiting economic viability. For example, separation of oxygenates and alkanes may contribute significantly in overall cost of the product. In addition, these cyclic oxygenates may inhibit the activity of the microorganism due to their toxic nature, thereby reducing overall alkane yield. On the contrary, alkanes produced from algae may not necessarily require separation (Chang et al. 2013).

5 Emerging Routes for Production of Commodity Chemicals from Algal Biomass

Development of alternative methods and technologies other than conventional methods for biofuel production leads to efficient utilization of biorenewable resources. The most promising route in this regard is decarboxylation–decarbonylation (DCO) leading to production of high value commodity monomer olefins and fuel grade hydrocarbons. Specially, the present stage when biofuel sustainability and feasibility is under question due to several environmental and economic reasons (Michel 2012). The progressive removal of oxygen via DCO may be considered as a promising route which seems more feasible in terms of overall economic of biofuel production technologies. Indeed, it is found to be most effective route for efficient utilization of third generation feedstock such as algae and free fatty acids obtained from algae. In addition, DCO route is equally effective for deoxygenation of carboxylic acids obtained from any source as well as non-edible vegetable oils consisting of mainly free fatty acids. Moreover, on integration with existing technologies for biomass processing, it is possible to convert organic acids obtained from various sources into fuel and value added products (Fig. 4). Furthermore, obtained chemicals can be further processed to produce a wide range of chemicals such as polymers, plasticizers, aldehydes, detergents, wax, and lubricants. Indeed, exploration of this area could lead to a new window of opportunity towards attaining goals of sustainability.

5.1 Decarbonylation–Decarboxylation of Algal Biomass Derived Fatty Acids

In general, DCO route involves removal of carbonyl group from free fatty acids, thus resulting into production of CO, CO_2, and H_2O as by-product besides alkane and alkenes. However, in case of product specific technologies such as for production of high value linear alpha olefins, decarbonylation is favored due to selective C─C bond scission whereas in case of decarboxylation to produce fuel range

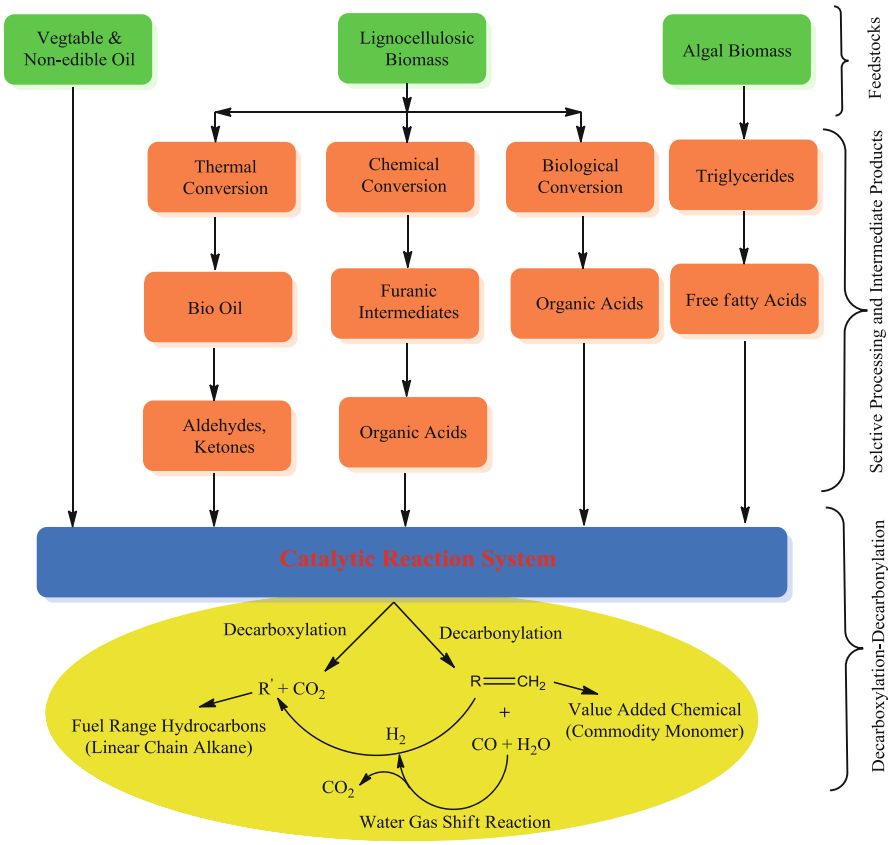

Fig. 4 Integrated approach for production of fuels and value added chemicals via DCO route from various algal biomass and other feedstocks

hydrocarbons such as linear chain alkane, additional hydrogen is required (Van Der Klis et al. 2015). Therefore, reaction mechanism which is considered to be one of the major factors responsible for determining final product selectivity is explored and correlated with operating process parameters and catalyst properties. Thermodynamic study suggests that both decarbonylation and decarboxylation are favored simultaneously at elevated temperatures above 300°C (Immer et al. 2010). Therefore, role of operating parameters is crucial. Albeit DCO route has been reviewed in past by some of prominent research groups (Santillan-Jimenez and Crocker 2012; Dawes et al. 2015; Gosselink et al. 2013), a detailed discussion towards advantages of oxygen free fuel range hydrocarbons production from algal biomass has not been reported. To be noted that oxygen free fuel range hydrocarbons obtained from biomass sources are also called as jet fuel or green diesel due to its structural analogy with fossil derived diesel. However, we expect that production of linear

alpha olefins (monomeric compounds) would be an emerging area of research due to its high value and capability to produce a wide range of commodity chemicals.

5.2 *Catalytic Production of Linear Chain Hydrocarbons from Algal Biomass*

At present, majority of transportation system uses linear chain hydrocarbons which is in contrast to the oxygen containing biofuel obtained from algal biomass. Possibly, due to this compositional difference bulk of biofuel ends up as a fuel additive rather than being directly used as a fuel. Interestingly, triglycerides obtained from algal biomass can be processed via different techniques such as hydrodeoxygenation to yield fuel range hydrocarbons. For example, hydrotreatment of triglycerides produces C_{13}–C_{20} hydrocarbons which have identical properties and composition to regular diesel. In addition, further processing steps such as catalytic cracking, reforming, and hydrocracking can be applied to upgrade the obtained hydrocarbons into jet fuel. In a typical hydrodeoxygenation process, unsaturated carbon chain of triglycerides obtained from algal biomass is saturated with hydrogen which helps to break the long chain carbon into smaller chain followed by oxygen removal via decarboxylation, decarbonylation, or simple deoxygenation reaction (Carlson et al. 2010). In general, hydrodeoxygenation is carried out at high temperature and high pressure in the presence of a suitable catalyst. The liquid product primarily consists of diesel range hydrocarbons whereas gaseous product contains CO, CO_2, methane, and propane. In general, linear chain fuel range hydrocarbons production from algal biomass is a two-step process. Firstly, triglycerides and bio oil are extracted from the algae via different thermal methods. Thereafter, catalytic hydrodeoxygenation reaction is done using a suitable noble metal or transition metal derived catalyst. On the contrary, recent reports suggest that this hydrodeoxygenation reaction can be performed using other catalysts such as zeolites as well (Wang et al. 2014).

Moreover, some interesting and less complex processes for fuel range hydrocarbons production from algal biomass or its derivatives have been reported in past few years. For example, Bala et al. have reported the production of C_{10}–C_{14} hydrocarbons from algal oil at 400°C temperature and 400 psi pressure in the presence of ceria supported zeolite (Bala and Chidambaram 2016). Consequently, the 98% algal oil conversion and 85% selectivity towards desired product were obtained. Moreover, the product obtained can be directly implied as an aviation or jet fuel. However, noteworthy that this process requires algal biomass with high lipid content because lipids primarily contain fatty acid esters and free fatty acids which become a feedstock for hydrodeoxygenation. Hydrodeoxygenation in general is not explored much as standalone and one pot process for fuel range hydrocarbons production from algal biomass. However, it is an important and emerging process that has potential to be scaled up further, possibly due to its

integration with existing technologies. For example, pyrolysis and liquefaction can be equipped with a hydrodeoxygenation unit to convert produced algal oil into fuel range hydrocarbons simultaneously. Alternatively, one pot hydrodeoxygenation process for production of fuel range hydrocarbons directly from algal biomass may be explored in near future.

5.3 Catalytic Process for Production of Aromatics from Algal Biomass

In general, majority of the processes employed for biofuel production from algal biomass either produced oxygenates or diesel range fuel. However, since gasoline range fuel essentially consists of low molecular weight hydrocarbons and aromatics, therefore it is essential to produce aromatics and light hydrocarbons from the algal biomass. Besides their application in gasoline and other fuel, aromatics are considered as important class of petrochemicals used to produce an array of chemicals. Thus, efforts have been made to produce oxygen free aromatics such as benzene, toluene, and xylene (BTX) from algal biomass to meet the growing petrochemicals demand. In addition, several other value added chemicals may be obtained simultaneously with production of biofuel.

For example, Wanga et al. have reported the production of aromatics and ammonia from the catalytic pyrolysis of algal biomass in the presence of a zeolite based catalyst. Authors have claimed to achieve 24% aromatics yield when experiments were performed using *Chlorella vulgaris* as feedstock at a temperature below 600°C. Interestingly, authors have claimed to recover to 53% nitrogen in the form of ammonia which is unlikely the case with other technologies where nitrogen is often discarded as waste product. Thus, this process has potential to reduce load of oil and gas industry, petrochemical industry, and supply feedstock for fertilizer industry simultaneously. Moreover, this process can further be improved for better utilization of remaining feedstock. For example, Gopakumar et al. have reported up to 25% wt% of carbon algal biomass converted into aromatics (Thangalazhy-Gopakumar et al. 2012). In addition, it is suggest by the authors that ammonia production may be improved by increasing the amount of HZS-5 catalyst applied for the production of aromatics from the algal biomass. However, this area is still under development and we anticipate a major breakthrough in algal biomass conversion technology if the aromatic yield can be improved further.

6 Opportunities and Challenges in Algal Biomass Conversion

In general, algal biomass can be considered as sustainable and promising source of energy and chemicals. It provides several opportunities over other biomass sources in terms of overall recycling of greenhouse gases, thus making it more environment friendly. Indeed, it is photoautotrophic organism capable of utilizing solar energy and carbon dioxide to produce chemicals in a short cultivation cycle (Guo et al. 2015). Thus, enabling efficient utilization of solar energy and reducing environmental load simultaneously. On the contrary, additional oxygen release during growth of algal biomass may serve as a source of oxygen. To be noted that algal biomass is fastest growing plant, which is unlikely to be the case with lignocellulosic biomass. In addition, it can be grown anywhere and does not necessarily require a fertile land, thereby avoiding conflict with food crop production. It can be used as mean to curb water pollution by growing algae in polluted water ponds and seaside, thereby preserving aquatic ecosystem. Similarly, algae cultivation may be considered as a means to restore degraded and contaminated areas. Moreover, oil yield (20,000–80,000 L) per acre of cultivated land which is 7–31 times than the sum of the best oil producing crops such as palm oil (Demirbas and Fatih Demirbas 2011).

Noteworthy that algal biomass can be used to produce a wide range of chemicals and fuel ranging from biodiesel, alcohols, renewable hydrocarbons, biogas, fertilizers, animal feed, surfactant, monomers for plastics to recovery of nutrients such as phosphorous, proteins, and other minerals. Moreover, application of algal biomass derived biofuel (biodiesel) helps to suppress environmental damages by capturing CO_2, SO_x, NO_x, and other toxic elements (Vassilev and Vassileva 2016). Besides biofuel, algal biomass derived fatty esters have found several applications in bio-lubricant industry. Interestingly, bio-lubricant market is estimated to be $40 billion at present which may escalate in future with development of sustainable technologies. Indeed, algal biomass derived fatty acid esters have potential to replace conventional food grade bio-lubricant sources such as vegetable oil. Development of such technologies would have great societal and economic impact.

On the contrary, high production cost of feedstock limits its application as a source of biofuel and commodity chemicals. In general, preparation of algal biomass feedstock for biofuel production typically involves cultivation, harvesting, and drying. Algal biomass feedstock cost is about 5–7 times higher than that lignocellulosic biomass (Huber et al. 2006). Furthermore, capital cost investment for algal biomass based biorefineries is quite high. National Renewable Energy Laboratory (NREL) has conducted techno-economic feasibility study, resource assessment, and life cycle assessment (Davis et al. 2012). On the basis of their assessment, it was suggested that increased production and high lipid content of algal biomass alone cannot make the process economically viable. Therefore, feedstock cost preparation and processing cost must be reduced to sustain such technologies. Interestingly, several methods such as thermal route, catalytic route, and biological routes, as discussed in this chapter, are implied for production of

biofuel. However, low crude oil prices at present situation remains a threat for further progress in this area. Other interesting process for production of diesel like hydrocarbons is hydrodeoxygenation which essentially requires additional hydrogen supply, thus limits the applicability for industrial processes. On the contrary, other techniques such as decarboxylation and decarbonylation are yet to be proven on a relatively larger scale.

