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Trends and novel strategies for enhancing lipid accumulation and quality in microalgae

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ABSTRACT

In order to realize the potential of microalgal biodiesel there is a need for substantial impetus involving interventions to radically improve lipid yields upstream. Nutrient stress and alteration to cultivation conditions are commonly used lipid enhancement strategies in microalgae. The main bottleneck of applying conventional strategies is their scalability as some of these strategies incur additional cost and energy. Novel lipid enhancement strategies have emerged to research forefront to overcome these challenges. In this review, the latest trends in microalgal lipid enhancement strategies, possible solutions and future directions are critically discussed. Advanced strategies such as combined nutrient and cultivation condition stress, microalgae–bacteria interactions, use of phytohormones EDTA and chemical additives, improving light conditions using LED, dyes and paints, and gene expression analysis are described. Molecular approaches such as metabolic and genetic engineering are emerging as the potential lipid enhancing strategies. Recent advancements in gene expression studies, genetic and metabolic engineering have shown promising results in enhancing lipid productivity in microalgae; however environmental risk and long term viability are still major challenges.

Contents

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1. Introduction

Microalgae have attracted significant interest from researchersas a biodiesel feedstock due to its capacity to accumulate substantial amount of lipids, high growth rate and environmental benefits. Production of value added by-products and utilization of lipid-extracted biomass (animal feed, aquaculture and biomethane production) have further strengthened the case for microalgae as a sustainable feedstock for biodiesel production. Despite many advantages, commercial realization of microalgal biodiesel is still a challenge owing to its high production cost [1–5]. Enhancement of microalgal lipid content could improve the economics of biodiesel production. Nutrient limitation and induction of stress by controlled cultivation have been the norm to improve lipid content in microalgae [1,6,7]. Recently, novel strategies have been explored to overcome the challenges of conventional approachesand to achieve maximum possible outcomes in terms of lipid yields, sustainability and cost effectiveness [8,9]. These strategies include a combination of stress factors, co-culturing with other microorganisms, addition of phytohormones and chemical additives [10–13].

Developing microalgal strains with high lipid accumulation capability using genetic and metabolic engineering tools has recentlygainedmomentum as alternative strategies for strain improvement [14,15]. The recent advancements in decoding the full genome of several microalgal strains and identification of key genes involved in lipid synthesis pathways makes genetic and metabolic engineering an alluring strategy to enhance lipid accumulation in microalgae [16]. The major challenges for genetically modified microalgal strain for high lipid accumulation are their long term viability and environmental risk assessment at open cultivation systems.

The present review deals with recent advancements and novel strategies for lipid enhancement in microalgae and their challenges.Molecular approaches for enhancing the lipid accumulation and quality are also described in detail (Fig. 1).

2. Nutrient regime and cultivation conditions to enhance lipid accumulation

Microalgae are able to survive in extreme environments as they can alter their metabolism according to varied environmental conditions. Under unfavorable conditions, microalgae have the tendency to accumulate neutral lipids to protect cells from photooxidation [7,17,18]. Lipid enhancement strategies involving alteration of the nutrient regime and cultivation conditions are widely applied in microalgal cultivation. Factors such as nutrient stress, light, temperature, CO_2, salinity etc. have been explored by several researchers to enhance lipid accumulation in microalgae [19,20].

2.1. Effect of nutrient regimes on lipid accumulation

Nutrients such as nitrogen, iron, phosphorus, magnesium, sulfur and silicon are very important for cellular mechanism viz, photosynthesis, cell division, respiration, intracellular transportation, protein synthesis etc in microalgae [6,21]. Under stressed conditions; microalgae tend to accumulate energy in the form of polysaccharides, and/or neutral lipids. This defense mechanism of the microalgal cell has been exploited widely for the production of neutral lipids, carotenoid, polysaccharides and many other metabolites [22–24].

Nitrogen starvation is the most widely used strategy to improve lipid accumulation. Nitrogen is provided in the form of nitrates, urea and ammonium salts. Uptake and utilization of these nitrogen forms by microalgae however vary; ammonia is utilized more efficiently compared to the other forms of nitrogen as it can be directly converted to amino acids [25–27]. Nitrogen deprivation conditions could lead to reduced cell division [28]. Reduced cell division shifts the lipid biosynthetic pathways to synthesize more neutral lipids than synthesizing membrane lipids required for the cell wall formation [29,30].Subsequent accumulation of NADH due to the slower photosynthetic rate inhibits enzyme citrate synthase and prevents acetyl CoA from entering into the TCA cycle. Elevated concentrations of acetyl CoA activate acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA. This irreversible conversion reaction is the rate limiting step infatty acid biosynthesis which leads to enhanced lipid accumulation in microalgal cells [31]. Tao et al. [32] studied the effect of nitrogen limitaion on lipid yields of *Chorococcum* spp. and *Scenedesmus destricola.* Under nitrogen deficent conditions, the lipid conent was increased from 31.6% to 40.7% in *Chlorococcum nivale* and 48% to 54% in

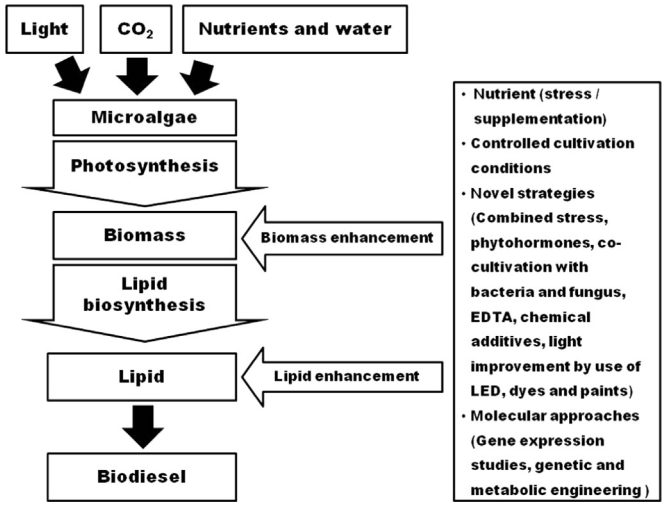

Fig. 1. Lipid enhancement strategies for sustainable microalgal biodiesel production.

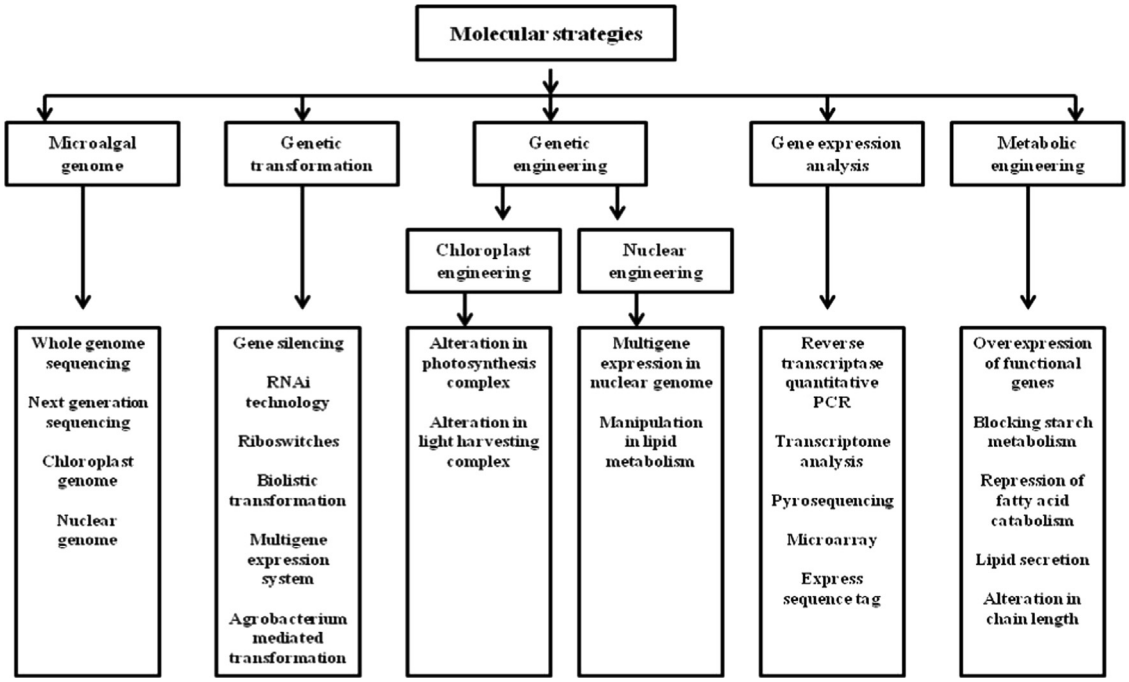

Fig. 2. Molecular strategies for enhancing lipid accumulation in microalgae.

Scenedesmus destricola. Biomass productivity of *Chlorococcum nivale* and *Scenedesmus destricola* was decreased from 0.40 $gL^{-1}d^{-1}$ to 0.38 $gL^{-1}d^{-1}$ and 0.48 gL^{-1} d^{-1} to 0.38 $gL^{-1}d^{-1}$ repectively under these conditions. Converti et al. [29] studied the effect of nitrogen limitation on lipid yields of *Nannochloropsis oculata* and *C. vulgaris*. Under nitrogen limited conditions, the lipid yield was increased to 15.31% and 16.41% in *Nannochloropsis oculata* and *Chlorella vulgaris* respectively. Similarly Gao et al. [33] reported increase in lipid accumulation from 23 to 46% and decrease in biomass productivity from 19.0 $mgL^{-1}d^{-1}$ to 12 $mgL^{-1}d^{-1}$ in *Chaetoceros muelleri* under nitrogen limitation. Altering the nitrogen source in the media may also lead to change in the quantity of lipid accumulation and fatty acid composition. Lin and Lin [34] reported that a combination of urea and sodium nitrate results in highest biomass productivity in *Scenedesmus rubescens*. Tao et al. [32] found that deficiency of nitrate and urea results in variation of lipid content and fatty acid composition of *Chlorococcum ellipsoideum*, *Chlorococcum nivale*, *Chlorococcum tatrense* and *Scenedesmus deserticola* strain (Fig. 2).

Phosphorous is an important macronutrient that contributes to various metabolic processes such as signaling pathways, energy generation, and photosynthesis. Under phosphate limited cultivation conditions, microalgal cells are known to accumulate neutral lipids. Xin et al. [25] reported a highest lipid accumulation of 53% under phosphate limitation (0.1 mgL^{-1}) in *Scenedesmus* sp. however; they observed low biomass of 0.13 gL^{-1} under the same conditions. Microalgal cells store phosphorus as polyphosphate under nutrient sufficient condition which are utilized by the cell during nutrient deficient conditions [35–37]. Although the above nutrient stress strategies are successful in terms of enhancing lipid content in microalgae; low biomass production is the main limitation as it affects the overall lipid productivity.

