Recent Advances and Future Prospects of Microalgal Lipid Biotechnology

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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

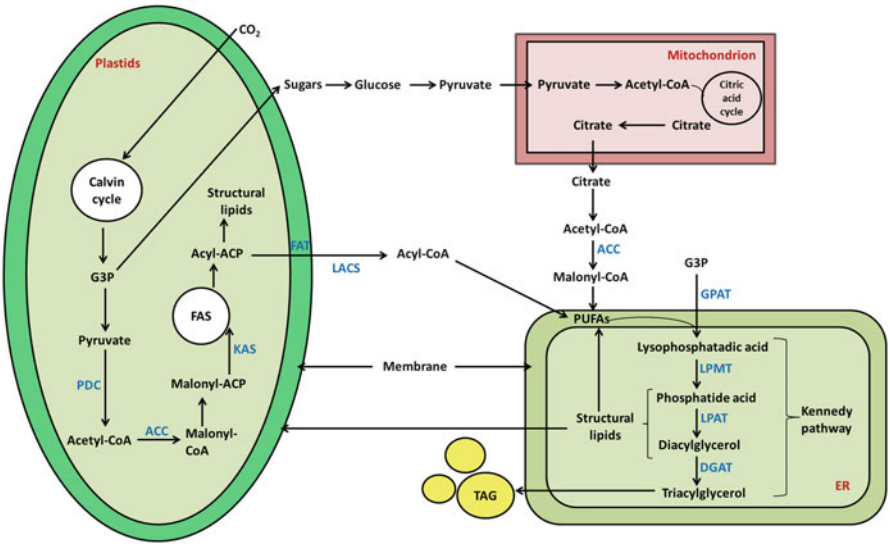

Fig. 1 Outline of lipid synthesis in microalgae (Adopted from Bellou et al. 2014). Abbreviations: *ACC* acetyl-CoA carboxylase, *ACP* acyl-carrier protein, *LACS* long-chain acyl-CoA synthetase, *ATP:CL* ATP-dependent citrate lyase, *CoA* coenzyme A, *DGAT* diacylglycerol acyltransferase, *ER* endoplasmic 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

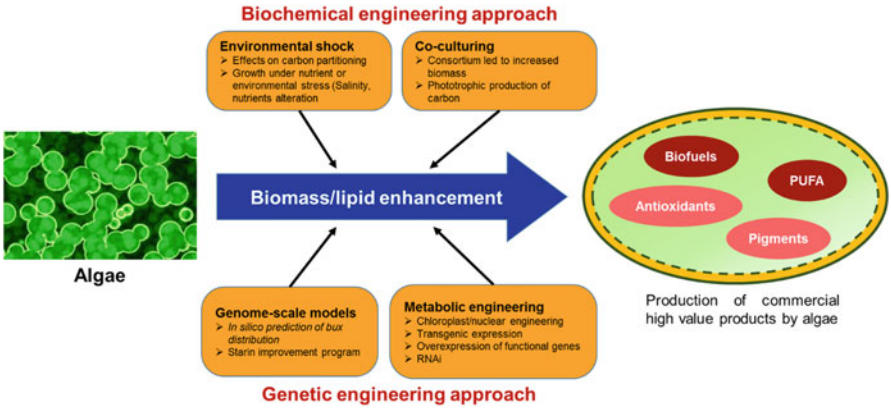

Fig. 2 Various strategic options available to enhance lipid production in algae

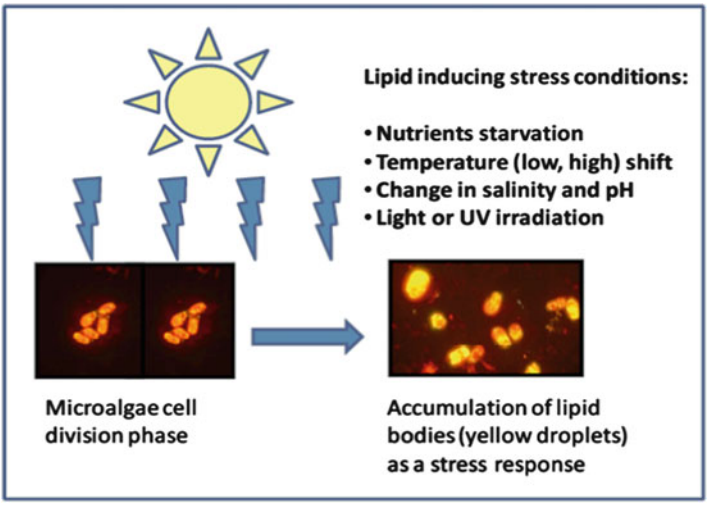

Fig. 3 Induction of algae lipids under unfavorable stress conditions (Adopted from Sharma et al. 2012)