Other important criteria for techno-economic viability of any process is the availability of feedstock. Although, algal biomass can be cultivated anywhere, seasonal effects may limit its availability. In addition, excessive cultivation of algal biomass for application in biofuel production may lead to water contamination and acidification, thus resulting into environmental damages. Furthermore, excessive algae cultivation may cause disturbance in the ecosystem which is in contrast to the overall of objectives of promoting biofuel production from algal biomass. Therefore, a more robust and sustainable methodology for algal biomass cultivation is required.

7 Summary and Outlook

Algal biomass is a promising substitute of conventional fossil fuel that has potential to meet enormous energy and chemical demands. Unlike lignocellulosic biomass, algal biomass produces a relatively higher yield of bio oil which makes it an interesting feedstock for biofuel industry. Moreover, several technologies have been established and proven authenticating the feasibility of biofuel production at commercial scale. Algal biomass can be directly used for the power generation in power plants via direct combustion or it can be co-fed with coal in the power plants to improve the overall plant efficiency by 20–40%. Moreover, depending upon the requirement, solid, liquid, or gaseous fuels can be produced. The gaseous products are primarily CO, CO_2, methane, and hydrogen whereas liquid product can be mixture of several oxygenates. On the contrary, solid product is char like material. Interestingly, all the products hold great fuel value and application to meet energy demands. At present, thermal routes such as combustion, gasification, pyrolysis, and liquefaction are predominant for the production of biofuel from algal biomass. However, these processes are energy intensive, thus catalytic routes have been explored. In contrast, biological routes are not energy extensive and can be applied to produce hydrogen, methane, alcohol, and alkanes. The major limitation of biological routes is their inability to produce the chemicals in bulk and huge space requirement for processing. Overall, algal biomass as source of biofuel and commodity chemicals remains an attractive choice due to its better oil yield and energy density as compared to lignocellulosic biomass.

Albeit the oil content and energy density of algal biomass is comparatively high. The oil content per acre of land of algae cultivation can further be enhanced by application of genetic engineering based technologies. It would be interestingly to mix algae DNA with some other species to escalate oil content and reduced cultivation time. It is anticipated that genetically modified crops would cheap and

economic having high oil content and require less cultivation area as compared to existing algal biomass. Alternatively, other emerging technologies such as photobioreactors can be used for continuous and sustainable production of algal biomass. Similarly, it would be interesting to produce long chain monomers from algal biomass derived fatty acids. These monomers may strengthen interdisciplinary research areas especially in biopolymer and polymer industry.

Nevertheless, many interesting and sustainable technologies for algal biomass conversion into biofuel have emerged. For example, hydrodeoxygenation, hydrotreatment, decarboxylation, and deoxygenation technologies have capability to produce oxygen free fuel range hydrocarbons. However, these processes are still under development and demonstrated at lab scale. Further scale up studies and process optimization can be a promising area to work in. Moreover, synthesis of non-transition metal and noble metal based catalysts such as zeolites or metal doped zeolites may find a great interest. Since crude oil prices have gone to a very low level, therefore techno-economic feasibility of such processes needs to be improved. Alternatively, efforts can be made to produce high value commodity chemicals such as linear alpha olefins via decarbonylation route. Decarbonylation may help to sustain the biofuel industry in future even if the crude oil prices are very low.

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Challenges and Opportunities in Commercialization of Algal Biofuels

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Contents

1 Introduction

Fossil fuels have remained to be the single largest driver of our socio-economic structure since the dawn of industrialization. However, the time has arrived to look beyond fossil fuels to meet our energy demands. Finite availability, uneven distribution of reserves, alarming rate of depletion, energy insecurity, climate

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change and environmental degradation are among the major concerns associated with consumption of fossil fuels. Biofuels, of late have emerged as a potential substitute for crude oil based fuels especially for meeting the fuel demand of transport sector. Depending on the feedstock used biofuels have been categorized as 1st generation biofuels (derived from edible crops, e.g., ethanol and biodiesel from corn and soybean, respectively), 2nd generation biofuels (derived from non-edible plants, e.g., ethanol and biodiesel from switchgrass and Jatropha, respectively) and 3rd generation biofuels (derived from algae, e.g., algal biodiesel, bioethanol, biohydrogen, etc.) (Sims et al. 2010). The problems raised by 1st and 2nd generation of biofuels and advantages offered by algae as feedstock over others have been discussed extensively (Chisti 2007; Singh and Gu 2010; Hannon et al. 2010). Use of edible crops for biofuel production can escalate the market price of food commodities as it will involve diversion of food crops from food market towards biofuel production (Ewing and Msangi 2009). Use of 2nd generation biofuel production technology overcomes the famous food v/s fuel dilemma associated with 1st generation of biofuels. However, since the yield and biomass productivity rate of lignocellulosic plants is rather low, unsustainably large area of land would be required for any significant scale displacement of fossil fuels. Owing to its high biomass productivity rate and limited space requirement, among other alternatives only biofuels derived from algae have the potential to entirely displace fossil fuel currently in use for powering our transport infrastructure (Murphy et al. 2011). The superiority of algae as biofuel feedstock can be gauged by the following advantages: high biomass and lipid productivity rate, high photosynthetic efficiency (PE), short biomass doubling rate, flexible carbon assimilation modes, short harvest period, availability of species capable of utilizing brackish, saline and wastewater as culture medium and nutrient source, relatively easy manipulation opportunity and several others. The productivity of oil by algae is reportedly 10–23 times greater than the highest oil yielding terrestrial plant "oil palm" (Demirbas and Demirbas 2011) and during exponential growth algae can double their biomass in period as short as 3.5 h (Chisti 2007). Algae can be used to produce bioenergy in the form of several biofuels (like-bioethanol, biodiesel, bio-hydrogen, biogas, green diesel, jet fuel, etc.), heat and electricity. Oil content of algal biomass commonly lies between 20 and 50% (dry weight basis) and it can further be enhanced to values as much as 80% by manipulation of growth and culture conditions (Metting 1996). High degree of genetic diversity offered by over 44,000 identified members of algae (Guiry 2012) necessitates a careful screening of species possessing an ideal combination of traits as desired by a biofuel production system that is both economically and environmentally sound. Freshwater, saline water and even wastewater (bioremediation of wastewater) have been used as culture medium for cultivation of algae. Use of freshwater (even when the water remaining after harvest is recycled) for large scale cultivation of algae for biofuel production is unsustainable (Yang et al. 2011). The current market of nitrogenous and phosphate fertilizers cannot absorb any additional demand for

large scale cultivation of algae for biofuel production (Chisti 2013). Use of saline water and wastewater can substantially reduce the water footprint and nutrient demand of algal biofuels. All three modes of algal growth (i.e., photo-autotrophic, heterotrophic and mixo-trophic) have been envisaged for biofuel production. The advantages offered by photo-autotrophic growth using atmospheric CO_2 include the free availability of sunlight and CO_2 however, the intensity and duration of sunlight has inherent spatio-temporal variability and the atmospheric concentration of CO_2 (close to 400 ppm) is very low for facilitating optimal growth of algae. CO_2 in concentrated form can be supplied by flue gas emanating from coal fired thermal power plants and cement industries (Pires et al. 2012). Only a fraction of sunlight reaching the earth surface is captured by photosynthetic pigments (photosynthetically active radiation). Alternatively, several artificial sources of light having a narrow emission spectra and high intensity can be used as a substitute. Heterotrophic cultivation of algae is facilitated by supplying organic carbon substrates (glucose, glycerol, acetate, etc.) and it is technically viable (Perez-Garcia et al. 2011). Abundant and cheap sources (e.g., lignocellulosic hydrolysates) of organic carbon substrate are required for improving the economic viability of heterotrophic mode of growth (Li et al. 2011). Mixo-trophic mode of growth takes advantages of both heterotrophic and autotrophic modes by combining them during diurnal cycle (for preventing any significant loss of biomass in the dark attributed to respiration) or different growth stages of algae; usually for improving lipid accumulation (Cheirsilp and Torpee 2012). In order to ascertain the overall feasibility and environmental sustainability of algal biofuels life cycle assessment (LCA) based approaches are critical (Kumar et al. 2015). The major sustainability indicators usually studied during LCA include energy return on investment (EROI), greenhouse gas (GHG) balance, and water footprint (WF). The values of sustainability indicators vary widely depending on species specific parameters (biomass productivity rate, lipid accumulation rate, PE etc.), technology employed, cultivation route, biomass harvesting and processing technique, biofuel produced, co-product allocation strategy, etc. Several LCA studies for algal biofuels have been reported but their comparability is limited due to procedural differences and assumptions. Although algae appear to be a promising feedstock for biofuel production, there are several hurdles (both economic and environmental) in its large scale implementation. Currently the cost of algal biofuels is significantly higher than their fossil fuel based counterparts but it is highly likely to come down in near future with dedicated research efforts aimed at examining the biosynthetic pathways and improving its economics (Singh et al. 2011). Genetic engineering of algae is perhaps the most promising strategy for improving the economic feasibility and environmental sustainability of algal biofuels. Table 4 depicts a comparison of different unit operations involved in algal biofuel production system.

2 Sustainability Framework

2.1 Algal Species/Species Selection

The benefits offered by algae as a biofuel feedstock over other feedstocks have been discussed extensively (Brennan and Owende 2010; Demirbas 2010; Wijffels and Barbosa 2010). Selection of an ideal species/strain is a critical factor which can significantly alter the overall economic feasibility and environmental sustainability of any algal biofuel production facility. Choosing one algal species over others is a complicated task. The definition of an ideal species varies depending on several factors including the desired biofuel type, growth mode to be adopted, culture conditions, etc. Ideally, we would like to have an algal species which is superior than others in terms of photosynthetic efficiency, biomass productivity rate, relative proportion of biomolecules in the biomass, cell density in the culture media, lipid productivity/accumulation rate and desired lipid profile, tolerance to fluctuations in climate and changing water chemistry, ability to outcompete wild and invasive species, ability to fix atmospheric N_2, tolerance to a varied degree of salinity, self-flocculation characteristics, fast productivity cycle, withstanding hydrodynamic stress common in open ponds/photo-bioreactors or fermenters, etc. Evidently, these are very demanding conditions and to date no known algal species fulfils all of these demands and therefore we are forced to settle with some compromises. Algae, although share the same CO_2 fixation mechanism but, in general have a higher photosynthetic efficiency than land based C3 plants attributed to its relatively simple structure (Chisti 2013). Thus, they are more efficient in transforming photosynthetically active radiation into chemical energy. The theoretical photosynthetic efficiency for C3 plants under normal atmosphere has been reported to be 4.6%. Algae, on the other hand, can be grown under CO_2 supplemented culture conditions with reported estimate between $5< $ photosynthetic efficiency $<8.3\%$ (Chisti 2013). As several factors play key roles in photosynthetic mechanism the realization of maximum photosynthetic efficiency under natural conditions is more often than not limited by one factor or the other. Realization of theoretical maximum efficiency even under carefully controlled conditions is very difficult at any significant scale. Besides photo-autotrophic growth, hetero-trophic and mixotrophic growth modes of algae are well established. Combination of photo-autotrophic and hetero-trophic modes can be used to overcome the respiratory biomass loss during nighttime when photosynthetically active radiation is unavailable. Algal species having higher biomass productivity rate are inherently more suitable as higher yields per unit of input cost is economically and environmentally sound. Even for a given species the biomass productivity rate greatly varies with operational condition. Nitrogen is a vital growth promoting nutrient for algae required in relatively large quantity. Species having an inherent capability of nitrogen fixation offers economic savings as they need lower inputs of synthetic nitrogenous fertilizers but since biological fixation of nitrogen is an energy intensive affair the biomass productivity is compromised: hence optimization of culture

and operational conditions is imperative. The relative proportion of biomolecules (carbohydrate, lipid and protein) in the biomass widely varies among species even when they are grown identically.

Certain algal species are known to secrete biofuel precursor molecules (Georgianna and Mayfield 2012) in the culture medium and this can exclude processing steps downstream of biomass harvest which are highly energy intensive and at times inefficient.

Unlike terrestrial plants algae can be manipulated to produce certain biomolecules in excess at the expense of the others. This facilitates large scale production of specific feed based biofuels (e.g., biodiesel from lipids and bioethanol from carbohydrates). Several microalgae are known which naturally accumulate >20% of their dry cell weight as lipids (oleaginous microalgae). Controlled manipulation of culture media by imposing certain type of nutrient starvation (particularly nitrogen) has been reported to greatly enhance lipid accumulation of certain microalgae (Li et al. 2008). However, several microalgae tend to accumulate very significant proportion of poly-unsaturated fatty acids and this results in poor oxidative stability and cold flow properties of produced biodiesel (Hu et al. 2008). Thus, use of neat biodiesel or its higher blends (>20%) with petroleum diesel in internal combustion engines can be troublesome. Self-flocculation character of species facilitates easy harvesting (Guo et al. 2013) which is otherwise highly energy intensive. However, self-flocculation character may require additional energy input for keeping the biomass in suspension for effective light penetration in open ponds prior to harvesting.