Metals such as iron are known to influence microalgal growth under nutrient stress conditions. It is one of the most important-metals in photosystem (I) and photosystem (II), and play roles in nitrogen assimilation, respiration and DNA synthesis [38]. Lipid accumulation in microalgae occurs mainlyduring the late log phase. Iron supplementation reduces the time period for the onset of stationary phase of microalgal growth thereby increasing both biomass and lipid accumulation [11,39]. Liu et al. [40] have reported 3–7 folds increase in lipid accumulation as compare to the control in *Chlorella vulgaris* under higher iron concentration. Baky et al. [41] cultivated *Scenedesmus obliquus* in N-9 medium with varying concentrations of $FeCl_3$ and lipid yields were found to increase with an increase in$FeCl_3$ concentration. Maximum lipid yield of 28.12% was observed in culture with $FeCl_3$ concentration of 20 mgL^{-1}.

Similarly, Magnesium ions are also found to influence the lipid accumulation in microalgae [42]. Magnesium ions promote the activity of acetyl-CoA, the enzyme that regulates the first committing step of microalgal lipid biosynthesis and are also required for chloroplast pyruvate dehydrogenase complex which provides acetyl-CoA and NADH for fatty acid synthesis [43,44]. Gorain et al. [45] observed an improvement in lipid content (1.44 fold rise) with the addition of magnesium (100 mgL^{-1}) into the media. Huang et al. [46] reported 1.25 folds increase in the cell density of *Monoraphidium* sp. FXY-10 with supplementation of 100 μM magnesium into the medium. Ren et al. [43] also observed increase in lipid content and growth with increasing magnesium concentration. Therefore an increase in Mg supply to the media could enhance the activity of these enzymes, leading to an increased cell division under nutrient stress conditions. Table 1 represents the conventional lipid enhancement strategies for sustainable microalgal biodiesel production.

Nutrient regime alterations are the preferred choice for the enhancement of lipid accumulation, because of its easy applicability at both lab and large scale cultivation. However, the main challenge for this strategy is the trade-off between biomass and lipid yields. Nutrients like nitrogen have significant influence on both the biomass generation as well as the lipid accumulation in the cell. Increase in the nitrogen concentration in the medium directly affects the biomass and inversely affects the lipid yields [17]. Nutrients such as nitrogen iron and phosphorous has been widely studied for microalgal lipid enhancement, however other nutrients also need to be investigated. The further in depth studies on actual physiological role of these nutrient stresses on the organism for better lipid accumulation are required. Other challenge includes identification of suitable and economical sources

Table 1
Conventional lipid enhancement strategies for sustainable microalgal biodiesel production.

Stress	Microalgal strain	Stress levels	Lipid content (%)	Biomass	Lipid productivity ($mgL^{-1}d^{-1}$)	Reference
Nutrient stress						
N	*Chlorella vulgaris*	375 mgL^{-1}	16.51	-	20.30	[29]
	Nannochloropsis Oculata	75 mgL^{-1}	15.51	-	16.41	[29]
	Chaetoceros muelleri	Nitrogen limited	46.32	12 $mgL^{-1}d^{-1}$	4 $mgL^{-1}d^{-1}$	[33]
	Chlorococcum ellipsoideum	2.9 mML^{-1}	40.5		236.9	[57]
	Scenedesmus deserticola	5.8 mML^{-1}	54.4	0.38 $gL^{-1}d^{-1}$	216.6	[57]
P	*Scenedesmus Sp.*	0.1 mgL^{-1}	53	0.13 gL^{-1}	-	[25]
	Chlorella sp	32 μM	23.6	1.8 gL^{-1}	15.67	[36]
	Chlorella vulgaris	P deficient	37.73	38.25 $mgL^{-1}d^{-1}$	19.50	[58]
Fe	*Scenedesmus obliquus*	20 mgL^{-1}	28.12	1.250 $mgL^{-1}d^{-1}$	95.25	[41]
	Chlorella vulgaris	1.2 × 10^{-5} mol L^{-1}	56.6	-	-	[40]
Mg	*Monoraphidium* sp. FXY-10	100 μM	58	28 $mgL^{-1}d^{-1}$	16.00	[46]
Cultivation conditions						
Light	*Nannochloropsis sp.*	100 μmol m^{-2} s^{-1}	31.3			[59]
Temperature	*Nannochloropsis oculata*	20–25 °C	7.42-14.9			[29]
CO_2	*Scenedesmus obliquus*	12% CO_2	33.14			[41]
	Nannochloropsis sp.	15 % CO_2			1.43 g L^{-1}	[52]
Salinity	*Dunaliella salina*	1.0 M	67			[55]

for the nutrients such as nitrogen, phosphorous, magnesium, iron etc.

2.2. Effect of cultivation conditions on lipid accumulation

Cultivation conditions such as light, temperature and salinity have known to influence microalgal growth [47–49]. Light is important for photosynthesis in autotrophic microalgae and variation in light intensity and photoperiod could alter the lipid biosynthesis. Wahidin et al. [50] studied the effect of light intensity and photoperiod on lipid accumulation in *Nannochloropsis* sp (marine microalga) and noticed an increase in lipid accumulation of up to 31.3% after 8 day cultivation under 100 μmol m^{-2} s^{-1} light intensity and photoperiod of 18 h light: 6 h dark cycle. A gradual decrease in cell density and growth rate was also noticed when the photoperiod cycles were extended to 24 h. It was reported that during low light intensities, microalgae tend to produce more polar lipids due to an increase in chloroplast membrane synthesis. A stepwise increase in light intensity on the other hand, tends to accumulate more neutral lipids without affecting the biomass yield [51]. Although the light intensity has shown a significant role in microalgal biomass and lipid accumulation, the approach is expensive and energy intensive to scale up.

Supply of CO_2 is crucial in autotrophic cultivation of microalgae. Microalgae fix CO_2 via photosynthesis to form various organic metabolites. It was observed that with the increased concentration of CO_2 supply, biomass and lipid productivities increase [28]. Jiang et al. [52] observed an increase in biomass productivity (0.39–1.43 gL^{-1}) and growth rate (0.33–0.52 d^{-1}) of *Nannochloropsis* sp. after CO_2 (15%) supplementation.

Microalgae can tolerate a wide range of temperatures depending upon the strain. Most of the algal strains grow well in the temperature range of 25–30 °C [53]. It has been well documented that an increase in temperature increases the saturated fatty acids composition in microalgae while decrease in temperature increases the unsaturated fatty acid composition of total cellular lipids [29,54]. The temperature effect on microalgal species can however be purely strain specific. The effect of temperature on lipid yields of *Nannochloropsis oculata* and *Chlorella vulgaris* was studied by Converti et al. [29] and have reported that an increase in temperature from 20 to 25 °C have resulted in two-fold increase in lipid yield (7.90–14.92%) for *Nannochloropsis oculata*, while an increase in temperature from 25 to 30 °C decreased the lipid yield from 14.71% to 5.90% for *Chlorella vulgaris*.

Similarly an increase in NaCl (salt stress) in the medium can lead to an increase in lipid content in microalgae [55,48]. An increased NaCl concentration from 0.5 to 1.0 M improved lipid content upto 67% in *D. Salina* [55]. High salinity in the culture medium creates oxidative stress to the microalgal cells which may induces increase in the lipid content. Increase in salt concentration can also have qualitative effect on microalgal lipids. Salinity stress also could facilitate in maintenances of saturated fatty acid in higher amounts in microalgal species. *Botrycococcusbraunii* grown under high salinity concentrations of 34 mM and 85 mM, showed 1.7–2.25 fold increase in the relative proportion of palmitic acid and 2 fold increases in oleic acid [48]. Considering the impact of salinity on quality and quantity of lipids, this approach can may be use to enhance lipid accumulation in microalgae. This strategy however, may only be feasible with halo-tolerant strains.

Though the cultivation conditions are easy to maintain in the lab, maintaining them at large scale is challenging and cost intensive. Controlling temperature and light is unfeasible in the open cultivation system and is cost intensive in the closed cultivation system [29,56]. Thesupply of CO_2 to both, closed or open cultivation systems is unfeasible because of technical and economical challenges. The choice of the cultivation system is also a long discussed topic. Table 1 depicts effect of conventional approaches on biomass, lipid content and lipid productivity of microalgae. Each system has its own pros and cons, however, the microalgal strain and economics of the process makes the choice easier. Further in depth investigation is required on the above discussed parameters, in order to decide on the economical, easy and scalable strategy for the enhancement of lipid accumulation in microalgae (Table 2).

3. Novel approaches for enhancing lipid accumulation in microalgae by altering culture conditions (nutritional and cultivation)

To improve the viability of producing lipids from microalgae, a more efficient approach is required, focusing on reducing the overall production cost and improved scalability. Some of the novel approaches include combined nutrient and abiotic stress, use of phytohormones and EDTA, chemical additives and bacteria–microalgae co-cultivation etc.

Table 2
Comparison of various approaches their advantage and challenges.

Strategies	Advantages	Challenges
Conventional approaches		
Nutrient stress		
N	High lipid accumulation	Low biomass productivity
P	High amount of saturated fatty acids	Low lipid productivity
Metals	High biomass	Single stress factor is not sufficient to improve lipid productivity
	High lipid productivity	
	High chlorophyll content	
Light	High biomass production due to efficient photosynthesis	Not easy to control at open cultivation systems
		High operational cost
Novel approaches		
Two stage cultivation	High biomass production at first stage	Large scale trails are required
	High lipid accumulation in second stage	
	High lipid productivity	
Combined nutrient and abiotic	High biomass and lipid productivity	Large scale trials are required
	Suitable fatty acid profile	Need to find cheap nutrient sources
	Easily scalability	
Phytohormones	High growth rate	High cost of phytohormones
	High biomass	
	High lipid productivity	
EDTA supplementation	Enhance metal uptake	High concentration of EDTA may cause growth inhibition
	High biomass production	
	High lipid productivity	
	Easily applied at large scale	
Co-cultivation of Microalgae-bacteria	High lipid productivity	Bacterial population may affect the fatty acid composition
	High growth	Need further research to understand mechanism
Chemical additives		
Azide	High lipid accumulation without compromising growth	Long term use is toxic to microalgal cell
	High lipid productivity	Variation in response from species to species
Brefeldin A	High lipid accumulation with normal growth	Toxic to cells after some time
	High biomass production	Need further research and optimization
Use of LED, dye and paints	High lipid yield	Cost intensive
	High biomass	Needs large scale trials
		Need further research to understand effect on lipid accumulation

3.1. Two-stage cultivation

To overcome the challenges of nutrient deprivation studies, alternate two-stage cultivation was suggested by many researchers to improve the lipid yield without affecting the biomass [60–62]. In two-stage cultivation, microalgae are initially grown under nutrient-sufficient conditions to obtain maximum biomass and thereafter the cultivation conditions are altered to trigger the accumulation of lipids. Mujtaba et al. [63] demonstrated the use of two-stage cultivation for improved lipid productivity in *Chlorella vulgaris*. In their study, *C. vulgaris* was grown under nutrient rich conditions in the initial stage of cultivation and thereafter the cultivation conditions were altered to provide nitrogen stress. They have noticed a lipid productivity of 71.1 $mgL^{-1}d^{-1}$ with two stage cultivation which was significantly higher than the control (31.5 $mgL^{-1}d^{-1}$). Similarly, Ratha et al. [64] have successfully employed two-stage cultivation approach to improve the lipid productivity in *Chlorella* and *Scenedesmus* sp. Alvarez-Diaz et al. [65] obtained an increase (36.5–45.5%) in lipid accumulation using two-stage cultivation of *Ankistrodesmus falcatus*. Yang et al. [66] reported, supplementation of high concentration of NaCl at late log phase enhances lipid productivity during two-stage cultivation of *Monoraphidium dybowskii* LB50. Despite of several economical benefits, the main limitation of the two stage cultivation is that most of these results are strain specific and its efficiency may vary from strain to strain. None of these studies have proved universal application of two-stage cultivation for all microalgal strain (Table 2).