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

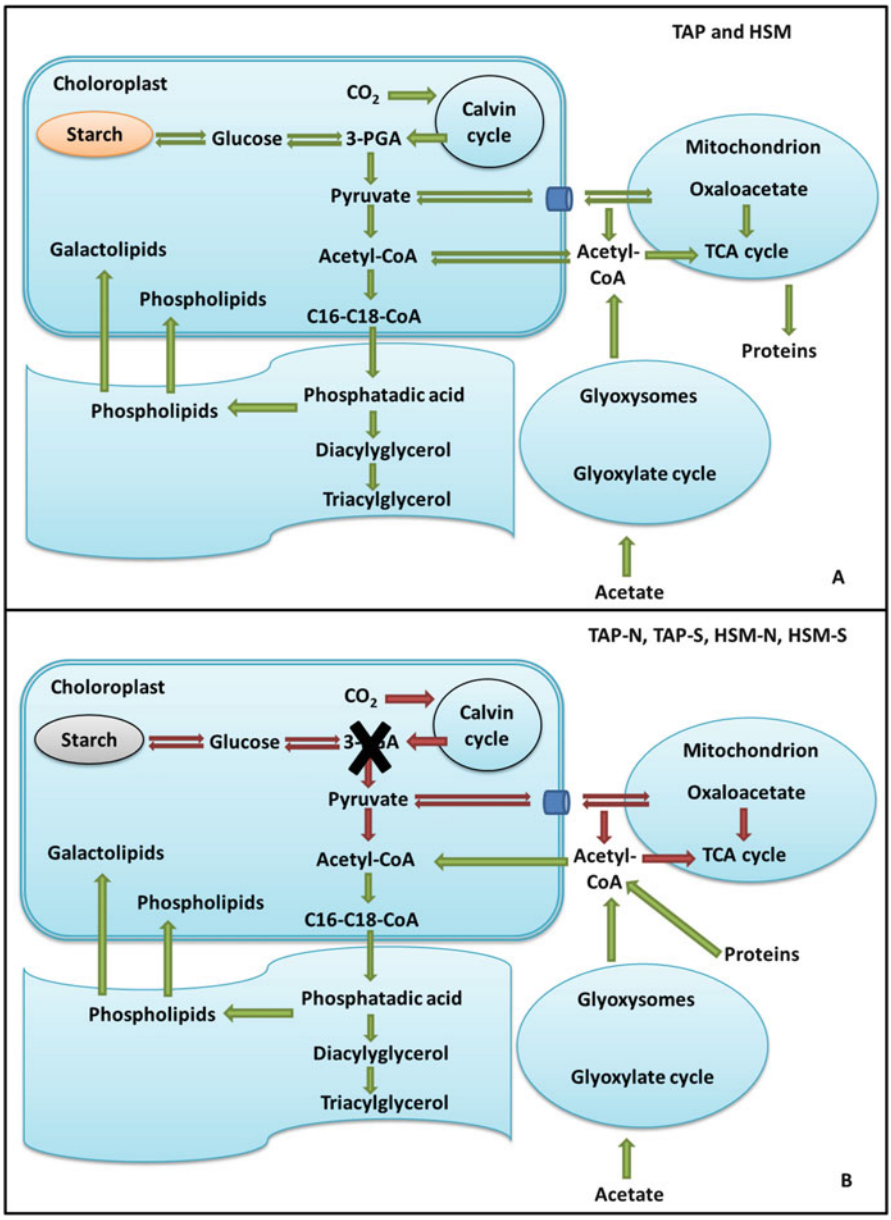

Fig. 4 Synthesis of TAG using (**A**) photosynthesis and acetate assimilation intermediates under nitrogen and sulfur sufficiency, and (**B**) acetate assimilation intermediates under nitrogen and sulfur deficiency (Adapted from Deng et al. 2011)

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).

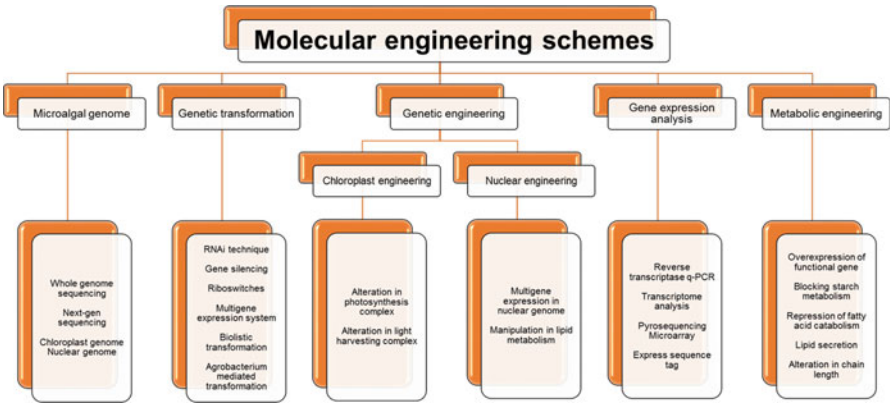

Fig. 5 Molecular schemes for enhancing accumulation of lipids in microalgae (Adapted from Singh et al. 2016)

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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