Using marine algal species can not entirely eliminate the use of fresh water but can significantly reduce the overall water footprint of algal biofuels. Identification of marine algal species having superior qualities is critical. Species that can tolerate high degree of alkalinity can be grown on soluble bi-carbonates as culture media since the low atmospheric concentration of CO_2 is a major limiting factor at times. Marine species cannot be cultured on soluble bi-carbonates as sea salts precipitates at pH >8 (Chisti 2013). Culturing marine species is only feasible in coastal areas. Wastewater resources can also be employed to culture algae which besides reducing the demand for freshwater can also reduce the demand for synthetic fertilizers and simultaneously effecting wastewater treatment (Pittman et al. 2011). The utilization of wastewater for maintaining healthy culture of algae is vastly dependent on the composition of wastewater which typically varies both spatially and temporally. The feedstock algae should be able to withstand the several hydrodynamic stresses common in open pond, photo-bioreactors or fermenters. It should be able to out-compete the wild species that may accidently find their place in culture medium. Although several characters of an invasive species like fast productivity cycle, tolerance to several types of stresses, competitive inhibition of other species present in the culture medium, etc. are desirable qualities for large scale biofuel production, it can create environmental nuisance if accidently released to the outside environment (Pullin et al. 2009). The heterogeneity within the group members has huge genotypic diversity on offer and a careful selection of traits combined with an efficient and well-defined cultivation system is vital.

2.2 Water Footprint

All classes of aquatic systems be it a freshwater body, brackish water body or a marine system support algal life. Algae being an aquatic organism (mostly) needs water as a growth medium and water forms the bulk of its biomass. Water footprint is an indicator of freshwater use for production of goods and services measured over the entire supply chain. Since available freshwater is a scarce resource every effort should be made for promoting its judicious use. Use of freshwater for culturing algae may give rise to drink v/s drive controversy. According to Gerbens-Leenes et al. (2014) the water footprint of any product or process consists of three components, namely blue (fresh surface and/or ground water component), green (rainwater component) and grey (freshwater required for waste dilution). Algal biofuel production usually does not involve the green water component. Water footprint of algal biofuels consists of two major components (1) evaporation loss (consisting of surface evaporation from open ponds, evaporation of sprayed water used for cooling the photo-bioreactors, loss of water during thermal drying, loss during pyrolysis of biomass, etc.) and (2) transformation of freshwater into wastewater. Cultivation of algae at any significant scale poses a huge demand for culture water. Utilization of freshwater algae does not seem to be a practical option as current competing demands for freshwater at global scale largely surpass its availability due to overexploitation and water pollution. Frequent input of freshwater for maintenance of water level and salinity is required in large open ponds due to evaporative loss which has a disproportionate effect on overall water footprint (Chisti 2007). The quantum of evaporation loss from open ponds has inherent spatio-temporal variability determined by factors such as latitude, season, time, pond depth, surface area, elevation, light intensity, temperature, wind velocity, relative humidity, etc. and thus care must be taken in locating open ponds for algae cultivation. In contrast, photo-bioreactors being a closed cultivation system have significantly lower water demand (Table 1). In addition to reduction in water footprint photo-bioreactors facilitate better control of culture and achieve highly dense culture per unit volume of water employed (Yang et al. 2011). But the trade-offs appear in the form of installation, operation and maintenance cost. In order to prevent overheating the photo-bioreactors often need water spray as evaporation has a cooling effect. Bioreactors or fermenters are another form of closed culture system suitable for non-autotrophic growth mode having similar freshwater demand as photo-bioreactors (Borowitzka 1999). Biodiesel has remained the preferred biofuel for ascertaining the water footprint of algal biofuels. Yang et al. (2011) in their study reported a total freshwater demand of 3726 kg kg^{-1} of biodiesel produced for freshwater alga *C. vulgaris*. The water remaining after harvest accounted for 84.1% of the initial water content of the cultivation medium. Recycling of harvested water limited the required input of freshwater to 591 kg kg^{-1} of biodiesel produced. Gerbens-Leenes et al. (2014) in their study reported that in order to meet just 3.5% of the total transportation fuel demand of the EU (28) using the most efficient algal biofuel (56% bioethanol and 44%

Table 1 Comparison of freshwater demand for different fuels and fuel production pathways

Fuel production pathways	Freshwater demand per unit of fuel	Reference
Freshwater algae in open pond (without recycling)	3726 kg kg^{-1} biodiesel	Yang et al. (2011)
Freshwater algae in open pond (with recycling)	660 kg kg^{-1} biodiesel	Yang et al. (2011)
Marine algae in open pond	370 kg kg^{-1} biodiesel	Yang et al. (2011)
Marine algae in open pond	216 L L^{-1} biodiesel	Harto et al. (2010)
Marine/freshwater algae using wastewater in open pond	280–400 kg kg^{-1} biodiesel	Chinnasamy et al. (2010)
Cultivation in photo-bioreactors	113–238 L L^{-1} biodiesel	Harto et al. (2010)
Soybean oil seed	13,676 kg kg^{-1} biodiesel	Yang et al. (2011)
Corn ethanol	138 L L^{-1} ethanol	Harto et al. (2010)
Non irrigated lignocellulosic ethanol	6.5 L L^{-1} ethanol	Harto et al. (2010)
Petroleum extraction and refining for gasoline production and its distribution and marketing	2–6 L L^{-1} gasoline	McArdle et al. (2009)

biodiesel) production system by employing fresh water as much as 15% of the existing fresh water (blue water) demand must be diverted toward algal biofuel production. Clearly, the utilization of freshwater for culturing algae at any significant scale is not feasible. Fortunately, there are species that can be grown using saline water and freshwater. If sea water or wastewater (preferably after secondary treatment) is employed as culture medium, the overall water footprint comes down by 90% (Yang et al. 2011). Chinnasamy et al. (2010) in their work on cultivation of marine and freshwater algae in open pond using carpet mill effluent dominated wastewater reported that total consumptive use of freshwater demand to be 280–400 kg kg^{-1} biodiesel. Although utilization of sea water and wastewater as culture medium is possible, this does not totally eliminate the use of freshwater as evaporative losses must be compensated by adding freshwater and harvested biomass may require washing with freshwater along with other demands for freshwater in downstream processing steps. Surface runoff from agricultural field contains vital nutrients for algal growth leading to algal bloom in receiving waterbody. Thus, surface runoff water from agricultural field can be a potential source of culture water and nutrients for culturing algae. Besides, rain water (green water) harvesting systems can be employed to meet some of the freshwater demand. The demand for freshwater per unit of biodiesel produced is lower for algae compared to the most widely used oil seed crop (soybean) in the US irrespective of whether freshwater or marine species are cultivated using freshwater, marine water or wastewater as culture medium with or without freshwater recycling (Table 1). But, freshwater demand is still very high when compared to petroleum based liquid transportation fuels. The total water consumption for producing gasoline has been reported to fall between 2 and 6 L L^{-1} gasoline which is likely to increase in future (US Department of Energy 2006). Table 1 lists freshwater demand for production of biofuels using different approaches and water demand for gasoline production using petroleum.

2.3 Carbon Substrate

As already discussed algae in general have higher photosynthetic efficiency than land based C3 plants and hence they are more efficient in converting sunlight reaching the earth's surface into photosynthates. Cultivation of algae via photo-autotrophic growth can capture up to 1.83 kg CO_2 kg^{-1} of algal biomass produced (Brennan and Owende 2010). But, photo-autotrophic cultivation of algae for large scale biofuel production is hampered by very low atmospheric concentration (0.0395% by volume) of CO_2 which at times becomes a limiting factor for high productivity due to mass transfer and solubility limitations. Relatively lower concentration of CO_2 in chloroplast of photosynthetic organisms with respect to O_2 can cause competitive inhibition of CO_2 fixation (photorespiration) and consequent reduction in carbon fixation efficiency by 20–30% (Zhu et al. 2008). However, algae have carbon concentrating mechanism in place in which carbon dissolved in water in the form of bi-carbonate (more than 50% of total organic at pH between 6.4 and 10.3) is pumped into the chloroplast and is subsequently converted to CO_2 so as to raise its concentration which competitively inhibits photorespiration. However, the current atmospheric CO_2 concentration level is rather low for realizing high growth potential of algae. Therefore, alternative concentrated sources of CO_2 are required. Point sources of concentrated CO_2 like coal fired thermal power plant exhaust (15–20% CO_2 by volume) and cement industry exhaust (15% CO_2 by volume) can be utilized effectively by locating algal based biofuel production facility closely (Pires et al. 2012). Algae cultivation can reduce the overall CO_2 load of exhaust by as much as 80–90% which makes algae highly efficient when CO_2 in concentrated form is supplied. Thus, the process can be employed in cultivation plants which are located near to a concentrated point source of CO_2 for reducing the overall carbon footprint of the CO_2 emanating industry for gaining carbon credits and the production of biomass for bioenergy simultaneously. However, there are several hurdles in using this synergy including the unavailability of point concentrated CO_2 sources in plenty, temperature of emanating exhaust, presence of toxic and noxious gases, etc. (Van Den Hende et al. 2012). Locating algae cultivation plants near concentrated point source can be difficult due to land, locality and climate. Further, photo-autotrophic carbon fixation can only take place during the daylight hours and it results in significant loss of photosynthates as Calvin cycle becomes dysfunctional while at the same time citric acid cycle continues in dark (Grobbelaar and Soeder 1985), therefore technologies for CO_2 capturing, its concentration and storage during night followed by its utilization after sunrise should be developed for preventing biomass after in the absence of light. Carbon can be provided in the form of soluble bicarbonates (White et al. 2013) for promoting photoautotrophic cultivation of algae as it can help overcome the limitations attributed to low concentration of CO_2 in the lower atmosphere as algae have bi-carbonate transports located on plasma membrane and on membranes enclosing the chloroplast which ultimately gets converted to CO_2 followed by its fixation in presence of light. Addition of bi-carbonate results in proportionate increase in pH of the medium and it can promote the activity of flocculants when

used for facilitating the harvest of algal biomass (via flocculation and sedimentation). But, sea salts tend to precipitate at pH >8 (Chisti 2013) and therefore bi-carbonates cannot be used for culturing algae adapted to survival and growth in saline water which have lower CO_2 solubility than freshwater. Carbon besides acting as the substrate can also act as source of energy for biomass productivity (provided by sunlight during photo-autotrophic growth). Several algal species possess a remarkable capability of assimilating carbon hetero-trophically using organic carbon substrates derived from previously fixed carbon. Carbon in the form of acetate, glycerol, and carbohydrates (glucose, lactose, fructose, starch, etc.) can be employed to facilitate heterotrophic growth of algae (Bhatnagar et al. 2011). Heterotrophic production of algae in closed systems has better scale-up potential for large scale cultivation of algae and unlike photo-autotrophic cultivation biomass productivity does not vary during the diurnal cycle. Several researchers have reported high biomass and lipid productivity for heterotrophic culture of algae than light dependent systems with or without imposing nutrient stress (Cheirsilp and Torpee 2012). Depending on the composition of municipal wastewater its organic load can be effectively utilized for heterotrophic cultivation of algae. C/N ratio of the culture media is an important factor which controls the switch between protein and lipid biosynthesis and nitrogen starved media promotes accumulation of lipid at the expense of protein and other biomolecules (Gordillo et al. 1998). Although technically more viable (Graverholt and Eriksen 2007; Xiong et al. 2008), the utilization of heterotrophic cultivation of algae is hampered by unavailability of cheap and abundant carbon substrates. Sugars obtained via hydrolysis of waste lignocelloulosic biomass appear to be a promising alternative but currently little knowledge is available on its techno-economic viability. A combination of heterotrophic and auto-trophic modes (mixo-trophic) of algal growth has also been envisaged by spatially and temporally separating the two modes of growth. Initial findings on photo-autotrophic cultivation of algae followed by heterotrophic cultivation under nitrogen starvation are promising but it complicates the overall setup and needs further investigation to ascertain its viability.