3.2. Combined nutrients and abiotic stress

Recent reports have presented the potential of combining nutrients and abiotic stress factors to improve the lipid productivity in microalgae. To obtain maximum yields, it is imperative to have the knowledge of the synergistic effects of these factors as well as significance of each factor with regards to the lipid accumulation [27,67]. Breuer et al. [51] investigated the effect of light, pH, and temperature on TAG accretion under nitrogen deficient condition and found pH and temperature to be major influencing factors for TAG accumulation. The highest TAG content (40%) was obtained at pH 7 and 27.6 °C which was independent of light variation. Similarly, Cao et al. [27] observed high lipid accumulation in *Chlorella minutissima* as a result of combined influence of iron, sodium chloride and nitrogen. Ji et al. [30] studied the effect of variation in temperature, light intensity and photoperiod on *Desmodesmus* sp. and observed high biomass production at a combination of temperature: 30 °C, light intensity: 98 $\mu mol\ m^{-2}\ s^{-1}$ and photoperiod: 14:10 (L:D Light: Dark photoperiod). Similarly, Pan et al. [54] have subjected four thermotolerant *Desmodesmus* strains to nitrogen starvation under different temperature regimes. Two of the isolated strains showed lipid production of 50% with 75% of TAG content at 45 °C. A combination of low nitrogen and high iron concentration also reported to increase lipid productivity of up to 74.07 $mgL^{-1}d^{-1}$in *Ankistrodesmus falcatus* [11]. The major advantage of combined strategy is that in this approach, one factor may compensate the negative effect of the other. Moreover, this approach can easily be employed for large scale production of microalgal biodiesel (Table 2).

3.3. Phytohormones

Plant growth hormones (Auxins, Cytokinins) have been identified in microalgae and play important role in many metabolic pathways [68,69]. Phytohormones and there derivatives are reported to have a stimulating effect on microalgal growth (biomass) and metabolite production (i.e., carotenoid, lipid, carbohydrate and protein) [10,70]. These hormones have also been reported to stimulate growth under unfavorable conditions such as high salinity, drought and nutrient deficiency [71]. Park et al. [9] reported an increase in the growth of *Chlamydomonas reinhardtii* when auxins were supplemented to nitrogen limited medium. Similarly, diethyl amino ethyl hexanoate (DAH) and indole acetic acid (IAA) have been reported to enhance growth (1.9 fold and 2.5 fold respectively) and poly-unsaturated fatty acid content (56% and 59%, respectively) of *Scenedesmus obliquus* [10]. The addition of phytohormones, 24-epibrassinolide (0.1 μM) and gibberellic acid (100 μM) also facilitated a 3-fold increase in TAGs as well as 2-fold increase in biomass [70]. Jusoh et al. [72] observed a change in the fatty acid composition of *Chlorella vulgaris* after supplementation of growth media with IAA (100 μM) where they observed an improvement in the quantity of palmitic and stearic acids and a decrease in linoleic and linolenic acids. High expression of β-ketoacyl ACP synthase I (KAS I) gene, with an increase in the levels of saturated fatty acids (C16:0 and C18:0) further confirmed the effect phytohormones on the metabolic pathways of *C. vulgaris*.

Assessment of input costs of applying phytohormones however, is important for the economic viability of the microalgal biodiesel production using this strategy. These are generally required in very small amounts for inducing high biomass and lipid generation in microalgae. Phytohormones such as IAA and DAH are required in very small quantity, 1.75–2.15 mgL^{-1} respectively, to achieve optimum biomass production in microalgae [10]. Park et al. [9] analyzed the economic viability of phytohormones as compared, to normal synthetic medium and acetate. The biomass production cost whilst using phytohormones was found to be 0.014US$/g (GA3) which was much more economical than without phytohormone (0.024US$/g) and acetate (0.017US$/g). In another study by Salama et al. [10] the costs of biomass production induced by IAA was found to be 0.39 US$/g and DAH was 0.30 US$/g which were lower when compared with normal synthetic medium (0.78 US$/g). Application of phytohormones thus has great potential as sustainable, scalable and economical lipid enhancement strategies (Table 2) and should be further investigated for a better understanding of the mechanisms and its application to large scale cultivations.

3.4. Supplementation of Ethylene-diamine-tetra-acetic acid (EDTA)

Ethylene-diamine-tetra-acetic acid (EDTA), a lewis acid with six binding sites and reacts with metals and forms a stable ion structure, and has been extensively studied in phytoremediation [73,74]. EDTA has been widely employed in microalgal growth media for enhancing the solubility of metals, and thereby facilitating their uptake into the microalgal cells. Recently, Ren et al. [43] reported an increase in the total lipid content (28.2%) and lipid productivity (29.7%) in microalgae *Scenedesmus obliquus*with an increase in EDTA concentration (0–1 mgL^{-1}) thereby revealing its potential as a lipid enhancer.Their effectiveness can further be improved by coupling EDTA supplementation with nutrient and metal stress[75]. Due to the comparatively small amount of additional EDTA required and inexpensive nature of the chemical, this approach could be easily adopted at large scale.

3.5. Co-cultivation of microalgae–bacteria

In the natural environment, microalgae co-exist with many other microorganisms including bacteria which may have influence microalgal growth [76,77]. The possible symbiotic relationships between microalgae and bacteria cannot be ignored in large scale open cultivation systems. Therefore, selection and characterization of microalgal growth-promoting bacteria could offer a new strategy for improving industrial scale microalgal cultivation [78]. Do Nascimento et al. [79] investigated the effect of *Rhizobium sp*, on the biomass, lipid and chlorophyll content of the microalgae *Ankistrodesmus* sp. In their study, they found that co-culturing of *Rhizobium* sp. strain resulted in an increase in the chlorophyll (from 9 to 30 μgml^{-1}) and biomass (699 to 1999 mgL^{-1}) contents of the microalgal strain. The overall lipid productivity of the strain was increased to 112 $mgL^{-1}d^{-1}$ after optimization. Le Chevanton et al. [13] isolated 48 bacterial strains associated with marine microalgae and studied their effects on growth of Dunaliella sp. Among these, two bacterial strains *Alteromonas* sp. and *Muricauda* sp. enhanced the growth of *Dunaliella* sp. during co-culturing. These bacterial strains have the ability to demineralize organic nitrogen which can be used by microalgae under nitrogen limited conditions. Zhao et al. [80] studied the effect of microalgae–bacteria consortia on lipid production and CO_2 fixation when cultivated using landfill leachate. The maximum biomass concentration of 1.58 gL^{-1} and highest lipid productivity of 24.8 $mgL^{-1}d^{-1}$ were obtained in 10% leachate spike ratio. Despite the potential benefits observed thus far, there is scope for further research to determine the degree of microalgae–bacteria interactions and mechanisms (Table 2).

3.6. Co-cultivation of microalgae–yeast

Microalgae–fungus symbiotic relation is very common in ecosystems (such as Lichen). During symbiosis, microalgae are oxygen producers while yeast is CO_2 supplier for photosynthesis. Both these strains are also used in bioremediation as they can converts complex substrates (industrial waste etc) into simpler forms. Mixed culture of microalgae and yeast (fungus) can be utilized to produce lipids by utilizing waste as growth substrate. Cheirsilp et al. [76] cultivated *Chlorella vulgaris* and *Rhodotorula glutinis* using the effluent from sea food processing plant and obtained higher biomass and lipid production as compared to the individual culture of both strains. Yen et al. [81] studied the mutual effect of yeast *Rhodotorula glutinis* and *Scenedesmus obliquus* on biomass and lipid accumulation of both yeast and microalgae. Co-culture improved biomass upto 40–50% and lipid content about 60–70% respectively as compared to individual culture of both strains. During co-cultivation yeast cells produces various acidic compounds which serves as carbon source for microalgal growth. Utilization of wastes, high biomass production, high and lipid production and suitable fatty acid profile would make this co-cultivation technique economical and efficient for large scale biodiesel production.

3.7. Improving light conditions using LED, dyes and paints

Light intensity and wavelength has great influence on microalgal growth and lipid accumulation. Improving the light conditions optimum for achieving maximum growth and lipid yields has been investigated by several researchers. Use of light emitting diodes (LED), dyes and paints are the recent strategies for providing light of desired intensity and wavelength to microalgal culture. The chlorophyll pigments absorb mainly in the range of blue (450–475 nm) and red (630–675 nm) light [82]. The performance of photosystem I and II can be enhanced by providing the

light in the range of red and blue wavelengths respectively [83]. LED can be used to provide light with narrow band spectrum and has several advantages over the conventional fluorescent lamps such as low power consumption, longer lifetime, low heat generation, high conversion efficiency. The life time of LED are 941% and intensity (Wm^{-2}) 500% more compared to the conventional fluorescent lamps [59,83]. Atta et al. [59] investigated the effect of blue LED and conventional white lights on the lipid yields of *C. vulgaris*. They observed a maximum lipid yield of 23.5% *C. vulgaris* was 23.5% in 8 days cultivation time under blue LED conditions, while the lipid content was 20.9% in 10 days cultivation time under white light conditions. Photosynthetically active radiations (PAR) account for less than 15% of the solar radiation spectrum.

Fluorophores such as organic dyes and fluorescent paints can alter the photons with high energy to the low energy light wavelengths [84,85]. Seo et al. [84] found that a mixture of rhodamine 101 and 9, 10-diphenylanthracene dye solution improved the lipid productivity of *C. vulgaris* by 2.3 times compared to the cultivation without using dye. In their study, they have also confirmed that individually these dyes have different effect on biomass and lipid production. Seo et al. [85] studied the effect of yellow, red, blue and green florescent paint solutions on the growth and lipid accumulation of *Chlorella* sp. In their study they found that the different color solutions have different effect on biomass growth and lipid accumulation. They observed maximum biomass production (1.7 gL^{-1}) with red paint and maximum lipid content (30%) with blue paint solution. Use of LED and fluorophores for improvingmicroalgal growth have shown promising results, however, these strategies needs to be investigated further including their feasibility for large scale cultivation.