2.4 Nutrients

Nitrogen and phosphorus are nutrients of prime importance for culturing algae and therefore must be supplied in relatively large quantity. Atmospheric nitrogen can be fixed synthetically (via Haber-Bosch process) for producing nitrogenous fertilizers but it involves use of fossil energy and consequent release of GHGs which can disrupt the overall energy and GHG balance of the algal biofuel production facility. Rock phosphate serves as the feedstock for all types of phosphorus based fertilizers hence the reserves of phosphorous is limited. Current supplies of nitrogen and phosphorus based fertilizers cannot absorb any sudden and significant increase in demand arising from cultivation of algae for large scale biofuel production. Chisti (2013) in his study reported that in order to meet merely 9 days of petroleum

demand (based on 2010 values) of the US from algal biofuels would require diversion of at least 44% of existing usage of nitrogenous fertilizers and a significant proportion of phosphorous based fertilizers in the US towards algal biofuel production. The global average nitrogen use efficiency is roughly 40% and must be improved substantially. Compared to nitrogen the phosphorus use efficiency of crops is higher (up to 90%) depending on the time scale used for efficiency analysis (Syers et al. 2008). Runoff from agricultural fields containing excess of nitrogen and phosphorus can be utilized to meet some of the water and nutrient demand for algae cultivation (Sharpley et al. 1991) which is otherwise known to promote algal bloom in receiving waterbody having detrimental effects on its health. Utilizing species having inherent N_2 fixing capability, culturing algae in nitrogen deficient media for increasing lipid accumulation, use of wastewater or runoff from agricultural field as culture medium, and nutrient recycling can reduce the demand for synthetic nitrogenous fertilizers usually provided in the form of urea, ammonium nitrate, monoammonium phosphate, urea ammonium nitrate, etc. Recycling of nutrients after harvest, extraction and processing of biomass is critical. Wastewater can be used as culture medium, source of organic substrates for facilitating heterotrophic growth of algae, and also for meeting the nutrient requirements for algae cultivation depending on its origin. Using freshwater as culture medium without its recycling required 3276 kg of water, 0.33 kg of nitrogen, 0.71 kg of phosphorus, 0.15 kg of sulphur and 0.58 kg of magnesium per litre of biodiesel produced (Yang et al. 2011). With 100% recycling of leftover water after biomass harvest the demand for fresh input of nutrient came down by 55%. Use of wastewater or saline water offers additional advantages in terms of reduction in freshwater demand by 90% and significant reduction in nutrient input requirement except for phosphorus based fertilizers. Energy independence and security act (EISA) 2007 of the US mandates production of one billion litres of algal biodiesel by 2022. The life cycle analysis of freshwater and nutrient demand for meeting this goal using freshwater algae *C. vulgaris* were reported and it could enhance the national usage of freshwater, nitrogenous and phosphorus based fertilizers by 9.7%, 8% and 22.7%, respectively, even if the leftover water after harvest is recycled (Yang et al. 2011). These statistics clearly suggest that large scale production of algal biofuels would require selection of species capable of growth and high productivity under conditions of limited nutrient availability, utilization of alternative sources of nutrients, and nutrient recycling coupled with improving the nutrient use efficiency.

2.5 Photosynthetically Active Radiation (PAR)

The ultimate source of energy for photo-autotrophic and heterotrophic modes of growth is sunlight. Sunlight provides the energy required for fixation of carbon and it is often a limiting factor in photosynthesis. Visible radiation (390–760 nm) of the electromagnetic spectrum emanating from the sun is responsible for facilitating photosynthesis especially the red (660–700 nm) and blue (420–450 nm) segments

of VIBGYOR known as photosynthetically active radiation. Infrared radiation has very little energy to facilitate any direct chemical change while the high energy content of UV radiation has the potential to damage algal cell. Algal pigment chlorophylls (a, b, c1, c2, d) and carotenoids (β-carotene, α-carotene, lutein, violaxanthin, fucoxanthin) preferentially absorb the red and blue bands of visible spectra. Phycobilins (phycocyanin, phycoerythrin, allophycocyanin) are hydrophilic pigments reflecting the blue band of visible spectra and along with chlorophyll are responsible for the characteristic blue-green colour of cyanobacteria. Unfortunately the available intensity of sunlight is highly variable in spatial and temporal terms and depends on factors such as geographical co-ordinates of the biofuel facility on earth surface, season, and time during diurnal cycle. Further, the efficiency of photo-autotrophic organisms in converting usable solar energy to chemical energy contained in photosynthates is very low and at best does not exceed 7.5–8%. However, recently photosynthetic efficiency of ca. 20% has been reported for *Phaeodactylum tricornutm*, *Chlorella* sp., and *Tetraselmis suecica* (Packer 2009). Amount and quality of PAR absorbed is greatly dependent on factors like the composition and relative proportion of pigments in algae, depth of the cultivation systems, turbidity of the medium, time, etc. Alternative sources of PAR like incandescent bulb, halogen lamp, fluorescent lamp, light emitting diode, and laser diode can also be used to provide energy for carbon fixation (Table 2). Artificial sources of light are usually employed during photo-autotrophic growth of algae in photo-bioreactors as supplying large open ponds with artificial PAR is not feasible. Sunlight and synthetic PAR can be used alternatively during day and nighttime, respectively, for improved biomass yield as it can reduce any significant loss of biomass in dark attributed to respiration. Alternatively, several authors including Contreras et al. (1979), Park and Lee (2001), and Tennessen et al. (1995) suggested using intermittent cycles of short light flashes of high intensity followed by long dark period instead of continuous supply for improving the light use efficiency and prevention of photo-inhibition. The rate of algal growth is highest at saturation intensity (Sorokin and Krauss 1958) but in nature the light intensity varies widely. Photo-adaptation/photo-acclimation is a character shown by algae which allows it to tolerate changing light intensity by means of morphological modification such as change in cell volume and change in density and number of thylakoid membranes or other metabolic means like change in number and type of pigments, respiration rate in dark, change in growth rate, or availability of some fatty acids (Fábregas et al. 2004). These mechanisms allow algae to survive in conditions of varying light intensity but have some associated metabolic cost. Synthetic sources of PAR can be employed for providing energy at appropriate (saturation) intensity. Light intensity is also known to affect the cellular composition and in general increase in light intensity (below saturation intensity) leads to enhanced production of triacylglycerol (substrate for biodiesel) and simultaneous reduction in polar membrane lipids (Brown et al. 1996; Khotimchenko and Yakovleva 2005; Carvalho and Malcata 2005). Even though not clearly understood, increase in triacylglycerol in response to increasing light intensity probably has a protective role (Sharma et al. 2012). The fatty acid profile of triacylglycerol also

Table 2 Comparative analysis of synthetic sources of PAR (Carvalho et al. 2011)

Types of artificial PAR source	% of energy emission b/w 400 and 500 nm	% of energy emission b/w 600 and 700 nm	Energy loss in the form of heat	Lumen efficiency (lm/W)	Lifetime (h)	Cost
Incandescent bulbs	0.5	3.8	Very high	10–18	750–2000	Low
Halogen lamps	0.3	3.3	High	15–20	3000–4000	Low
Fluorescent lamps	25.0	20.7	Low	35–100	10,000	10× incandescent bulbs
Gro-lux fluorescent lamps	18.9	37.9	Low	35–70	15,000	3–10× incandescent bulbs
Light emitting diodes (LEDs)	0.004–0.008	87.6–98.3	Very low	26–64	35,000–50,000	2–10× fluorescent lamps
Laser diodes	–	–	Negligible	30–45	Up to 100,000	2× LEDs

varies among algal species in response to variation in light intensity and generally the degree of unsaturation increases with increasing light intensity. High light intensity can bring about oxidative damage of poly unsaturated fatty acids (PUFAs) (Sharma et al. 2012). Careful control of temperature of the culture medium is equally important as optimal temperature for different species is different. Besides light intensity, temperature increase of the culture medium is also known to increase the degree of unsaturation in fatty acids. Careful control of light supplied to algal culture in terms of wavelength, intensity, photon flux density, temperature and length of photoperiod is only feasible in photo-bioreactors by employing synthetic sources of PAR. Synthetic sources of PAR for photo-bioreactors should be able to provide radiation of desired wavelength (depending on the type and relative proportion of pigments) at desired intensity, concentrate and uniformly distribute energy, provide sufficiently large lifetime coupled with steady radiation intensity over time, and should be rather cheap in order to prevail over sunlight. Heterotrophic mode of growth offers better scale up opportunities as it is not dependent on sunlight in a direct way but it ultimately depends on carbon that has been fixed recently via a highly inefficient process of energy transformation (photosynthesis).

2.6 Demand for Land

In the present era of industrialization and rapidly growing population there are several competing demands for land be it arable or marginal. Unlike terrestrial plants algae do not need arable land for its cultivation and thus can be cultivated on marginal land/wasteland or land with limited productivity. This coupled with higher productivity, and short biomass doubling period in comparison to other biofuel feedstocks at the same time significantly reduce the land area required for large scale biofuel production. According to Chisti (2007) meeting 50% demand of liquid transport fuels in the USA by algal biodiesel would require only 1.1–2.5% of the existing cropped area in the USA (for oil content of 30–70%). When oil palm (highest oil content among terrestrial oil seed plants) based biodiesel is considered for meeting 50% of the demand 24% of the land area under cultivation must be diverted towards oil palm cultivation. The figures are even higher for other feedstocks (soybean-326%, canola-122%, jatropha-77% and coconut-54%). In order to produce biodiesel from microalgae for meeting 3.5% of the liquid transport fuel demand of the EU (28) by 2030 land area required would be close to 17,000 km^2 which is equivalent to only 1% of the total cropped area and if the entire fleet of transport were to be fuelled by algal biodiesel 28% of the existing area under agriculture would be sufficient (Gerbens-Leenes et al. 2014). Direct and indirect land use and land cover changes (LULCC) associated with 1st and 2nd generation of biofuel feedstock cultivation and consequent release of GHGs can get significantly reduced when algae serve as feedstock for biofuels (3rd generation of biofuels). Further it does not give rise to the notorious food v/s fuel controversy. Evidently algae are far better feedstock than other oil seed plants when only land

area requirement is considered. The productivity in photo-bioreactor per unit area is usually greater than open pond (Pulz 2001) and its compact design can further lower the land area requirement. Locating open pond or photo-bioreactors entails a careful consideration of climate for minimizing loss of water via evaporation and also for providing optimal growth conditions. Locating open ponds in humid coastal areas can reduce the evaporative losses of water. Topographic factors such as slope and geological factors like compactness, porosity and permeability of the land are other important parameters to be taken into account while selecting a site for establishing open ponds or photo-bioreactors.

2.7 Energy Return on Energy Invested (EROI)

Yield based parameters such as rate of biomass productivity, rate of CO_2 uptake and lipid accumulation have traditionally been used to ascertain the efficiency of algal biofuel production systems. Although these parameters are important, for any holistic assessment of algal biofuel production systems the encumbrances of achieving these yields must also be taken into account. Energy return on energy investment (EROI) is one such parameter. It is an important matrix for ascertaining the desirability of fossil fuel alternatives and it denotes the ratio of energy contained in one unit of the alternatives produced to the energy that goes into their production. The EROI of any fuel determines the amount of energy available for economic activity and its value for fossil fuels is declining at a fast pace. Unfortunately most of the renewable and un-conventional alternatives have EROI values substantially lower than fossil fuels (Hall et al. 2014). EROI values of algal biofuel production systems depend on a multitude of factors and selective manipulation of certain factors may have a disproportionate effect on EROI. Important factors that directly or indirectly affect EROI include strain of algae, production system employed, mode of growth, source of carbon and other nutrients, source of water and illumination, biomass harvesting technique, biomass drying technique, lipid extraction technique, biofuel type and biomass conversion route employed, residual biomass processing, co-product allocation strategies, nutrient recycling, material and energy synergy, etc. Since, different life cycle stages of algal biofuel production can be modelled using unit operations having multiple alternative routes and use of different set of assumptions by different researchers a highly variable result of EROI values ranging from 0.09 to 4.3 for algal biofuels (Table 3) has been reported in the literature (Sills et al. 2012). Comparability of different studies is limited due to the fact that different researchers have employed different set of assumptions about the growth and productivity parameters of the selected algal strain, co-product allocation strategies, use of residual biomass, technologies employed during the life cycle stages of biofuel production from cultivation of algae to processing of biomass to biofuels or up-to combustion of biofuels to generate energy (final use). Energy inventory of the entire life cycle stages of algal biofuel production system has shown that some of the stages such as harvesting of biomass,

Table 3 EROI values of different biofuels

Biofuel type	EROI	Conditions	Reference
Algal biodiesel	1.075	GREET modelling (well to pump) of *Nannochloropsis* using a photobioreactor architecture	Batan et al. (2010)
Algal biodiesel	0.975	Based on extrapolation of lab scale data, for production of 1 kg of biodiesel by cultivating *Chlorella vulgaris* in open raceways, flocculated, dried using belt drier, solvent extraction followed by transesterification and combustion of biodiesel	Lardon et al. (2009)
Algal biodiesel	3.545	Based on extrapolation of lab scale data, for production of 1 kg of biodiesel by cultivating *Chlorella vulgaris* in open raceways, flocculated, wet extraction, solvent extraction followed by transesterification and combustion of biodiesel	Lardon et al. (2009)
Algal biodiesel	1.247	Based on extrapolation of lab scale data, for production of 1 kg of biodiesel by cultivating *Chlorella vulgaris* in open raceways under N starved condition, flocculated, dried using belt drier, solvent extraction followed by transesterification and combustion of biodiesel	Lardon et al. (2009)
Algal biodiesel	4.343	Based on extrapolation of lab scale data, for production of 1 kg of biodiesel by cultivating *Chlorella vulgaris* in open raceways under N starved condition, flocculated, wet extraction using hexane, followed by transesterification and combustion of biodiesel	Lardon et al. (2009)
Algal oil	3.05	Open raceway pond, 29.6% lipid content	Jorquera et al. (2010)
	1.65	Flat plate photobioreactor	
	0.07	Tubular photobioreactor	
Algal biomass	8.34	Open raceway pond, 29.6% lipid content	Jorquera et al. (2010)
	4.51	Flat plate photobioreactor	
	0.20	Tubular photobioreactor	
Bio-oil	6.67:1	Hydrothermal liquefaction of *B. braunii* at 300 °C, HHV of 45.9 MJ kg^{-1}	Dote et al. (1994)
Bio-oil	2.94:1	Hydrothermal liquefaction of *Dunaliella tertiolecta*, HHV of 34.9 MJ kg^{-1}	Minowa et al. (1995)

drying and dewatering of harvested biomass and lipid extraction often have a disproportionate energy demand. Lardon et al. (2009) in their study on biodiesel production from *Chlorella vulgaris* reported that out of total energy required 90% of the demand was attributed to drying (up to 90% solid content) of harvested biomass followed by extraction of lipids using hexane as a solvent. Energy demand reportedly came down by 20% when wet algal biomass was employed for lipid extraction. Based on data obtained from pilot scale studies Liu et al. (2013) concluded that the EROI of algal biofuels produced through hydrothermal liquefaction lies close to unity. They expect the figure to reach close to 2.5–3 in. near future. Jorquera et al. (2010) in their comparative LCA study on production of algal oil and algal biomass using raceway pond, and flat, tubular photobioreactors calculated the EROI values for three different scenarios (Table 4). However their

Table 4 Comparison of different unit operation involved in algal biofuel production systems

Options		Advantages	Disadvantages
Cultivation system	Open pond	Cost effective	Poor control, frequent cleaning required, low productivity
	Photo-bioreactor	Easy control and manipulation opportunity, high productivity, low water demand, high cell density, easy harvest	Costly, scale-up issues
	Fermenter	Better scale-up possibility	Costlier than open ponds
Growth mode	Photoautotrophic growth	Uses naturally available sunlight and CO_2, or artificial PAR and CO_2 from flue gas	Biomass loss in dark, artificial sources of PAR are somewhat expensive, high temperature and presence of toxic components in flue gas
	Heterotrophic growth	High lipid accumulation, technically viable	Expensive carbon substrate
	Mixo-trophic growth	Combines the advantages of photo-autotrophic and heterotrophic modes of growth	Combines the disadvantages of photo-autotrophic and heterotrophic modes of growth
Water	Fresh water	Several freshwater species having high productivity are known	High water footprint
	Saline water	Low water footprint	Soluble bi-carbonates cannot be used as carbon substrate, location specificity
	Waste water	Simultaneous bioremediation of wastewater	Only wastewater from certain operations having appropriate composition can be used
Nutrients	Biological N_2 fixation	Reduction in demand of synthetic nitrogenous fertilizers	Biological N_2 fixation is an energy intensive affair
	Synthetic fertilizers	Fertilizers used in agriculture can be used	Low availability for emerging demands, low fertilizer use efficiency, manufacturing requires energy and release GHGs
	Saline water/waste water/agricultural runoff/nutrient recycling	Minimal input of most synthetic nutrients is required, improves nutrient use efficiency, use of agriculture runoff prevents algal bloom in water bodies	Composition varies widely

PAR	Natural	Freely available	Only a fraction of sunlight is photosynthetically active, spatio-temporal variability, high intensity of sunlight causes photo-inhibition
	Artificial	Narrow bands of radiation can be provided, can reduce photo-inhibition, highly efficient light sources are available	Somewhat expensive
Carbon	Atmospheric CO_2	Freely available, relatively constant quantity	Concentration is low, mass transfer limitations
	Flue gas	Can serve as concentrated source of CO_2	Very hot at times, presence of toxic components
	Soluble bi-carbonates	Bi-carbonate transporters are present on plasma membrane	Raises the pH of the culture media
Biomass harvest	Self-flocculation	Low input requirement for biomass harvest	May require continuous function of paddlewheels in order to keep biomass in suspension for effective light utilization during growth
	Mechanical/chemical	Effective harvesting	May require energy and chemical input
Drying and dewatering	Solar insolation	Available for free, fossil energy is not required	Slow, less efficient and needs large drying area
	Roller drying, freeze drying, spray drying, etc.	More efficient than solar drying, needs less drying area	Highly energy intensive, adds to overall cost and GHG emission of algal biofuel production
Extraction	Heated oil extraction	Wet algal paste can be used	Involves hazardous extraction solvent
	Mechanical extraction	No chemicals are involved, 70–75% extraction	Energy intensive
	Ultrasonic-assisted extraction	Lower extraction time, reduced solvent consumption, 76–77% extraction	Involves energy and solvents both
	Solvent extraction	Reproducible results, relatively inexpensive	Low extraction efficiency 60–70%
	Supercritical fluid extraction	Extracts almost 100%, extract is free of solvent	May need high temperature and pressure depending on the choice of solvent
	Enzymatic extraction	Further fractionation of extracts is possible	Costly

(continued)

Table 4 (continued)

Options		Advantages	Disadvantages
Biomass processing techniques	Gasification	Syn gas produced can be burned directly or used as a fuel for gas engine/turbine	Only marginally positive energy balance has been reported
	Direct combustion	Co-generation using biomass reportedly improves the GHG balance of power generation	Storage of energy produced is not a viable option
	Transesterification	Very high conversion and yield can be obtained, produces drop-in-fuel	Uses hazardous acidic and alkaline chemicals, biodiesel has 10% less energy than mineral diesel on volumetric basis
	Anaerobic digestion	Can process high moisture biomass	C/N ratio of feedstock is a critical factor
	Alcoholic fermentation	Produces drop-in-fuel	Lower energy density than petrol
	Hydrothermal liquefaction	Wet biomass can be processed, EROI > 2.9 have been reported	Requires high temperature and pressure
	Pyrolysis	Very high biomass to oil conversion is possible	Produces highly viscous, acidic and unstable oil fraction

study was limited only to biomass/oil production and did not include upstream processes of biomass harvesting, drying, extraction, processing and combustion (well to gate analysis). Further, the only energy that was considered as input for EROI calculation was that required for air pumping, mixing and mass transfer. Hirano et al. (1998) estimated a marginal positive EROI of 1.1 for methanol production from *Spirulina* biomass through biomass gasification. The harvesting step involving centrifugation was reportedly the major factor responsible for low EROI. Availability of multiple routes for individual unit operations provides ample scope for manipulation so as to improve the EROI of algal biofuels. Thus a careful selection of unit operations having minimal energy input requirement without compromising the calorific value of the biofuel produced is critical. EROI values correlate directly to the electricity used in the production process and thus alternative low power consuming operations favour a higher EROI. Table 3 reports EROI values of different algal biofuels.

2.8 Green House Gas (GHG) Balance

For any alternative to be a viable alternative to fossil fuels it should emit comparatively less CO_2 and should at least provide EROI >1. One of the most talked about benefit of using algae as biofuel feedstock is their capability to sequester large amount of CO_2 with somewhat higher photosynthetic efficiency in relatively very short span of time. Several studies have reported that cultivation of algae for biofuel production does not result in any net emission of CO_2. However, the overall GHG balance of any algal biofuel production system is dependent on several factors. GHG balance indicates the amount of CO_2 that is sequestered or released over a product or process life cycle chain taking into account all the input and outputs of greenhouse gases. All the emissions of different GHGs are reported in terms of kg of CO_{2eq}/functional unit chosen for LCA study. In general, the GHG balance is directly dependent on the quantum of electricity and heat that goes into the production chain and the GHG balance of algal biofuel production systems is greatly dependent on the energy mix of the country where the production facility is located. The GHG balance of algal biofuels is also dependent on the source of carbon for fixation into biomass by algae. Several researchers have analysed the GHG balance of algal biofuels by employing flue gas CO_2 from thermal power plants and cement industries as carbon substrate for photoautotrophic cultivation of algae. Stephenson et al. (2010) in their study on algal biodiesel production by using CO_2 from flue gas of gas fired power plant reported a reduction in GWP of exhausts by around 80% (relative to fossil diesel) when cultivation of algae was carried out in open raceway pond. In the same study they reported the GWP of exhausts to be significantly higher than that of fossil diesel if instead of open raceway, air lift photobioreactor was to be used for culturing algae. Using GREET as a modelling tool for estimating the net GHG emission of algal biodiesel Batan et al. (2010) reported a net reduction in GHGs by 75 g of CO_2 equivalent per MJ of energy

generated. Brentner et al. (2011) compared the base case and best case scenario for algal biodiesel production using a functional unit of 10 GJ and according to them the GHG emission of best case scenario (805 kg CO_{2eq}) for algal biodiesel production comprised only 14% of the base case scenario (5340 kg CO_{2eq}). The best case scenario was assumed to have significantly lower energy demand than the base case scenario. Minimization of energy use, co-product allocation by different means, biorefinery approach of material and energy transfer and nutrient recycling after biomass harvest or by using anaerobic digestate can have positive effects on overall GHG balance. Besides release of CO_2 upon combustion of fossil fuels and organic matter decomposition, several other GHGs such as methane (product of anaerobic digestion of biomass) nitrous oxide (released during production of fertilizers, fossil fuel combustion, and organic matter degradation) and ozone (produced upon reaction between VOCs and NOx in presence of sunlight) are also taken into consideration for calculation of GHG balance. Fertilizer production for culturing algae is an indirect but significant contributor to the emission of GHGs from algal biofuel production systems. Depending on the fertilizer type and its nitrogen content emission of GHGs during production of nitrogenous fertilizers for cultivation of algae reportedly range from 2.6 to 16 kg CO_{2eq} Kg^{-1} N (Handler et al. 2012). Therefore every effort should be made to recycle nutrients.

2.9 Biomass Processing Techniques

Processing of biomass after harvest for production of different biofuels is a critical step which affects the overall desirability of such fuels. The selection of a particular processing technique should be based on the relative proportion of different bio-molecules in the biomass. It should lead to high yield and conversion of biomass to target biofuels, should be feasible at large scale, should involve minimum energy conversion routes, and should provide a high calorific value fuel and high EROI values. There are several biomass processing routes which are broadly classified as thermo-chemical (pyrolysis, hydrothermal liquefaction, gasification, and direct combustion), biochemical (fermentation, anaerobic digestion, and biophotolysis) and chemical routes (transesterification and hydrotreatment). Biomass processing techniques (hydrothermal liquefaction, anaerobic digestion, etc.) which are capable of processing wet algal biomass are inherently more suitable in terms of energy savings and consequent GHG balance than those requiring drying (except solar drying) of biomass prior to its processing (direct combustion, gasification, etc.). Although solar drying of biomass has been studied, its efficiency is reportedly rather low. High yield and conversion of biomass or individual biomolecules to respective biofuel(s) is required for high atom economy. In case of biomass to liquid fuel conversion, pyrolysis technology has been deemed to have potential for entirely displacing fossil fuels currently in use throughout the world as high yield and conversion of biomass to bio-oil having high calorific value can be

achieved (Bridgwater 2007). Both pyrolysis and hydrothermal liquefaction have been reported to be a viable option for bio-oil production. Dote et al. (1994) carried out thermochemical liquefaction of *B. braunii* biomass at 300 °C and obtained bio-oil up to 64% (dry wt. basis) having HHV of 45.9 MJ kg^{-1} with positive energy balance. Flash pyrolysis of biomass is a very promising technology for displacement of fossil fuels by bio-oil derived from biomass and up to 95.5% yield of bio-oil has been reported (Demirbas 2006). However, the bio-oil needs upgradation prior to its use for purposes other than heating as it is typically highly acidic, viscous, unstable and contains dissolved water (Chiaramonti et al. 2007). Direct combustion of algal biomass has also been reported but it needs prior drying and dewatering of algal biomass (moisture content should be <50% dry wt. basis), grinding and chopping but produced energy must be used immediately as storage is unviable (Clark et al. 2008). Gasification involves partial breakdown of biomass at high temperature (800–1000 °C) and produces a combustible mixture of gases known as syn gas which is a low calorific value gas (4–6 MJ m^{-3}) (McKendry et al. 2002). For lipid based biofuels, extraction of intracellular lipid from wet algal biomass is preferable than extraction techniques requiring dry biomass. Compared to hydrotreatment, transesterification based processing of lipid needs very moderate reaction conditions and also produces glycerol as a by-product. Anaerobic digestion can be used to process wet algal biomass for production of biogas, having calorific value of about 20–40% lower than the heating value of the feedstock. It has been reported that anaerobic digestion of biomass can recover as much energy as that contained in intracellular lipid of biomass (Sialve et al. 2009). The digestate can be used as a fertilizer for supporting algal culture. However, the overall effectiveness of anaerobic digestion as an energy recovering step depends greatly on the C/N ratio of the feedstock. The residual biomass after lipid extraction can also be used to produce energy or energy carriers. Microalgae rich in starch can be used as a substrate for alcoholic fermentation and up to 65% conversion efficiency has been reported in literature (Hirano et al. 1997). Algal biorefinery is an emerging but highly a promising concept analogous to petroleum refinery which integrates the concept of industrial symbiosis for production of multiple biofuels, feeds and chemicals from algal biomass by utilizing waste material and/or energy from individual unit operations for other useful purposes. The concept of algal biorefinery promises to be a sustainable, environment friendly and economically feasible system.