3.8. Chemical additives

3.8.1. Azide

Azide is known to induce oxidative stress in plants by inhibiting various metabolic pathways such as respiration, oxidative evolution of PS II and ATP-synthase. In recent investigations, azide stress is reported to induce lipid accumulation in microalgae. Zalogin and Pick [86] compared the effect of sodium azide and nitrogen stress on growth and lipid accumulation of *Chlorella desiccata.* Maximum lipid accumulation (60–70%) was obtained with the addition of 20 μM sodium azide stress with minor reduction in growth. They suggested azide treatment is better than nitrogen deprivation as high lipid can obtain with high biomass production. Mechanism of azide stress on lipid induction without compromising growth in microalgae was explained by Zalogin and Pick [87]. Sodium azide inhibits nitrogen assimilation by reducing activity of nitrogen reductase enzyme and results in high lipid accumulation. However, optimum azide concentration required to enhance lipid accumulation is strain specific, which might affect the economics of biodiesel production. More research is therefore required to know the exact mechanism behind lipid accumulation by azide treatment, economic feasibility analysis for large scale production and to establish a universal strategy for all microalgal strains.

3.8.2. Brefeldin A (BFA)

Brefeldin A is a chemical that induces stress in endoplasmic reticulum (ER) and interrupt transportation of newly synthesized molecules (protein and lipid) from ER to golgibody and thus accumulates lipids and proteins [88]. Brefeldin A is reported to induce lipid droplets in *Saccharomyces scerevisiae* [89]. Kim et al. [88] has reported Brefeldin A induced an increase of 34% lipid in microalgae, *Chlamydomonas reinhardtii* with concentration of 75 μgML^{-1}. They also observed up-regulation of ER stress marker gene and reduction of cytosolic lipid (betaine lipid diacylglyceryl N,N,N-trimethylhomoserine). These results proved that Brefeldin induces stress in ER of microalgae that causes the change in lipid composition and converts phospholipids and sterol into neutral lipids. This mechanism is independent of nitrogen stress induced lipid accumulation. Thus, this strategy can improve lipid accumulation without compromising biomass and lipid productivity. However, economic feasibility of utilizing this chemical and its toxic nature that can reduce biomass production needs further investigation before application of this method on commercial scale can be considered (Table 2).Thus further research is required to understand the effect BFA on lipid biosynthesis pathway.

3.8.3. Surfactants

Surfactants are widely used in household and industrial processes. These compounds denature the proteins in cell wall structure and alter its permeability. Polyoxyethylenesorbitanmonooleate (Tween-80) is non-ionic surfactant which is known to improve nutrient availability to cell by improving permeability of cell membrane. Taoka et al. [90] observed supplementation of 1% tween-80 in the medium lead to a 2 fold increase in biomass and 1.15 fold increase in lipid content of marine protist *Thraustochytrium aureum* ATCC 34304. This approach can be applied to microalgae for enhanced lipid accumulation. This approach can be applied individually or in combination with other nutrient stress. However, microalgae have shown varying sensitivity towards the surfactants and in higher concentrations it could be toxic for microalgal growth [91].

4. Molecular approaches for enhancing lipid accumulation and quality

Advancements in molecular biology in the recent past, has led to the speculation that if the genomes of oleaginous microalgae are appropriately modified, it is likely to have a greater impact on improving the economics of biofuel production [92–95]. Various molecular strategies have been studied to enhance the lipid yield of microalgae as well as improvement in its quality of the lipids. Some of these recent strategies include engineering of fatty acid synthesis pathway in microalgae towards more appropriate lipid profiles, secretion of lipids from cells to the media, engineering of the Kennedy pathway for over-expression of the major enzymes involved in the biosynthesis of TAG, increasing the availability of precursor molecules and inhibition of competing pathways of lipid biosynthesis and catabolism [96]. Adaptability of microalgal strains towards genetic manipulation is crucial for lipid enhancement studies. Suitability of a strain for genetic manipulation is determined by various factors such as; ease of application, transformation capabilities and availability of fully sequenced genomes [47].

4.1. Microalgal genome and tools for genetic transformations in microalgae

Most of the previous genetic modification studies on microalgae were based on *Chlamydomonas* genome as little information was available for microalgae [19,97]. However, at present whole genome sequence for many microalgaeincluding some of the biodiesel feedstock strains are available in public domain which includes *Chlorella vulgaris, Nannochloropsis, Phaeodactylum tricornutum, Coccomyxa sp*. C-169, *Micromonas CCMP* 1545 *Ostreococcuslucimarinus* CCE9901, *Ostreococcustaur, Volvox carteri,* and *Thalassiosira pseudonana* [19,97]. Genomic information of these sequences might facilities the scope of genetic improvement of strain by transformation. This information could open new paths for improved genetic engineering studies in microalgae as evidenced in recent literatures

Insertion offoreign DNA molecule/s into the host cell (chloroplast/mitochondria/nuclear genome) and maintaining its viability are the most important steps for a successful transformation. Several methods have been developed to transfer particulargenes of interest into the microalgae. The first successful transformation was achieved in *Chlamydomonasreihardtii* by using glass beads where the cell wall free *Chlamydomonasreihardtii*was agitated in the presence of DNA, glass beads and polyethylene glycol (PEG) [98]. This method is further successfully applied to transform genes in few other microalgal strains viz, *Amphidium*, *Sybiodium* [99]. However, requirement of cell wall deficient host strain is the main drawback of this method as most of the hyper lipid containing microalgal strains such as *Chlorella, Scenedesmus* etc. are reported to have thick and complicated cell wall structure [100,101]. A more applied electroporation technique was successfully employed by different authors for transformation studies in various microalgae viz., *Chlamydomonasreihardtii, Chlorella eliopdodeia, Chlorella vulgaris* [102], *Chlorella sp. MACC/C95* [103], *Phaeodactylum* [104], *Dunaliella salina* [105] and *Nannochloropsis oculata* [106]. This technique involves application of electric current to disrupt the lipid bilayers in the cell wall structure leading to an efficient molecular transport across the plasma membrane. Ahmad et al. [107] successfully used electroporation method to improve lipid accumulation *C. reinhardtii* (CC-125) where they have transformed diacylglycerol aceyltransferease (BnDGAT2) gene from *Brassica* in to *Chlamydomonas reinhardtii.* The engineered strain, *Chlamydomonas reinhardtii* showed higher lipid content (18.76%) than the wild strains (12.33%). Efficiency of the technique however depends on many factors including field strength, pulse length, medium composition, temperature and membrane characteristics as well as the concentration of DNA [108].

Among the different techniques developed, bombardment with micro-particles loaded with genetic material is the preferred method for chloroplast and nuclear genome transformation as it allows for the delivery of multiple copies of recombinant DNA through both the cellular and chloroplast membranes, escalating the chance for a successful mixing regime to occur [105]. This method can thus be applied directly to transform metabolic pathways regulated by chloroplast and nucleus such as fatty acid biosynthesis and TAG synthesis and is successfully applied for stable nuclear and chloroplast transformation of *C. reinhardtii*, *Chlorella sorokiniana, Chlorella ellipsoidea, Chlorella kessleri* and diatom *Phaeodactylum tricornutum* [104]. These microalgal strains are already known for their high lipid content which can be further enhance by using this transformation method. Bombardment is even effective for transformation of algal strains with complex cell wall structure such as *Gonium pectoral* [106] which makes this technique suitable for transformations related to lipid enhancement. In another study, Muto et al. [109] established a transformation system for high lipid producing microalgae *Fistulifera* sp. (61%) using microparticle bombardment methods.

Agrobacterium tumefaciens mediated transformation is the most widely used technique for plants [108,110] due its natural ability to transfer inter-kingdom DNA transfer. This method can be utilized to improve lipid content in microalgae by expressing exogenous genes related to lipid metabolism. Cheng et al. [110] transformed (gfp gene) *Schizochytrium* using *Agrobacterium tumefaciens* transformation method; the transformed strains have shown similar lipid and biomass content as wild strain.

Though there have been substantial developments with respect to the transformation studies in microalgae, the challenge being the approach is not uniform for all algal strains [111].

4.2. Genetic engineering

4.2.1. Chloroplast engineering

Microalgal chloroplast is an attractive platform for routine genetic manipulation as the genetic system is well suited to targeted insertion into the genome that results in high level expression of foreign genes [97,115]. In microalgae, this organelle is the site for major biosynthetic pathways including fatty acid biosynthesis, photosynthesis, xanthophylls cycle etc which can be targeted and manipulated to enhance lipid, biomass and pigments production [94]. Chloroplast transformation have been successfully done in several high lipid producing microalgal strains such as *Chlamydomonas reinhardtii, Haematococcus pluvialis* [116], *Dunaliella* sp. [106], and *Scenedesmus* sp. [112]. Genetic engineering of chloroplast have resulted in high lipid accumulation in transformed strain, however, decreased cell growth was reported in engineered strains [115,117–119]. Wobbe and Remacle [4] have observed improved photosynthetic efficiency by alteration in light-harvesting complexes (LHC) in *Chlamydomonas reinhardtii*using RNAi technology. In their study, the transformed stains showed reduction in the risk of photo inhibition thereby resulting in improved biomass yields.Lipid accumulation and biomass production both can be enhanced simultaneously by chloroplast engineering which will increase the overall volumetric lipid productivity for economical biodiesel production.

4.2.2. Nuclear engineering

Nucleus is the most attractive platform for genetic manipulations of microalgal strain to improve lipid accumulation and quality of biodiesel in microalgae. The whole genome analyses of microalgal strains have provided substantial evidence of presence of lipid biosynthesis genes in different cell organelles [120,121]. It is now known that the microalgal nuclear genome contains around 6% of total genes involved in lipid biosynthesis [121]. Most of these genes are related to TAG synthesis (DGAT), fatty acid chain termination, membrane lipid synthesis and therefore alteration of these genes could improve both quantity and quality of the microalgal lipids in engineered strains. Advancements in biotechnological tools, knowledge on suitable markers and promoters facilitated microalgal nuclear transformations to a greater extent. Nuclear transformations in microalgae have been successfully carried out in several high lipid accumulating microalgal strains such as *Phaeodactylum tricornutum*, *Nannochloropsis oceanica* CCMP1779 and *Fistulifera sp*. thus far [109,113,122].

Most success stories of microalgal nuclear transformations are based on single gene insertion. Recently, Noor-Mohammadi et al. [123] reported a new method for multi-gene expression in nuclear genome of *Chlamydomonas reinhardtii.* In their study, they have developed a new method for construction of multiple-gene pathway in *Saccharomyces cerevisiae* and integrated this to the nuclear genome of *Chlamydomonas reinhardtii.*Using this method, they were successful in co-expressing three reporter proteins (Ble, AphVIII, and GFP) concurrently in the nucleus of *C. reinhardtii*. The multi-gene expression studies has tremendous potential in GE of microalgae and can be applied to improve both quantity of TAG synthesis and quality of fatty acid synthesis simultaneously which will have greater impact on the lipid productivity and fuel properties of microalgal biodiesel. The above technique may also be utilized to express more functional genes of lipid synthesis pathway as well as biomass generation to improve lipid productivity (Table 3).