2.10 Life Cycle Assessment (LCA)

LCA has emerged as the preferred analytical choice for ascertaining the environmental friendliness of algal biofuels over fossil fuels and also over biofuels derived from non-algal feedstocks. Numerous LCA studies have been carried out for algal biofuels (especially for algal biodiesel) using several modelling systems such as GREET model developed by Argonne National Laboratory, USA and GaBi

software program developed by Thinkstep. To date no algal biofuel production facility has been established and therefore these models are either based on extrapolation of lab scale data or data obtained from pilot scale studies. The comparability of different LCA studies is limited by the selection of different functional unit, cut-off criteria, co-production allocation strategy, incorporation of different set of assumptions, different impact categories, etc. Inclusion of different factors by different researchers has resulted in wide ranging values for sustainability indicators. Sills et al. (2012) have emphasized on the need for conducting uncertainty analysis for better understanding of the wide variability in values of sustainability indicators. Typically included impact categories are EROI, water footprint, GHG balance, and nutrient balance. LCA studies can help in identifying hotspots (if any) in a production or process chain which requires most of the attention (Gasafi et al. 2003). LCA study scrutinizes all the unit operations involved in a production system for their input and output and thereby facilitates comparison of environmental burdens associated with different alternative operations/activities.

2.11 Genetic Engineering

The genotypic diversity offered by close to 44,000 members of algae identified so far (Guiry 2012) is enormous but selection of species possessing an ideal combination of traits is a daunting task. At present the biosynthetic pathways leading to enhanced productivity and high accumulation of lipid are not clearly understood (Beer et al. 2009). Increase in genetic library database can dramatically enhance our understanding of mechanism behind biosynthetic pathways and the role of regulatory factors. Although *Chlamydomonas reinhardtii* has attracted most attention for studying the genetic regulation of biosynthetic pathways, sequencing has been completed for several species (*Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, *Ostreococcus tauri*, *Cyanidioschyzon merolae*, *Micromonas pusilla*, *Ostreococcus lucimarinus*, etc.) and that of several species (*Pseudo-nitzschia*, *Fragilariopsis cylindrus*, *Thalassiosira rotula*, *Chlorella vulgaris*, *Botryococcus braunii*, *Dunaliella salina*, *Galdieria sulphuraria*, *Micromonas pusilla*, *Porphyra purpurea*, *Aureococcus anophageferrens* and *Volvox carteri*) are currently underway (Radakovits et al. 2010). Traditionally expression of genes for enhanced production of biofuel or its precursor has been achieved indirectly through control of culture conditions, by selective breeding or by selection and adaptation. Cultivation of algae under sub-optimal and stressful conditions is known to enhance accumulation of neutral lipid triacylglycerol as a protective measure. But, these techniques (imposed stress) result in highly variable yield depending on species/species and several interdependent factors. Natural process of selection and adaptation is a time taking affair and the conventional methods of breeding are not firmly established. Genetic engineering of microalgae perhaps possesses the greatest potential for producing significant quantities of biofuels or its precursors

by overexpressing certain genes responsible for biosynthesis of desired products, knockdown of other genes that directly or indirectly play a role in diversion of carbon and energy towards other metabolic or physiological processes of little importance, introduction of foreign genes that encode enhanced production of biofuel precursors and improving the overall photosynthetic efficiency; economically and in an environmentally sustainable way. Unlike higher plants, algae lack cell differentiation and vegetative stage of algal growth is characterized by haploid genetic makeup and therefore genetic manipulation and propagation of superior traits in algae is relatively simpler and at the same time very tempting. More than 30 species belonging to Chlorophyta, Phaeophyta, Rhodophyta, Euglenoids and Dinoflagellates have been engineered till date. The results obtained are promising as stable expression of target genes has been achieved for several species however, for few species only transient expression was reported. Radakovits et al. (2010) suggested use of endogenous promoters and proper use of codons among other methods to improve the stability of expression. Genetic engineering of algae for increasing tolerance to various kinds of stresses common in natural systems is invaluable. Other techniques like secretion of biofuel precursors by genetically engineered microalgae and in-vivo transesterification/esterification of triacylglycerol and fatty acids by introducing genes that encode enhanced production of ethanol and facilitate transformation of fatty acids and ethanol into fatty acid ethyl esters (Biodiesel) simultaneously have also been envisaged. In-vivo production of fatty acid ethyl ester by *E. coli* upon introduction and overexpression of ethanol producing gene from *Zymomonas mobilis* and gene encoding acyl-CoA-diacylglycerol acyltransferase or wax ester synthase (DGAT/WS) from *Acinetobacter baylyi* species ADP1 simultaneously were reported by Kalscheuer et al. (2006). Such approaches can also be employed for algae. Roessler (1990) was the first to isolate acetyl-CoA carboxylase (ACC) catalysing the first committing step in fatty acid biosynthesis (production of malonyl CoA by carboxylation of acetyl-CoA) from *Cyclotella cryptica* which was later introduced into the genome of *C. cryptica* and *Navicula* saprophila (Dunahay et al. 1995). The gene responsible for ACC production (*acc*1) was overexpressed with two to threefold increase in activity of ACC. Besides, acyl-ACP thioesterase are highly specific enzymes that determine the chain length of fatty acids. The chain length and degree of unsaturation in fatty acids can be altered by introducing and overexpressing genes obtained from organisms having the desired fatty acid profile (Voelker and Davies 1994).

Since algae can accumulate significant quantities of biomass as fatty acid based lipids they can also be engineered to produce alkane molecules via sequential breakdown of fatty acids to aldehyde which is finally broken down to alkanes as drop in fuel replacement for petroleum products. The precise mechanism and clear identity of enzymes involved in breakdown of aldehydes is yet to be ascertained and therefore production of such drop in fuels by large remains an exciting goal (Radakovits et al. 2010). Algae can also be engineered to accumulate starch (precursor for bioethanol by fermentation) or for introduction and/or

overexpression of genes that facilitate accumulation and secretion of soluble sugars rather than polysaccharides as processing of simple sugars to ethanol is easier (Radakovits et al. 2010). Hydrogenase enzymes which catalyse reversible reduction of protons generated during the bio-photolysis of water in order to produce bio-hydrogen are sensitive to O_2. Hydrogenase engineering is already underway to develop oxygen tolerance in hydrogenases (Hankamer et al. 2007; Melis 2007). Such hydrogenases can enhance the algae mediated production of hydrogen by as much as 400% (Chandler 2011). Further biotechnological alteration in genetic makeup of algae can improve the nutrient and water use efficiency and thereby can substantially improve the overall sustainability of algal biofuel production facility.

Apparently genetic engineering of algae can help overcome the existing hurdles in large scale production of algal biofuels in an economical and sustainable manner. Although genetic engineering seems to have a disproportionate effect on overall economic feasibility of algal biofuels compared to bacteria, fungi and other eukaryotes research on genetic manipulation of algae lags behind and several important questions remain to be answered (Chisti 2008).

3 Economic Feasibility

The economics of fossil fuels affect our day to day life in several direct and indirect ways. Local price of petroleum derived fuels varies widely depending on the prevailing price of crude oil and changing taxation regime. The gap between demand and supply at any given time determines the price of crude oil basket. At present, the prevailing price of crude oil is at its lowest level since 2004 and it is likely to persist in near future until production is cut down substantially or the demand increases significantly to balance the demand and supply gap. The prevailing price of crude oil also affects the economic feasibility and acceptability of fossil fuel substitutes as low price of crude oil can prevent long term investment in renewable energy since renewable alternatives in general are costlier than fossil fuel based counterparts. Like many other renewable sources of energy, the idea of large scale production of algal biofuels as a fossil fuels substitute is flawed unless its production is economically superior or at least close to the prevailing price of petroleum based fuels. Production of algal biofuels by employing the state-of-the-art technologies is currently unviable as suggested by several techno-economic studies (Davis et al. 2011; Davis et al. 2014). However, the cost is likely to come down dramatically in near future as reported by various studies aimed at improving the economics of algal biofuels (Luque 2010; Darzins et al. 2010; Griffiths and Harrison 2009). The several life cycle stages of algal biofuel production (cultivation, harvest, drying, extraction and processing) incur cost in terms of capital investment for developing infrastructure, labour, energy and material input, operation and maintenance cost. Each of the life cycle stage can be completed using multiple approaches and hence the overall cost varies depending on the

technology employed. Approaches that envisage elimination of certain steps downstream of algae cultivation are invaluable. For a fixed capital investment utilization of species having higher biomass productivity and/or high lipid accumulation capacity may have a disproportionate impact on the overall economic feasibility. Benemann and Oswald (1996) reported that the overall cost of algal biodiesel might come down by 26.3–31.6% with twofold increase in biomass productivity and even higher reduction in cost (40.7–42.3%) was suggested by Nagarajan et al. (2013). Davis et al. (2011) estimated the minimum selling price for algal oil and green diesel (produced by hydrotreatment of algal oil) in order to achieve 10% rate of return. The estimated minimum selling price for algal oil and green diesel produced using open pond and photo bioreactor were 8.52\$ gal^{-1} and 9.84\$ gal^{-1}, respectively, while for green diesel it was reported to be 18.10\$ gal^{-1} and 20.53\$ gal^{-1}, respectively. Photo-bioreactors in general incur more cost (in terms of installation, operation and maintenance cost) than open cultivation system but inherently offer better control opportunities and higher productivity. Higher cell density can be achieved in photo-bioreactors which reduces the cost attributed to harvesting of biomass. Minimization of nutrient input requirement by improving the nutrient uptake and utilization efficiency, nutrient recycling, use of wastewater or saline water, genetic engineering, etc. can improve the economics of biomass production. There are several available techniques for extraction of biomass precursors (lipids) each having variable extraction efficiency and affect the overall economics differently. Biomass conversion techniques capable of using wet algal biomass (hydrothermal liquefaction, anaerobic digestion, fermentation) reduce the energy and capital required for drying and dewatering of algal biomass. By product valorization by various means (in energetic, environmental or economic terms) can improve the economic feasibility and environmental sustainability of the algal biofuels. Very recently the idea of using algal biorefinery (a system analogous to petroleum refinery) has emerged which involves synergy of material and energy (industrial symbiosis) between different unit operations and it appears to be a superior and viable approach (Subhadra 2010). Although traditional approaches such as selection of algae having desirable combination of traits such as high productivity and lipid accumulation by intensive screening and control of culture conditions so as to manipulate bio-synthetic pathways are currently underway, genetic engineering of algae appears to be the most promising strategy for improving the near and long economics of algal biofuels. Although several studies based on techno-economic modelling are available use of different techniques and different set of assumptions make their comparison difficult. Concerns related to energy insecurity and growing awareness about the negative impacts of fossil fuels is somewhat driving the worldwide growth of biofuel industry but policy interventions (by providing tax exemption, subsidy, etc.) are currently required for keeping biofuel price low and also for attracting investments in the field of biofuels.

4 Conclusion

The whole idea of using biofuels derived from algae as fossil fuel substitute is futile unless its industrial scale production is environmentally sustainable and economically feasible. To date there is no industrial scale setup for large scale algal biofuel production and hence real life data in unavailable for ascertaining the suitability of algal biofuel production system over other alternatives. Several modelling based LCA studies have been conducted for determining the EROI, GHG balance, water footprint, and nutrient demand of such systems. These studies have also highlighted the unit operations that need most of the attention. Genetic engineering for improved productivity and the concept of algal biorefinery appear to be the most promising options for achieving a sustainable and economically feasible biofuel production system.

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Ecological, Economical and Life Cycle Assessment of Algae and Its Biofuel

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1 Introduction

Algae belong to the eukaryotic organisms which show polyphyletic origin. They exhibit diverse nature in their origin, food and morphology as they range from unicellular species of *Chlorella* to the multicellular form of sea weeds (e.g. *Chara*). These are mostly aquatic and possess phototrophic (e.g. *Chlamydomonas pyronoidium, Chlorella vulgaris*, etc.), mixotrophic (e.g. *Chlorella minutissima, Scenedesmus bijuga*, etc.), heterotrophic (e.g. *Crypthecodinium, Galdieria*, etc.) and parasitic (e.g. *Cephaleuros*) natures to obtain their food from different sources of energy. Most of the algae show plant-like food habits and depend on photosynthesis for their food preparation while lacking some photosynthetic pigments and coverings around their reproductive cells which differentiate them from prokaryotes except Cyanobacteria. However, algae have highly complex interrelations among eukaryotes and Eubacteria as these are biologically and ecologically similar to plants. They both algae and plants lack common ancestors and their metabolism and biochemistry are distinct. Algae also lack some tissues and cells of plants such as stomata, xylem and phloem that are essential structures of most terrestrial plants.

A major feature of all algae is that they have the potential to adapt to extremely harsh and unfavourable conditions. In such environments they have evolved to produce different compounds, secondary metabolites and chemicals. Eukaryotic and prokaryotic algae can thus be found in diverse ecosystems such as pristine water, polluted freshwater and saltwater, seashores, glaciers, thermal hot springs, and even in places where other vegetation cannot grow. Approximately 72,500 species of algae contribute to about 7–80% of the earth's photosynthesis each year and substantially contributes to reduction in concentration of greenhouse gases in the atmosphere. Algae are microscopic to macroscopic photosynthetic autotrophs, and do not have the parts like plants such as leaves, stems, roots, and vascular tissue, and they reproduce by simple methods. Algae have been used for various purpose products and services. They play significant roles in aquatic ecosystems, including the basis of aquatic food webs as autotrophs.

Macroalgae, also known as seaweeds or kelp, are macroscopic algae found in saltwater habitats. They are often classified on the basis of photosynthetic pigment content and are commonly called brown (chlorophyll a and c, carotene, xanthophylls, and fucoxanthin), red (chlorophyll a and b, carotene, and phycoerythrin), and green (chlorophyll a & b) seaweeds. The average carbon sequestration potential of macroalgae is around 1.8 kg C $m^{-1}year^{-1}$. Kelp are well adapted to the sea water

habitat due to normally high availability of nutrients required for their growth. Microalgae are small (microscopic), unicellular to multi-cellular, prokaryotic and eukaryotic aquatic photosynthetic organisms. They are also known as microphytes. Approximately 50% of global CO_2 is captured by microalgae (Packer 2009). Micro- and macroalgae produce various kinds of valuable products including pharmaceuticals, nutraceuticals, antioxidants, polymers, and fatty acids (Richardson 1993; Plaza et al. 2008; Kim et al. 2011; Rodolfi et al. 2008).

2 Ecological Significance of Algal Species

2.1 Carbon Sequestration

Urbanization and industrialization have caused excess production of greenhouse gases, especially carbon dioxide (CO_2), and are also responsible for the reduction of the sink of these gases. Generally, sinks of CO_2 may be grouped into terrestrial plants, ocean water, and aquatic (phytoplankton). Aquatic flora, e.g. plants, macrophytes as well as phytoplankton absorb CO_2 and convert it into valuable products, i.e. carbohydrates which is the main (primary) food source of aquatic fauna.

In the aquatic systems, algae (both micro- and macro-) have significant roles in the sequestration of carbon. This carbon is absorbed by the algae in the form of CO_2 or HCO_3. Carbon is the building block for the carbohydrates, lipids, proteins, and nucleic acids in algae. The stored oils and other organic compounds can be harvested for the production of biodiesel, biohydrogen, bioethanol, polyunsaturated fatty acids, and other bioproducts (Spolaore et al. 2006; Milledge 2011; Razzak et al. 2013; Klinthong et al. 2015). Ono and Cuello (2003) compared several species of algae for their CO_2 tolerance; *Cyanidium caldarium* was found as the most CO_2-tolerant species (Fig. 1).

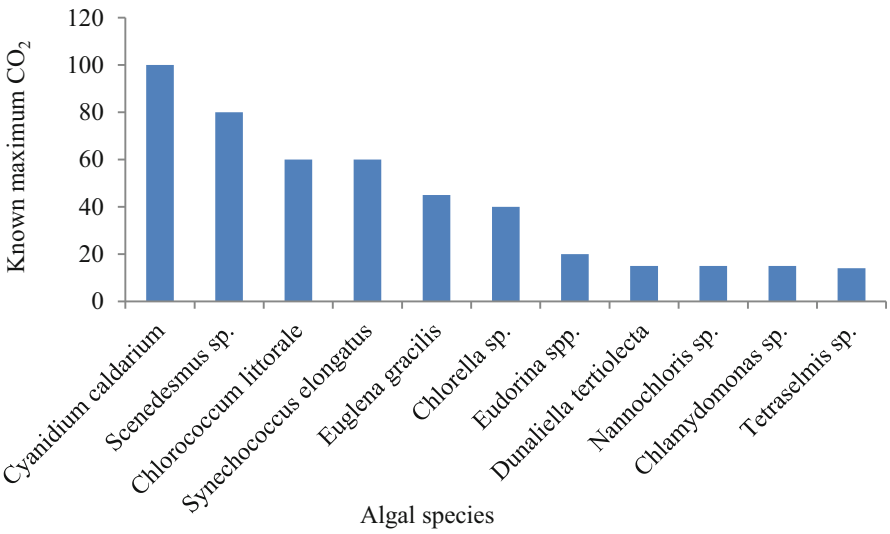

Fig. 1 Carbon dioxide (CO_2) tolerance of various species of algae (Ono and Cuello 2003)

Table 1 Use of algae for the remediation of heavy metals from contaminated aquatic ecosystems.

Metal removed	Algae	References
Zn and Cd	*Nostoc linckia* and *N. rivularis*	El-Enany and Issa (2000)
Cu and Ni	*Chlorella vulgaris*	Mallick (2003)
Trace element	*Spirulina* sp.	Chojnacka et al. (2004)
Cr and Ni	*Aulosira fertilissima*	Banerjee et al. (2004)
Cu, Pb, Co and Mn	*Nostoc muscorum* and *Anabaena subcylindrica*	El-Sheekh et al.(2003)
Cu and Pb	*Cladophora fascicularis*	Liping et al. (2008)
Cr	*Lyngbya* and *Gloeocapsa*	Kiran et al. (2008)
Cu	*Spirulina platensis* and *Aphanothece flocculosa,*	Cain et al. (2008)
Cd	*Gloeothece* sp. Strain PCC 6909	Micheletti et al. (2008)
Cu and Cd	*Oscillatoria* sp. NTMS01 and *Phormidium* sp. NTMS02	Kamaraj et al. (2011)
Zn and Cd	*Sargassum wightii* and *Caulerpa racemosa*	Tamilselvan et al., 2012
Cu and Ni	*Nostoc muscorum*	Dixit and Singh (2014)

2.2 *Pollution Remediation*

Use of algae for pollution removal is not a new approach; it has been in practice for several decades and popularized as phycoremediation (Afkar et al. 2010; Rawat et al. 2011; Olguín and Sánchez-Galván 2012; Prajapati et al. 2013; Chakravarty et al., 2015; Malla et al. 2015). Algae require light, CO_2, and nutrients for their growth. Light and CO_2 are sufficiently available in most habitats, but due to their capability for high uptake of nutrients, these may need to be supplied by added medium (Bajguz 2000; Subramaninan and Uma 1996; Shashireka et al. 1997). Various studies have been done to explore the remediation feature of algae (Table 1).This feature of algae has gained enormous interest for phycoremediation of polluted industrial wastewater because of its fiscal and environmental positives.

Chakravarty et al. (2015) reviewed the phycoremediation of dyes from wastewater using various species of algae. Several researchers found that the functional groups present on the surface of algal cell (such as carboxyl, phosphate, hydroxyl and many other charged groups) make the algae efficient at binding the molecules of dyes and removal of other pollutants from wastewater (Srinivasan and Viraraghavan 2010; Çelekli and Geyik 2011; Çelekli and Bozkurt 2011).

The available technology for treatment of wastewater includes ion exchange, electrochemical treatment, filtration, lime precipitation, chemical oxidation or reduction, osmosis, and membrane methods. However, these techniques possess some limitations like high cost, less significant removal of lower concentration of

pollutants, high initial cost, and generation of other ("secondary") pollutants (Chong and Volesky. 1995). Therefore, use of biological agents such as algae is vital to reduce contamination of environmental components. This biological remediation is relatively inexpensive because it only requires space and supplemental nutrients and CO_2 to grow, and the algal biomass can be harvested for various by-products.

2.3 Algae as Green Fertilizer (Biomanure)

Some Cyanobacteria (blue-green algae) such as *Nostoc* sp., *Tolypothrix tenuis, Aulosira* sp., *Anabaena* sp., *Azolla* sp., *Oscillatoria* sp., *Scytonema* sp., *Spirulina* sp., and *Plectonema* sp. can be used to improve the quality and quantity of agricultural crops because of their nitrogen fixing ability (conversion of inert atmospheric nitrogen gas into ammonia). This added biofertilizer reduces the need to add synthetic forms of nitrogen fertilizers to agricultural lands. They also help improve soil structure by reducing soil alkalinity and thus enhance the yield of crops. India has large areas of alkaline and sterile soils which need to be remediated. Chemical methods of land reclamation are neither cheap nor environment friendly. Bioremediation through blue-green algae seems like a more practical approach.

3 Economic Significance of Algal Products

3.1 Algae as Food and Fodder

The use of algae as food is not a new aspect; it has a long history. Several species of macroalgae like *Porphyra tenera, Enteromorpha intestinalis, Laminaria japonica, Undaria pinnatifida, Monostroma nitidum, Rhodymenia palmata,* and *Gracilaria* sp. are common components of the diet of people living in China, Japan, Scotland, and West Indies. Algal species like *Spirulina, Nostoc, Chlorella,* and *Dunaliella* are rich in food supplements, beta-carotene, thiamine, riboflavin, and vitamin B12 (Jeraci and Vansoest 1986). *Spirulina* has an excellent composition of dietary nutrients like 60–70% protein, 20% carbohydrate, 5% lipid, and 7% minerals (Chacoón-Lee and González-Mariño (2010). Becker (2007) compared different species of algae for their nutritional composition of proteins, carbohydrates, and lipids (Fig. 2.)

N-3 fatty acids obtained from microalgae are used in the agri-food industry (Simopoulos 1999). Bio-available protein and mineral elements present in algae are very useful for hyman consumption and recommended for daily nutritional consumption (Becker 1994).

Tokuşoglu and Una (2003) showed that the available carbohydrate of *Isochrisis* sp. was higher (16.98%) than that of the other microalgae they studied. Protein

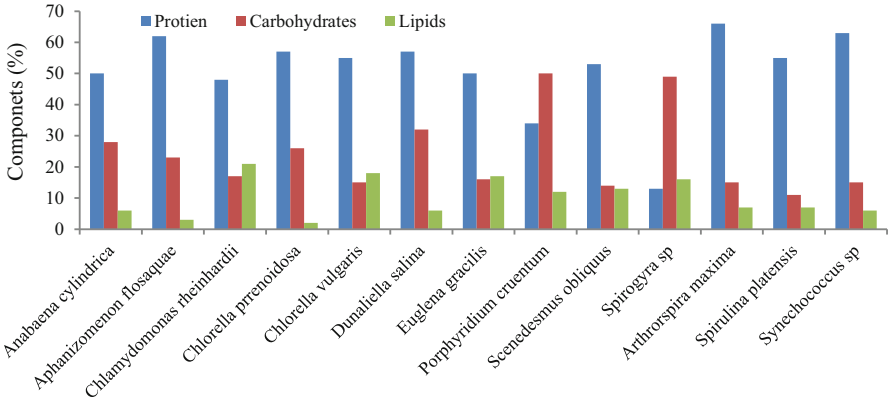

Fig. 2 The approximate amount (%) of protein, carbohydrates and lipids in different species of algae (Becker 2007)

content in *Spirulina* sp. was 63.0%, followed by *Chlorella* sp. (47.82%) and *Isochrisis* sp. (26.99%). Total lipid content of *Isochrisis* sp. was 17.16%, *Spirulina* was 7.53%, and *Chlorella* sp. had 13.32%. The major fatty acids were oleic acids (18:1n-9) for *Spirulina* sp. with 34.44%, followed by *Chlorella* with 33.14% of total fat present in the algal species. Currently, the high content of microalgal protein seems preferable to people who prefer to take natural food additives. Many studies show that algae are rich in basic nutrients like proteins, carbohydrates, and lipids (Fig. 3), yet algae are not widely acceptable as essential components of human food. The major drawbacks of algal food are its form of consumption in powder form. Its black colour after drying and its fish-like smell limit its incorporation in food materials. However, some processes like heating, baking, and mixing are being done by industries to modify algal materials in food items.

In developing countries algal ingredients in food face socio-ethnological barriers; however, it solves the need for protein and carbohydrate shortages by incorporating it with conventional food items. Despite these benefits, another major factor which limits algae ingredients in food is its high production cost.

3.2 As Animal Feed

In many areas of the world algae are preferably used for animal feed due to their high nutritional values of protein, carbohydrates and fats. These have become suitable substitutes for traditional animal feeds like rice bran, fish meal, and soy meal. At many places of the world algae are preferable to soy meal as feed of poultry, aquaculture, and small animals due to their higher carbohydrate, protein, and lipid content.