4.3. Expression analysis of genes involved in lipid biosynthesis

Lipid biosynthesis pathway in microalgae has been explained by several researchers [27,124,125]. Lipid biosynthesis is a multistep

reaction, catalyzed by an enzyme complex (acyl carrier protein, fatty acid synthase) [124]. It is important to understand the mechanism and behavior of these enzymes under different environmental conditions. Under favorable (optimum nutrients and cultivation parameters) conditions, microalgal growth is endorsed by increased translation and transcription processes which results in high growth rates and biomass production [19,126]. Under nutrient limited conditions the growth of microalgae is inhibited as most of the anabolic machinery is generally retarded. Fan et al. [127] investigated the effect of nutrient stress (phosphorus, iron, nitrogen) on the expression of various functional genes encoding the key enzymes involved in lipid synthesis of *Chlorella pyrenoidosa* (Table 4). Similarly, Wan et al. [128] investigated the effect of iron on lipid accumulation in *Chlorella sorokiniana*and observed that the expression of genes such as accD and rbcl was up regulated at higher concentrations of iron resulting in high lipid productivity (179 mgL^{-1}). Table 4 represents the effect of environmental conditions on the expression of functional genes related to lipid biosynthesis. These expression analyses of the key metabolic genes may give more insight into the actual mechanism of lipid accumulation in the microalgae in response to cultivation conditions [72,127]. Recently evolved molecular techniques such as transcriptome analysis, microarray analysis and full-length or EST (expressed sequence tag) transcript sequencing, can generate knowledge of how lipid biosynthetic genes are expressed under different stress conditionsand give insight into the key genes involved in triggering lipid accumulation [15,16,129]. The gene expression analysis therefore could assist us in improving the existing stress strategies and also to develop novel strategies for better lipid yields in microalgae. Gene expression analysis also reveals the key functional genes involved in lipid biosynthesis and this knowledge could be exploited for the improved applicability of molecular techniques such as metabolic and genetic engineering.

Table 3
Molecular tools and techniques for transformation of microalgae.

Microalgae	Tools and technique	Expressed gene	Reference
Chlamydomonas	Bombardment	atpB	[108]
Chlamydomonas Sp.	Agrobacterium		[108]
Scenedesmus obliquus	Agrobacterium	β-glucuronidase (uidA), green fluorescent protein (gfp) hygromycin phosphotransferase (hpt)	[112]
Schizochytrium	Agrobacterium mediated	green fluorescent protein (gfp)	[110]
Scenedesmus obliquus	Electroporation	green fluorescent protein (gfp)	[112]
Nannochloropsis sp.	Electroporation	GUS	[113]
Phaeodactylum Tricornutum	Biolistic transformation	Acyl-ACP thioesterases	[114]

4.4. Metabolic engineering

4.4.1. Overexpression of enzymes of lipid biosynthesis

Lipid metabolism involves (Fatty acid and TAG biosynthesis) several chemical conversion steps catalyzed by various enzymes. Acetyl-CoA (ACCase) carboxylase is the key enzyme involved in lipid biosynthesises and the most exploited enzyme for lipid enhancement studies both in plants and microalgae. Overexpression of ACCase is one of the successfully implemented strategies for improved fatty acid synthesis in plants and microorganisms which can be applied for strain improvement in microalgae. It was established by various authors that with the over expression of ACCase, enhanced availability of malonyl CoA is ensured in the chloroplast for the onset of increased fatty acid biosynthesis [130–132]. Davis et al. [133] observed 6 times increase in the rate of fatty acid synthesis while co-expressing ACCase (encoded by accA, accB, accC, accD) and thioesterase I (encoded by the tesA gene) in *E. coli* strain. These results confirmed that ACCase catalyzes the committing step in lipid biosynthesis which is undeniably the rate-limiting step for the fatty acid biosynthesis in *E. coli* [133]. The overexpression of ACCase gene has improved the lipid accumulation in *Brassica napus* to 5% [134].In microalgae, under certain nutrient limited cultivation conditions, the expression of ACCase tend to increase [127]. However, it was also reported that the overexpression of ACCase gene does not reflected into higher lipid yields; such as overexpression of acetyl-CoA carboxylase (ACCase) from *Cyclotellacryptica* in

Table 4
Expression analysis of the functional genes related to lipid synthesis.

Microalgae	Cultivation Condition	Targeted enzymes	Selected genes	Effect on relative gene expression	Lipid content/fatty acid content/ lipid productivity	Reference
Chlorella pyrenoidosa	Nitrogen deficient	Rubisco	Rbcl	4 times decrease		[127]
Chlorella pyrenoidosa	Nitrogen deficient	PEPC	pepc g6883	Slight increase	50.4 % 34.42 mgL^{-1} d^{-1}	
Chlorella pyrenoidosa	Nitrogen deficient	PEPC	pepc g8086	Slight increase		
Chlorella pyrenoidosa	Nitrogen deficient	Malic	me g6562	4.04 folds increase		
Chlorella pyrenoidosa	Nitrogen deficient	ACCase	accA	3.5 folds increase		
Chlorella pyrenoidosa	Nitrogen deficient	ACCase	accD	38.9 folds increase		
Chlorella pyrenoidosa	Nitrogen deficient	DGAT	dgat 3280	24.5 folds		
Chlorella pyrenoidosa	Phosphorus	Malic	Me 6562	8.43	19.60 mgL^{-1} d^{-1}	[127]
Chlorella pyrenoidosa	Heterotrophic	PEPC	pepc g6883	3.1 times increase		[127]
Chlorella pyrenoidosa	Continuous Light	ACCase	accD accA	2.5 folds increase	34.7%	[127]
Chlorella pyrenoidosa	Heterotrophy to photo trophy	DGTA	dgta g7566	47.6 folds increase		[127]
Chlorella sorokiniana	Mixotrophic	ACCase	accD	Increase in expression level	51%	[128]
Chlamydomonas reinhardtii	Nitrogen stress	DGTA	DGTT1	Increase in expression level	Increases in saturated fatty acids	[26]

Cyclotellacryptica and *Naviculasaprophila* did not show any effect on lipid accumulation [135]. This may be due to the fact that the ACCase activity isnotrate limiting step in the lipid biosynthesis pathway.

Similarly, the expression of Acyl-Co: diacylglycerol acyltransferase (DGAT), a key enzyme in the Kennedy pathway (TAG biosynthesis), has been exploited for its expression studies in many plants like *Brassica napaus*, *Arabidopsis thalia*na and *Nicotiana tabacum* [131,136]. Few other enzymes that are targeted for enhanced fatty acid and TAG synthesis in plants and microalgae includes fatty acid synthatase (FAS), lysophosphatidate acyltransferase (LPAT), acetyl-CoA synthase (ACS), malic enzyme (ME) and ATP: citrate lyase (ACL) [95,137]. The overexpression of malic enzyme for enhanced lipid accumulation was studied by Xue et al. [138] and the transgenic microalgae *Phaeodactylum tricornutum* showed increased lipid content (2.5 fold) and enzymatic activity without affecting the cell growth.

Hsieh et al. [139] over expressed the functional gene Acyl-CoA: glycerol-3-phosphate aceyltransferease (GPAT), acyl-CoA:lysophosphatidicaceyltransferease (LPAAT), acyl-coA:diacylglycerolaceyltransferease (DGAT) in *C. Minutissima*UTEX 2219 by using multiple gene expression system. The highest lipid content (2-fold) was found in transgenic microalgal strain. The multiple-gene expression can be effectively utilized to maximize lipid accumulation as well as biomass generation by targeting respective genes, for sustainable biodiesel production.

4.4.2. Blocking competitive pathways

Knocking down the competitive pathways such as carbohydrate and lipid catabolism is an effective approach to improve lipid accumulation in microalgae [132,140–142]. Carbohydrate metabolism is most important pathway for accumulation and storageof carbon in the form of starch employed by many microalgae [143,144]. Suppressing the starch metabolism may therefore will results in carbon flow towards lipid biosynthesis. Breuer et al. [145] reported an increase in TAG accumulation of upto 51% in the starchless mutant of *Scenedesmus obliquus* (0.217 $gmol^{-1}$) as compared to wild type (0.144 $gmol^{-1}$) under similar cultivation conditions. They observed no difference in photosynthetic behaviour of both the mutants and the wild type and concluded that the mutation only affected the division of available carbon between metabolic pathways and not photosynthetic performance. Astarchless mutant strain of Chlamydomonas showed a 10 fold increase in TAG accumulation as compared to its wild type which was achieved by deactivation of ADP-glucose pyrophosphorylase that catalyses the committing step in the starch metabolism [146]. These findings supported the potential of lipid enhancement strategy by redirecting carbon from starch synthesis to lipid accumulation by knocking down the key genes in lipid biosynthesis pathway. However, knock down of important genes involved in starch synthesis may results in decreased growth rate which would result in low biomass production and ultimately lower lipid productivity.

Suppression of lipid catabolism is one of thestrategies employed to increase the lipid accumulation in microalgae. A mutant strain of *Thalassiosira pseudonana* has shown 3.5 times higher lipid content after alteration in the lipid catabolism by knocking down the regulation of multifunctional enzymes lipase/phospholipase/acyl transferase [15] (Table 5).These genes in microalgae could also be targeted for manipulation for high lipid accumulation without compromising growth.

4.4.3. Altering fatty acid chain length for improved lipid quality

The microalgal lipidscomposition dictates the properties of subsequently produced biodiesel. Thus, it is important to develop strategies to improve the quality of microalgal lipids so that biodiesel can meet the standard specifications [147,148]. The most suitable fatty acids for biodiesel production are saturated and mono-unsaturated fatty acids preferably with carbon length 12:0, 14:0, 16:0, 16:1, 18:0 and 18:1. Acyl-ACP thioesterases regulates the fatty acid chain length by releasing the fatty acid chain from fatty acid synthase [149]. Thioesterasesfrom different organisms are specific for particular chain lengths of fatty acids as per the metabolic needs of that organism. Transformation of thioesterases genes from other organisms into microalgae, could significantly improve the fatty acid composition to obtain the desired fuel

Table 5
Molecular approaches to enhance lipid accumulation in microalgae.

Molecular approach	Microalgae	Targeted trait /pathways	Result	Reference
Chloroplast engineering (RNAi technology)	Chlamydomonas reinhardtii	Light harvesting complex	Increase in biomass productivity	[4]
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	[151]
Malic enzyme (ME)	*Phaeodactylum tricornutum*	Pyruvate metabolism	Increase in expression and enzyme activity 2.5 fold increase in total lipid content	[138]
Glycerol-3-phosphate aceyl transferase (GPAT), Lyso phosphatidic aceyl transferase (LPAAT), Diacyl glycerol aceyl transferase (DGAT)	*Chlorella minutissima*	Kennedy pathway	2-fold increase in lipid content	[139]
Blocking competitive pathways				
Starch less mutant	*Scenedesmus obliquus*	Starch metabolism	51% increase in mutant strain as compared to wild type	[145]
ADP-glucose pyrophosphorylase	*Chlamydomonas*	Starch metabolism	10 folds increase in lipid content	[146]
Knockdown of enzymes lipase/phospholipase/acyl transferase	*Thalassiosira pseudonana*	Lipid catabolism	3.5 fold increase in lipid content	[15]
Alteration in fatty acid chain length				
Acyl-acp thioesterases	*Phaeodactylum tricornutum*	Fatty acid chain termination (TE)	Increase quantity of in the Short chain length Lauric and Myristic acid fatty acid	[114]
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	[150]

properties. Radakovits et al. [114] transformed two shorter chain length fatty acid acyl-ACP thioesterases from *Cinnamomum camphora* and *Umbellularia californic* into *Phaeodactylum tricornutum*. The fatty acid profile of transgenic *Phaeodactylum tricornutum* showed an increase in the percentage composition of lauric (C12:0) and myristic (C14:0) acids (Table 5). This molecular strategy for improving the microalgal lipid quality has significant potential for the production of microalgal biodiesel with the required specifications.

4.4.4. Lipid secretion

Harvesting and extraction of biomass and lipids are challenging, energy and cost intensive steps in the microalgal biodiesel synthesis process. Genetic modification of microalgae which enables them to secrete the lipids into the medium can positively influence the economics of biodiesel production. Liua et al. [150] genetically modified the cyanobacteria *Synechocystis* sp. PCC6803 wild type (SD100) to produce and secrete fatty acids by transforming it with acyl-acyl carrier protein thioeseterase gene. In the wild type *Synechocystis* sp. (SD100), fatty acid secretion was limited to $1.8 \pm 0.06\ mgL^{-1}$ whilst the secretion of $197 \pm 14\ mgL^{-1}$ was achieved in the transgenic *Synechocystis* sp. (SD277). Various metabolic pathways have been altered such as deletion of competitive genes to enhance fatty acid synthesis and deletion of genes related to formation of surface protein to improve secretion of fatty acid (Table 5). This strategy could not only make the overall process more energy efficient but also avoids the use of toxic chemicals for harvesting and minimize the usage of organic solvents used in the extraction process. Despite of several advantages, low biomass production is main drawback of this technique. Deletion of genes related to surface protein results in cell fragility and reduced CO_2 aeration that affects photosynthesis of the cell. For industrial scale production,this technique needs further modification for better fatty acid yield.

4.5. Environmental risk and challenges of molecular approach

Genetic engineering approaches in microalgae have tremendous scope in enhancing lipid production; however, there may be major limitations during the scale up. The major risk related to genetically modified (GM) microalgae can be divided into two sections. The first section is related to the human health, while the other is related to the environmental impacts. Biofuels from microalgae are commodity products and thus require large scale cultivation; preferablyopen cultivation systems are adopted to reduce the production cost. Therefore, unlike other industrially important GM organisms which are cultivated in closed systems for high value products, the risk associated with GM microalgae could directly impact human health and environment. The escape or release of GM microalgae to the environment is of foremost concern as microalgae are primary producers in most of the ecological systems. In open cultivation systems, GM microalgae can be released to the environment by individual cell escape via wind, water, birds, animals, natural calamities or accidents. Specific impacts could be GM species dominance over native microalgae, change in microalgal diversity in the ecosystem, distorted food chains and biogeochemical cycles, production of toxins and allergens, and horizontal GM gene transfer to wild species [47,152]. Some of the microalgae are consumed as human food supplement, while some are used as main feed by fish in natural environment [153]. Release of GM microalgae can therefore cause harm to human health as well as the environment; however, there is no substantial data available on the magnitude of the risks and their impacts [154]. GM microalgae related studies are still in its early stages for its commercial release into outdoor cultivation systemsuggested that government regulations on GM microalgae are inevitable considering theirpossible negative impact on human health, environment and economy. Rigorous monitoring and risk assessment studies are therefore required to design the regulations for GM microalgae. Some non GM genetic strategies used for enhancing lipid yields does not necessarily fall under the definition set by governments for GM microalgae but could have ecological risks. This warrants need for athorough assessment of such type of genetic strategies for associated risks [155].

Another major challenge is the suitability of transgenic microalgae for large scale cultivation. Upon exposure to large-scale cultivation conditions, the strains experience situations that are more diverse from the controlled lab conditions, which subsequently affect its transgenic property. Consequently, productivity in outdoor cultivation, never reaches that of the optimized laboratory conditions. The stability of the mutant strain can also be a challenging during commercial level cultivationas the mutants are prone to reversion [106,154]. Though the use of algal transgenic for enhanced lipid production is beginning to be realistic due to the advancements in molecular techniques, there are major environmental risks that are need to be addressed prior to the use of these transgenicfor largescale cultivation.

5. Impacts of microalgal lipids on biodiesel properties

Biodiesel properties are principally dictatedby the lipid content of the feedstock oil. For the commercial utilization of biodiesel, it has to satisfy the quality specifications set by agencies like American Society for Testing and Materials (ASTM) D 6751 (USA); DIN 51606 (Germany); European Organization (EN 14214) and India (IS 15607) [1]. Biodiesel is mainly characterized for the ester content, physical properties such as density, viscosity and fuel properties flash point, oxidation stability, cetane number, calorific value and others. There is very little information available on the biodiesel derived from the microalgal lipids. Microalgal lipids constituents include triglycerides, free fatty acids, hydrocarbons, sterols, wax, sterol esters, alcohols etc [3]. Other components than triglyceride and free fatty acids not only interfere with the biodiesel conversion process but also have influence on the fuel properties. Hydrocarbons present in the microalgal lipids can lead to biodiesel with high cetane number, calorific value and low viscosity [1]. Balance between the saturated and unsaturated fatty acids in total lipids is important for the oxidation stability and cold flow properties of the biodiesel. Both these properties has an inverse relation, high saturated fatty acids favors the oxidation stability, while high unsaturated fatty acids are considered good for the excellent cold flow properties of the biodiesel fuel [156]. Song et al. [157] investigated 10 microalgal strains for their fatty acid composition and biodiesel properties. Their study indicates that fuel properties like kinematic viscosity, specific gravity, cetane number and higher heating value of all the 10 microalgal strains were almost complying with the ASTM D6751 and EN 14214 specifications. In their study they found that with the increase in the unsaturated fatty acids, cold flow properties show the improvement however, the cetane number and oxidation stability decreases. Microalgae grown with the various lipid enhancement strategies have shown biodiesel properties complying with the standards. Baky et al. [41] studied the CO_2 and iron supplementation for lipid enhancement in *Scenedesmus obliquus* and investigated the biodiesel properties of the final biodiesel. In their study they reported that 12% CO_2 resulted in saturated fatty acid composition of 59.01% and unsaturated fatty acid composition of 40.99% while the degree of unsaturation was 0.52. They found that the density (kgm^3, at 15 °C), viscosity (mm^2s^{-1}, at 40 °C), acid value (mg KOH g^{-1}) and iodine value (mg I_2 100 g^{-1}) for the biodiesel obtained from *Scenedesmus obliquus* grown with 12% CO_2 were 0.894, 4.56, 0.41 and 67 respectively. With iron

supplementation of 20 mg L^{-1}, the saturated fatty acid composition was 57.17% and unsaturated fatty acid composition was 42.56% while degree of unsaturation was 0.56. The density, viscosity, acid value and iodine value were 0.895, 4.53, 0.4 and 69 respectively of biodiesel obtained from *Scenedesmus obliquus* grown with 20 mg L^{-1} iron supplementation. In both the cases the biodiesel properties were comparable to the properties of the diesel fuel and also met the specifications set by ASTM (ASTM D6751) (Table 6). Under the stress conditions that are mainly applied for lipid enhancement in microalgae, unsaturated fatty acids tends to undergo oxidative cleavage, which results in higher saturated fatty acids in the microalgal lipids. Higher saturated fatty acids give oxidative stability, high heating values and cetane number. In a study by Singh et al. [11] of using combined stress condition of nutrients (N, P and Fe) for enhancing lipid accumulation in *A. falcatus* the composition of unsaturated fatty acids was 76.2% without any stress condition, and was decreased to 67.04% under stress conditions. They also observed increase in saturated fatty acid composition (32.96%) under stress conditions as compared to unstressed condition (23.8%).

Microalgal lipids have shown C12–C18 fatty acids as the major contributing fatty acids, these fatty acids are considered suitable for the biodiesel synthesis (Table 7). C12–C18 fatty acids have the viscosity in the range of 2.43–5.85 mm^2s^{-1}, and thus the resulting biodiesel also has lower viscosity values and satisfy the ASTM D6751 specified range of 3.5–5 mm^2s^{-1}. The calorific value of biodiesel from *chlorella protothecoides* was 41 MJ kg^{-1} [158]and 40 MJ $kg^{-1}$for the biodiesel from fresh water microalgae [159]. The calorific value of microalgal biodiesel is comparable to that of thediesel fuel (40–45 MJ kg^{-1}) [158]. Overall microalgal biodiesel has shown close compliance with most of the international specifications, which is possible because of suitable lipid profile of microalgae. However, a very few studies have reported the properties of microalgal biodiesel, indicating need of further exploration of this area. While applying various lipid enhancement strategies it becomes imperative to investigate the quality of the lipids so that final product i.e. biodiesel meets the specifications of standards.

6. Conclusion and future perspective

This manuscript distinguishes itself from other reviews in the fields since it encompasses the most recent advances in lipid enhancement strategies including molecular approach. To cater the need of renewable transportation fuels colossal amount of biodiesel is required. Economical production of biodiesel from microalgae can be achieved by ensuring high lipid accumulation in microalgal cells. Conventional approaches such as altering nutrient regime and cultivation conditions are either expensive or associated with overall low lipid productivity. To address these challenges, several novel approaches have been investigated in the

Table 6
Fuel properties of microalgal biodiesel.

Biodiesel characteristics	Cetane number	Calorific value	Density	Methyl ester content	Linolenic acid methyl ester content	Acid value	Iodine number	Cold filter plugging point (CFPP)	oxidative stability	Sulfur	Ref
Units	–	MJ kg^{-1}	kg m^{-3}	%	%	mgKOH g^{-1}	g100 g^{-1}	°C	h	wt%	–
ASTM 6751	min 47	–	860–900	–	–	max 0.5	-	–	min 3	max 0.05	–
EN 14214	min 51	–	860–900	min 96.5	max 12	max 0.5	max 120	–	min 6	–	–
Chlorella protothecoides	–	41	864	–	–	0.374	–	−11	–	–	[160]
Scenedesmus obliquus	51.77	37.67	877	90.81	11.17	0.42	98.68	4.9	3.54	< 0.001	[161]
Nannochloropsis sp.	–	–	854	92.2	–	0.46	–	–	1.53	0.06	[162]
Spirulina platensis	70	45.63	863	–	–	0.75	102	–	–	nil	[163]
Chlorella protothecoides	–	–	882	97.7	–	0.29	112.2	−13	4.52	–	[162]

Table 7
Lipid content and fatty acid composition of different microalgae.