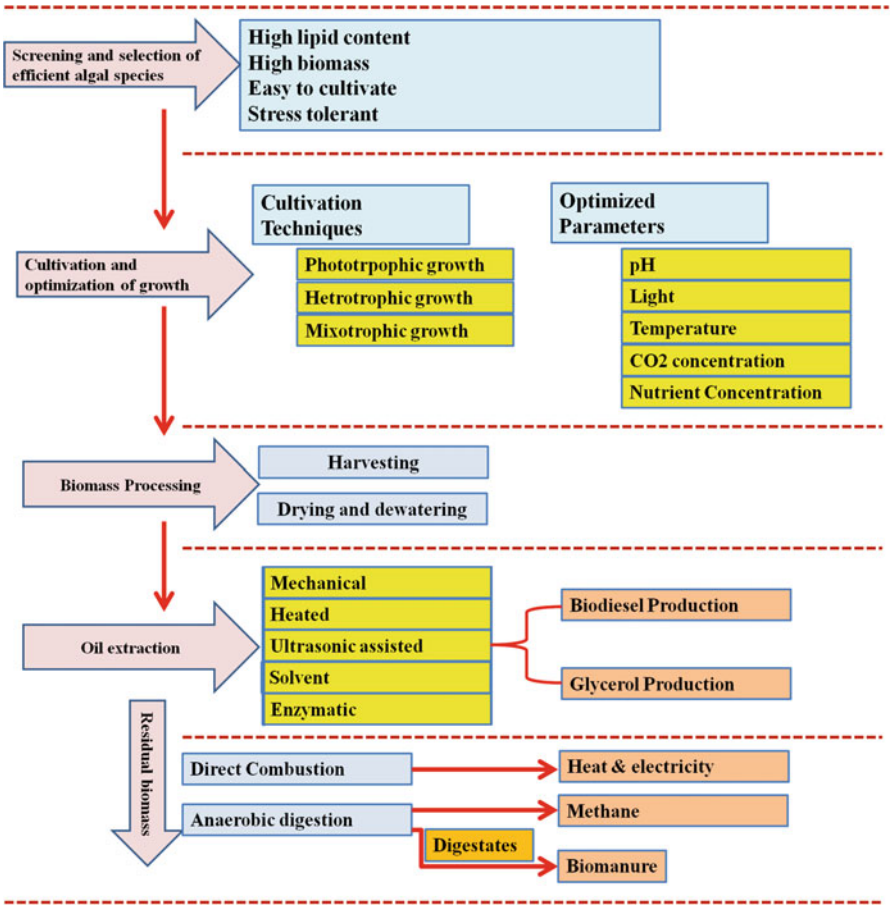

Fig. 3 Life cycle assessment of algal biodiesel (Kumar et al. 2015)

3.3 Use of Algae in Pharmaceuticals

Algae are the source of many compounds having antioxidant, anticancer, and antiviral properties. Several studies (Kim and Karadeniz 2011; Devi et al. 2011, Kim et al. 2011) have been done to evaluate the medicinal benefits of algal products. They are found effective in reducing fever, inflammation, and also treatment of cancer, ulcers, hemostasis, and blood pressure. Polyphenols, phycobiliproteins, and some vitamins found in algae have antioxidant properties (Plaza et al. 2008). A study done by Richardson (1993) suggested that some compounds act as scavengers for removing free radicals and active oxygen that promotes formation of cancer cells.

Some brown, and to a lesser extent a few red macroalgae have chemicals like polyphenols that work as highly effective antioxidants and are known as

phlorotannins. Some sulphated polysaccharides found in marine algae also show scavenging activities. Several algae have antimicrobial, antiviral, antifungal, antiallergic, anticoagulant, antifouling, anticoagulant, and anticancer properties (Na et al. 2005; Dayong et al. 2008; De Felício et al. 2010; Bouhlal et al. 2011). Algae produce a variety of metabolites and chemicals that protect them from other organisms in the water like bacteria, fungi, and viruses. These may eventually be used to cure different diseases (Bhadury and Wright 2004). Studies have shown that extracts from many algal species inhibit herpes viruses (Serkedjieva 2004).

3.4 Applications in Cosmetic Products

Algae are the source of a wide variety of cosmetic products including whitening, aging, moisturizing, tanning, pigment synthesis, and treatment of many skin diseases such as bacterial and fungal infections. Microalgal-based cosmeceuticals are in great demand. Many polysaccharides are being used in cosmetic products. Marine macroalgae are the most abundant source of natural polysaccharides like fucoidans obtained from brown algae, carrageenans from red algae, and ulvans from green algae (Table 2). These polysaccharides have a large number of cosmetic functions such as rheology modifiers, suspending agents, hair conditioners, and moisturizing hydrates. Polysaccharides derived from algae such as *Saccharina*

Table 2 Uses of various algal species in cosmetics

Algae	Uses	References
Porphyra umbilicalis	Contains substantial amounts of mycosorine, e.g. amino acids (MMAs) which can absorb UV light and also act as sun screams	Shick and Dunlap (2002)
Corallina pilulifera	Methanol extract attenuated matrix metalloproteinase (MMP), MMP-2 and MMP-9	Ryu et al. (2009)
Laminaria japonica	Suppression of thyrosinase activity	Thomas and Kim (2013)
Laurencia pacifica	Applied in treatment of bacterial function on skin	Fenical (1976)
Chlorella vulgaris	Wrinkles reduction and tissue regeneration	Stolz and Obermayer (2005)
Saccharina japonica	Extract used as skin moisturizer	Wang et al. (2013)
Chondrus cripus	Extract used in water distributions in skin	
Haematococcus pluvialis	Source of Astaxanthin carotenoids	Bolin et al. (2010)
Scendermus sp. *Chlorella* sp. *Spirilina* sp.	Contains Lutin which protects skin from UV-induced damage	Sánchez et al. (2008) Hallmann (2007)

japonica and *Chondrus cripus* are cheap and more environmentally benign, and thus are a substitute for petrochemicals (Wang et al. 2013).

Progerin is a protein that is accumulated in skin and causes aging. Consumption of the edible seaweed *Alaria esculanta* has been found to reduce the amount of progerin in skin cells (Takeuchi and Rünger 2013; Verdy et al. 2011). Tyrosinase inhibitors are used as skin whitening agents. Natural tyrosinase inhibitors can be obtained from marine macroalgae such as *Laminaria japonica* (Thomas and Kim 2013). Extracts from *Chlorella vulgaris* are also used as skin care products for collagen synthesis, tissue regeneration, and reduction in wrinkles (Stolz and Obermayer 2005) (Table 2).

4 Life Cycle Assessment of Algal Biodiesel

Phases in the life cycle assessment of algal biodiesel like screening of efficient algal species, cultivation and optimization of growth conditions of efficient algae, biomass processing, and oil extraction (Fig. 3) are important.

4.1 Selections of Microalgae for Biofuel Production

The adoptability of algae for commercial biofuel production is a big task felt across the globe. The biomass productivity and lipid yield cited in the literature is not feasible at large scale cultivation for commercial applications because factors such as temperature, pH, nutrients, light, carbon dioxide, herbivory, and contamination risk influence the growth and productivity of microalgae. Several algal species having higher biomass and lipid productivity are reviewed by Rodolfi et al. (2008). Important criteria in choosing optimum algal species are (1) high biomass productivity, (2) high lipid productivity per unit biomass, (3) pollutants tolerance to cultivation conditions, and (4)harvesting. Harvesting is a laborious, energy intensive, and costly process that increases the per unit production cost. It is highly desirable to choose the algal species which can be harvested with ease, which depends on the size, specific gravity, and the properties of auto-flocculation (Borowitzka 1997). Researchers have been adopting the integrated biorefinery-based approach to lower production cost of biofuel where production can be lowered by producing economically important nutraceuticals, fine chemicals, and other important co-products.

4.2 Cultivation Techniques and Optimum Conditions

Light intensity, temperature, pH, CO_2, nutrient concentrations, and other environmental and chemical factors not only increase or retarded the growth of microalgae, but they also influence the metabolic process of the microalgae, so researchers

working with algae are challenged with maintaining the physiological status of algae. Although different species of algae are adapted to a wide range of habitats, slight changes in nutrient concentrations affect their metabolic processes. The major factors which affect microalgal growth are discussed in detail below.

4.3 Cultivation Methods and Techniques

The high biomass and high lipid yielding algae are the primary species desired for biofuel production, but the growth conditions are also very important. The optimum growth and culture conditions must be known for each species of microalgae used. Four cultivation methods are in practice—phototrophic, heterotrophic, mixotrophic, and photo-heterotrophic.

4.3.1 Phototrophic Growth

Phototrophic growth is the most common globally used technique for cultivation of algae where only light, carbon dioxide, nutrients, and water (medium) are supplied. Algae build their carbon skeleton without the need for external organic carbon.

4.3.2 Heterotrophic Growth

In heterotrophic growth, algae use external carbon sources for their growth and development. Since some microalgal species do not grow well under phototrophic conditions, their biomass production can be improved by supplementing the external carbon sources in the growing medium. This is the practical and efficient method for higher biomass productivity. The most commonly used carbon sources for algal biomass production are glucose, sucrose, acetate, and fructose. The major disadvantage of heterotrophic growth is that chance of contamination is high.

4.3.3 Mixotrophic Growth

In mixotrophic growth, microalgae are supplied with external carbon sources and light. The microalgae use the external organic and inorganic carbon sources simultaneously.

4.3.4 Light

Light is the primary source of energy for photosynthetic organisms. The light intensity affects the photosynthetic process in the microalgae. Microalgae can be grown utilizing normal sunlight or artificial light. Highlight intensity can retard

algal growth through inhibiting the photosynthesis process, while low light intensity leads to poor growth by decreasing the photosynthetic rate. Researchers must therefore plot the light response curve as suggested by Richmond (2007) for optimum growth of microalgae at specific light intensities. In general, highest specific growth is found at the light intensity of 150 $\mu Em^{-2}\ s^{-1}$ and photoinhibition is observed at 200 $\mu Em{-}^{2}\ s^{-1}$.

4.3.5 pH

Several metabolic processes of microalgae are dependent on pH. The effects of pH change on microalgae have been studied in detail by Khalil (2010). In general, the optimum pH for the cultivation of freshwater microalgae is 7.5. Variation growing medium pH may lead to the decrease or increase in the cellular composition of microalgae.

4.3.6 Temperature

Temperature is the most crucial environmental factor influencing the growth of microalgae and their cellular composition. Variation in temperature influences the level of unsaturated fatty acids converted into glycolipids. Increased temperature is responsible for lower amounts of unsaturated lipids in membranes (Nishida and Murata 1996). Carbon dioxide solubility is dependent on temperature; increased temperature significantly decreases the rate of carbon dioxide uptake. Increased temperature is also responsible for the increased photorespiration. Some studies also reported that increased temperature leads to increased lipid content (Converti et al. 2009).

4.3.7 Carbon Dioxide and Nutrient Concentrations

Carbon dioxide is the natural carbon source for photosynthetic autotrophs. Each mole of carbon dioxide produces 1 mole of carbohydrate, three ATP, with 2 moles of $NADPH_2$ (Richmond, 2007). Microalgae are also able to grow on organic carbon sources such as glucose, acetate, and ethanol.

4.4 Biodiesel Production

Microalgae are advantageous for biodiesel production in comparison to traditional plant-based feedstocks like jatropha, corn, soya, and rapeseeds because they are easy to cultivate, require less attention, and different species can be grown in freshwater, saltwater, and polluted industrial and sewage water. Algae also require

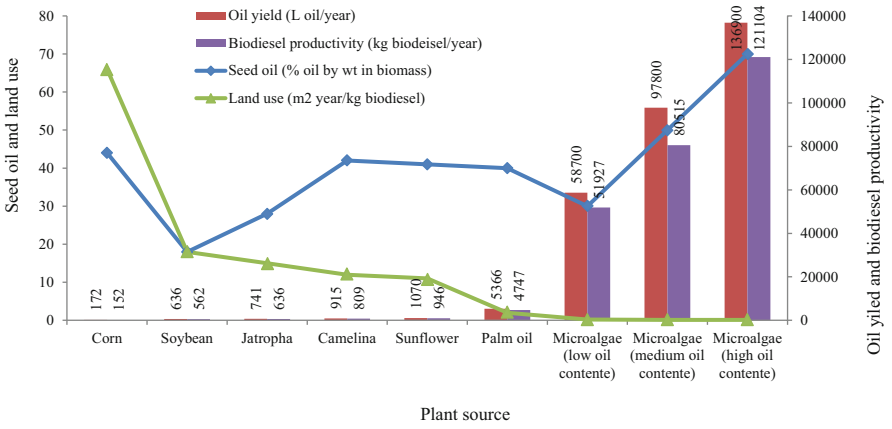

Fig. 4 Comparison of different biodiesel production feedstocks (Kunjapur and Eldridge 2010)

less area per unit biomass than agricultural crops and don't compete with agricultural plants for arable land or food vs. fuel.

Algal biodiesel has more advantages than petroleum diesel as it has no sulphur content and can be used directly or with slight modifications in internal combustion (IC) engines, and thus generate less emission of CO, NOx, Sox, and hydrocarbons (Tokusoglu 2001). Current research and development has focused on maximizing biodiesel yield from suitable algal species. There is a worldwide effort to obtain most effective and cheap technology to enhance the production of biodiesel from algae. Other biodiesel feedstocks include agricultural biomass, lignocellulosic material, and oil-yielding plants such as jatropha and karanja, but the cost of processing these feedstocks is more than algae because they need to be pretreated prior to the process for biodiesel production (Delucchi 2003). Algae are a preferable biodiesel feedstock over corn, soybean, sunflower, and palm oil in terms of oil content, yield, land used, biodiesel productivity, and cost per unit biomass (Fig. 4; Kunjapur and Eldridge 2010).

5 Conclusions and Future Recommendations

Algae have enormous potential for various uses such as food, fodder, fertilizer, medicines, cosmetics, biofuel, and other by-products. Ecological importance of algae is also very high as they greatly contribute to the fixation (sequestration) of CO_2, remediation of pollutants, and can be used as green fertilizer which may help to achieve the goal of sustainable development by reducing greenhouse effects, cleaning up the environment, and reducing the dependency on synthetic fertilizers, respectively. However, the most essential benefit of algae is their lipid content that makes it suitable for biodiesel production and economic by-products. Comparison

of biodiesel production of algae vs. other feedstocks also supports their value for bioenergy harvesting. Algae thus seem to be the overall best future asset for combined environmental improvements and economic products.

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