Microalgal strain	Lipid content (%)	Fatty acid composition (%)										Ref.
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA	
Chlorella vulgaris	17.3	–	14.55	1.18	10.51	23.62	13.8	32.1	25.06	24.8	45.9	[164]
***Scenedesmus* sp.**	30–36	–	18.42	2.31	3.43	49.64	11.30	8.26	21.85	51.95	22.82	[162]
***Chlorella* sp., BUM11008**	31.2	0.3	0.41	2.41	25.94	6.85	16.17	0.99	68.21	12.03	19.76	[31]
Dunaliella sp.	22	–	9.19	0.8	4.27	22.51	3.84	44.31	13.47	24.74	48.15	[164]
***Nannochloropsis* sp.**	–	5.37	28.83	32.93	0.98	21.16	2.24	–	35.18	54.09	8.57	[162]
Chlamydomonas reinhardtii	18.9	–	23.77	1.94	4.41	19.73	6.58	25.49	18.18	22.88	32.07	[164]
Dianoflagellate	--	6.01	16.65	3.35	–	2.10	–	–	25.31	5.45	66.14	[162]
***Chlorococcum* sp**	7.1	–	19	4	3	63	4	–	–	–	–	[165]
Monoraphidium sp. FXY-10	56.80	–	22.6	–	1.2	–	4.2	42.2	23.8	–	68	[166]
Neochloris oleoabundans	10–15	–	23–30	0.6–3.5	0.9–11	30–43	18–23	5–12	–	–	–	[167]
Selenastrum capricornutum	27.08	–	45.62	5.96	–	4.27	11.75	–	≈ 48	≈ 10	≈ 17	[157]
Phaeodactylum tricornutum	61.43	11.68	0.13	22.34	1.49	7.42	0.81	0.25	≈ 15	≈ 30	≈ 42	[157]
Isochrysis sphacrica	24.28	28.17	39.39	–	–	22.26	–	–	≈ 67	≈ 21	–	[157]
***Chlorella* sp.**	12	2.12	16.36	6.09	1.22	33.69	11.96	12.74	19.7	39.9	37.47	[168]
***Nannochloris* sp.**	21	2.03	25.28	2.36	0.98	5.83	19.65	23.24	28.29	8.19	49.5	[168]

recent past. Strategies like combined nutrient stress and addition of EDTA can be efficiently applied to commercial scale microalgal cultivation system. The lipid enhancement strategies such as addition of phytohormones and chemical additives, co-cultivation with other microorganisms can be effectively coupled with utilization of wastewater as a growth substrate for reducing the biomass generation cost. These novel approaches have shown promising results for successfully enhancing biomass and lipid productivity. However, most of these reports are based on laboratory scale investigations and are yet to be proven for universal applicability for all microalgal strains. Large scale trials and economic feasibility studies of these techniques are still required to validate their application. Molecular approaches have shown potential to enhance lipid accumulation, however environmental risk and long term viability are still considered as limitations. Recent developments in molecular approaches are able to not only induce high lipid accumulation but also achieve the desired quality for standard biodiesel production. Genetically modified strains that could secrete lipids directly into the medium can greatly reduce the harvesting and extraction cost. The advancements and novel approaches for lipid enhancement in microalgae are therefore certainly moving towards the economical and sustainable biodiesel production.

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References

[1] Singh B, Guldhe A, Rawat I, Bux F. Towards a sustainable approach for development of biodiesel from plant and microalgae. Renew Sust Energ Rev 2014;29:216–45.
[2] Pulz O, Gross W. Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 2004;65:635–48.
[3] Sharma YC, Singh B, Korstad J. A critical review on recent methods used for economically viable and eco-friendly development of microalgae as a potential feedstock for synthesis of biodiesel. Green Chem 2011;13:2993–3006.
[4] Wobbe L, Remacle C. Improving the sunlight-to-biomass conversion efficiency in microalgal biofactories. J Biotechnol 2014.
[5] Singh B, Guldhe A, Singh P, Singh A, Rawat I, Bux F. Sustainable production of biofuels from microalgae using a biorefinary approach. In: Kaushik G, editor. Applied Environmental Biotechnology. India: Present Scenario and Future Trends: Springer; 2015. p. 115–28.
[6] Klok AJ, Martens DE, Wijffels RH, Lamers PP. Simultaneous growth and neutral lipid accumulation in microalgae. Bioresour Technol 2013;134:233–43.
[7] Jiang Y, Yoshida T, Quigg A. Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. Plant Physiol Biochem 2012;54:70–7.
[8] Li Y, Fei X, Deng X. Novel molecular insights into nitrogen starvation-induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*. Biomass Bioenerg 2012;42:199–211.
[9] Park WK, Yoo G, Moon M, Kim CW, Choi YE, Yang JW. Phytohormone supplementation significantly increases growth of *Chlamydomonas reinhardtii* cultivated for biodiesel production. Appl Biochem Biotechnol 2013;171:1128–42.
[10] Salama S, Kabra AN, Ji MK, Kim JR, Min B, Jeon BH. Enhancement of microalgae growth and fatty acid content under the influence of phytohormones. Bioresour Technol 2014;172:97–103.
[11] Singh P, Guldhe A, Kumari S, Rawat I, Bux F. Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae *Ankistrodesmus falcatus KJ671624* using response surface methodology. Biochem Eng J 2015;94:22–9.
[12] Vila M, Diaz-Santos E, de la Vega M, Rodriguez H, Vargas A, Leon R. Promoter trapping in microalgae using the antibiotic paromomycin as selective agent. Marine Drugs 2012;10:2749–65.
[13] Le Chevanton M, Garnier M, Bougaran G, Schreiber N, Lukomska E, Bérard JB, et al. Screening and selection of growth-promoting bacteria for *Dunaliella* cultures. Algal Res 2013;2:212–22.
[14] Larkum AW, Ross IL, Kruse O, Hankamer B. Selection, breeding and engineering of microalgae for bioenergy and biofuel production. Trends Biotechnol 2012;30:198–205.
[15] Trentacoste EM, Shrestha RP, Smith SR, Gle C, Hartmann AC, Hildebrand M, et al. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proc Natl Acad Sci USA 2013;110:19748–53.
[16] Tabatabaei M, Tohidfar M, Jouzani GS, Safarnejad M, Pazouki M. Biodiesel production from genetically engineered microalgae: future of bioenergy in Iran. Renew Sustain Energ Rev 2011;15:1918–27.
[17] Adams C, Godfrey V, Wahlen B, Seefeldt L, Bugbee B. Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous green microalgae. Bioresour Technol 2013;131:188–94.
[18] Zhang YM, Chen H, He CL, Wang Q. Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3. PloS One 2013;8:69225.
[19] Merchant SS, Kropat J, Liu B, Shaw J, TAG Warakanont J. You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. Curr Opin Biotech 2012;23:352–63.
[20] Sharma KK, Schuhmann H, Schenk PM. High lipid induction in microalgae for biodiesel production. Energies 2012;5:1532–53.
[21] Zhang C, Zhang Y, Zhuang B, Zhou X. Strategic enhancement of algal biomass, nutrient uptake and lipid through statistical optimization of nutrient supplementation in coupling *Scenedesmus obliquus*-like microalgae cultivation and municipal wastewater treatment. Bioresour Technol 2014;171:71–9.
[22] Lamers PP, Janssen M, De Vos RC, Bino RJ, Wijffels RH. Carotenoid and fatty acid metabolism in nitrogen-starved *Dunaliella salina*, a unicellular green microalga. J Biotechnol 2012;162:21–7.
[23] Stehfest K, Toepel J, Wilhelm C. The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant Physiol Biochem 2005;43:717–26.
[24] Yu X, Chen L, Zhang W. Chemicals to enhance microalgal growth and accumulation of high-value bioproducts. Front Microbiol 2015;6:56.
[25] Xin L, Hu HY, Ke G, Sun YX. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour Technol 2010;101:5494–500.
[26] Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, et al. Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. Phytochem 2012;75:50–9.
[27] Cao J, Yuan H, Li B, Yang J. Significance evaluation of the effects of environmental factors on the lipid accumulation of *Chlorella minutissima* UTEX 2341 under low-nutrition heterotrophic condition. Bioresour Technol 2014;152:177–84.
[28] Amaro HM, Guedes AC, Malcata FX. Advances and perspectives in using microalgae to produce biodiesel. Appl Energ 2011;88:3402–10.
[29] Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process 2009;48:1146–51.
[30] Ji F, Hao R, Liu Y, Li G, Zhou Y, Dong R. Isolation of a novel microalgae strain *Desmodesmus* sp. and optimization of environmental factors for its biomass production. Bioresour Technol 2013;148:249–54.
[31] Praveenkumar R, Shameera K, Mahalakshmi G, Akbarsha MA, Thajuddin N. Influence of nutrient deprivations on lipid accumulation in a dominant indigenous microalga *Chlorella* sp., BUM11008: evaluation for biodiesel production. Biomass Bioenerg 2012;37:60–6.
[32] Tao L, Linglin W, Aifen L, Chengwu Z. Responses in growth, lipid accumulation, and fatty acid composition of four oleaginous microalgae to different nitrogen sources and concentrations. Chin J Oceanol Limnol 2013;31:1306–14.
[33] Gao Y, Yang M, Wang C. Nutrient deprivation enhances lipid content in marine microalgae. Bioresour Technol 2013;147:484–91.
[34] Lin Q, Lin J. Effects of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga. Bioresour Technol 2011;102:1615–21.
[35] Qi H, Wang J, Wang Z. A comparative study of maximal quantum yield of photosystem II to determine nitrogen and phosphorus limitation on two marine algae. J Sea Res 2013;80:1–11.
[36] Liang K, Zhang Q, Gu M, Cong W. Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella sp.* J Appl Phycol 2012;25:311–8.
[37] Chu FF, Chu PN, Shen XF, Lam PK, Zeng RJ. Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. Bioresour Technol 2014;152:241–6.
[38] Zhang C. Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. Protein Cell 2014;5:750–60.
[39] Yeesang C, Cheirsilp B. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresour Technol 2011;102:3034–40.
[40] Liu ZY, Wang GC, Zhou BC. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour Technol 2008;99:4717–22.

[41] Baky Hhae El-Baroty GS, Bouaid A, Martinez M, Aracil J. Enhancement of lipid accumulation in *Scenedesmus obliquus* by Optimizing CO2 and Fe3+ levels for biodiesel production. Bioresour Technol 2012;119:429–32.

[42] Sirin S, Clavero E, Salvado J. Potential pre-concentration methods for *Nannochloropsis gaditana* and a comparative study of pre-concentrated sample properties. Bioresour Technol 2013;132:293–304.

[43] Ren Hy Liu Bf, Kong F, Zhao L, Gj Xie, Ren Nq. Enhanced lipid accumulation of green microalga *Scenedesmus* sp. by metal ions and EDTA addition. Bioresour Technol 2014;169:763.

[44] Plank DW, Gengenbach BG, Gronwald JW. Effect of iron on activity of soybean multi-subunit acetyl-coenzyme A carboxylase. Physiol Planta 2001;112:183–94.

[45] Gorain PC, Bagchi SK, Mallick N. Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. Environ Technol 2013;34:1887–94.

[46] Huang L, Xu J, Li T, Wang L, Deng T, Yu X. Effects of additional Mg^{2+} on the growth, lipid production, and fatty acid composition of *Monoraphidium* sp. FXY-10 under different culture conditions. Ann Microbiol 2013;64:1247–56.

[47] Ho SH, Ye X, Hasunuma T, Chang JS, Kondo A. Perspectives on engineering strategies for improving biofuel production from microalgae – a critical review. Biotechnol Adv 2014.

[48] Rao AR, Dayananda C, Sarada R, Shamala TR, Ravishankar GA. Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. Bioresour Technol 2007;98:560–4.

[49] Rawat I, Ranjith Kumar R, Mutanda T, Bux F. Biodiesel from microalgae: a critical evaluation from laboratory to large scale production. Appl Energ 2013;103:444–67.

[50] Wahidin S, Idris A, Shaleh SRM. The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp. Bioresour Technol 2013;129:7–11.

[51] Breuer G, Lamers PP, Martens DE, Draaisma RB, Wijffels RH. Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*. Bioresour Technol 2013;143:1–9.

[52] Jiang L, Luo S, Fan X, Yang Z, Guo R. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2. Appl Energ 2011;88:3336–41.

[53] Roleda MY, Slocombe SP, Leakey RJ, Day JG, Bell EM, Stanley MS. Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy. Bioresour Technol 2013;129:439–49.

[54] Pan YY, Wang ST, Chuang LT, Chang YW, Chen CN. Isolation of thermotolerant and high lipid content green microalgae: oil accumulation is predominantly controlled by photosystem efficiency during stress treatments in *Desmodesmus*. Bioresour Technol 2011;102:10510–7.

[55] Takagi M. Karseno, Yoshida T. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J Biosci Bioeng 2006;101:223–6.

[56] Christenson L, Sims R. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotech Adv 2011;29:686–702.

[57] Li T, Wan L, Li A, Zhang C. Responses in growth, lipid accumulation, and fatty acid composition of four oleaginous microalgae to different nitrogen sources and concentrations. Chin J Ocean Lim 2013;31:1306–14.

[58] Chu FF, Chu PN, Cai PJ, Li WW, Lam PK, Zeng RJ. Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency. Bioresour Technol 2013;134:341–6.

[59] Atta M, Idris A, Bukhari A, Wahidin S. Intensity of blue LED light: a potential stimulus for biomass and lipid content in fresh water microalgae *Chlorella vulgaris*. Bioresour Technol 2013;148:373–8.

[60] Doan YTT, Obbard JP. Two-stage cultivation of a *Nannochloropsis* mutant for biodiesel feedstock. J Appl Phycol 2014.

[61] Farooq W, Lee YC, Ryu BG, Kim BH, Kim HS, Choi YE, et al. Two-stage cultivation of two *Chlorella* sp. strains by simultaneous treatment of brewery wastewater and maximizing lipid productivity. Bioresour Technol 2013;132:230–8.

[62] Xia L, Ge H, Zhou X, Zhang D, Hu C. Photoautotrophic outdoor two-stage cultivation for oleaginous microalgae *Scenedesmus obtusus* XJ-15. Bioresour Technol 2013;144:261–7.

[63] Mujtaba G, Choi W, Lee CG, Lee K. Lipid production by Chlorella vulgaris after a shift from nutrient-rich to nitrogen starvation conditions. Bioresour Technol 2012;123:279–83.

[64] Ratha SK, Prasanna R, Prasad RBN, Sarika C, Dhar DW, Saxena AK. Modulating lipid accumulation and composition in microalgae by biphasic nitrogen supplementation. Aquaculture 2013;392–395:69–76.

[65] Alvarez-Diaz PD, Ruiz J, Arbib Z, Barragan J, Garrido-Perez C, Perales JA. Lipid production of microalga *Ankistrodesmus falcatus* increased by nutrient and light starvation in a two-stage cultivation process. Appl Biochem Biotechnol 2014;174:1471–83.

[66] Yang H, He Q, Hu C. Lipid accumulation by NaCl induction at different growth stages and concentrations in photoautotrophic two-step cultivation of *Monoraphidium dybowskii* LB50. Bioresour Technol 2015;187:221–7.

[67] Sun X, Cao Y, Xu H, Liu Y, Sun J, Qiao D, et al. Effect of nitrogen-starvation, light intensity and iron on triacylglyceride/carbohydrate production and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two-stage process. Bioresour Technol 2014;155C:204–12.

[68] Tarakhovskaya ER, Maslov YI, Shishova MF. Phytohormones in algae. Russ J Plant Phys 2007;54:163–70.

[69] Kiseleva AA, Tarachovskaya ER, Shishova MF. Biosynthesis of phytohormones in algae. Russ J Plant Phys 2012;59:595–610.

[70] Mekhalfi M, Amara S, Robert S, Carriere F, Gontero B. Effect of environmental conditions on various enzyme activities and triacylglycerol contents in cultures of the freshwater diatom, *Asterionella formosa* (Bacillariophyceae). Biochimie 2014;101:21–30.

[71] Bajguz A, Piotrowska-Niczyporuk A. Synergistic effect of auxins and brassinosteroids on the growth and regulation of metabolite content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Physiol Biochem 2013;71:290–7.

[72] Jusoh M, Loh SH, Chuah TS, Aziz A, Cha TS. Indole-3-acetic acid (IAA) induced changes in oil content, fatty acid profiles and expression of four fatty acid biosynthetic genes in *Chlorella vulgaris* at early stationary growth phase. Phytochem 2015;111:65–71.

[73] Chen Y, Li X, Shen Z. Leaching and uptake of heavy metals by ten different species of plants during an EDTA-assisted phytoextraction process. Chemosphere 2004;57:187–96.

[74] Luo C, Shen Z, Li X. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. Chemosphere 2005;59:1–11.

[75] Viipsi K, Sjoberg S, Tonsuaadu K, Shchukarev A. Hydroxy- and fluorapatite as sorbents in Cd(II)-Zn(II) multi-component solutions in the absence/presence of EDTA. J Hazard Mater 2013;252–253:91–8.

[76] Cheirsilp B, Suwannarat W, Niyomdecha R. Mixed culture of oleaginous yeast Rhodotorula glutinis and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. New Biotechnol 2011;28:362–8.

[77] Cho D-H, Ramanan R, Heo J, Lee J, Kim B-H, Oh H-M, et al. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. Bioresour Technol 2015;175:578–85.

[78] Olguin EJ. Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotech Adv 2012;30:1031–46.

[79] Do Nascimento M, Dublan Mde L, Ortiz-Marquez JC, Curatti L. High lipid productivity of an *Ankistrodesmus – Rhizobium* artificial consortium. Bioresour Technol 2013;146:400–7.

[80] Zhao X, Zhou Y, Huang S, Qiu D, Schideman L, Chai X, et al. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. Bioresour Technol 2014;156:332.

[81] Yen HW, Chen PW, Chen LJ. The synergistic effects for the co-cultivation of oleaginous yeast-*Rhodotorula glutinis* and microalgae-*Scenedesmus obliquus* on the biomass and total lipids accumulation. Bioresour Technol 2015;184:148–52.

[82] Teo SH, Islam A, Yusaf T, Taufiq-Yap YH. Transesterification of *Nannochloropsis oculata* microalga's oil to biodiesel using calcium methoxide catalyst. Energy 2014.

[83] Choi YK, Kumaran RS, Jeon HJ, Song HJ, Yang YH, Lee SH, et al. LED light stress induced biomass and fatty acid production in microalgal biosystem, *Acutodesmus obliquus*. Spectrochimica acta Part A, Mol Biomol Spect 2015;145:245–53.

[84] Seo YH, Lee Y, Jeon DY, Han JI. Enhancing the light utilization efficiency of microalgae using organic dyes. Bioresour Technol 2015;181:355–9.

[85] Seo YH, Cho C, Lee JY, Han JI. Enhancement of growth and lipid production from microalgae using fluorescent paint under the solar radiation. Bioresour Technol 2014;173:193–7.

[86] Zalogin TR, Pick U. Inhibition of nitrate reductase by azide in microalgae results in triglycerides accumulation. Algal Res 2014;3:17–23.

[87] Zalogin TR, Pick U. Azide improves triglyceride yield in microalgae. Algal Res 2014;3:8–16.

[88] Kim S, Kim H, Ko D, Yamaoka Y, Otsuru M, Kawai-Yamada M, et al. Rapid induction of lipid droplets in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* by Brefeldin A. PloS One 2013;8:e81978.

[89] Fei W, Wang H, Fu X, Bielby C, Yang H. Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in Saccharomyces cerevisiae. Biochem J 2009;424:61–7.

[90] Taoka Y, Nagano N, Okita Y, Izumida H, Sugimoto S, Hayashi M. Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of *Thraustochytrium aureum* ATCC 34304. J Biosci Bioeng 2011;111:420–4.

[91] Pavlic Z, Vidakovic-Cifrek Z, Puntaric D. Toxicity of surfactants to green microalgae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* and to marine diatoms *Phaeodactylum tricornutum* and *Skeletonema costatum*. Chemosphere 2005;61:1061–8.

[92] Zheng J, Tashiro Y, Wang Q, Sonomoto K. Recent advances to improve fermentative butanol production: genetic engineering and fermentation technology. J Biosci Bioeng 2014.

[93] Sharon-Gojman R, Maimon E, Leu S, Zarka A, Boussiba S. Advanced methods for genetic engineering of *Haematococcus pluvialis* (Chlorophyceae, Volvocales). Algal Res 2015;10:8–15.

[94] Napier JA, Haslam RP, Beaudoin F, Cahoon EB. Understanding and manipulating plant lipid composition: metabolic engineering leads the way. Curr Opin Plant Biol 2014;19:68–75.

[95] Sarkar D, Shimizu K. An overview on biofuel and biochemical production by photosynthetic microorganisms with understanding of the metabolism and by metabolic engineering together with efficient cultivation and downstream processing. Bioresour Bioproces 2015:2.

[96] De Bhowmick G, Koduru L, Sen R. Metabolic pathway engineering towards enhancing microalgal lipid biosynthesis for biofuel application—a review. Renew Sust Energ Rev 2015;50:1239–53.

[97] O'Neill BM, Mikkelson KL, Gutierrez NM, Cunningham JL, Wolff KL, Szyjka SJ, et al. An exogenous chloroplast genome for complex sequence manipulation in algae. Nucleic acids Res 2012;40:2782–92.

[98] Kindle KL. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 1990:87.

[99] Wijffels RH, Kruse O, Hellingwerf KJ. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Curr Opin Biotechnol 2013;24:405–13.

[100] Misra R, Guldhe A, Singh P, Rawat I, Bux F. Electrochemical harvesting process for microalgae by using nonsacrificial carbon electrode: a sustainable approach for biodiesel production. Chem Eng J 2014;255:327–33.

[101] Voigt J, Stolarczyk A, Zych M, Malec P, Burczyk J. The cell-wall glycoproteins of the green alga Scenedesmus obliquus. The predominant cell-wall polypeptide of *Scenedesmus obliquus* is related to the cell-wall glycoprotein gp3 of *Chlamydomonas reinhardtii*. Plant Sci 2014;215–216:39–47.

[102] Niu YF, Zhang MH, Xie WH, Li JN, Gao YF, Yang WD, et al. A new inducible expression system in a transformed green alga, *Chlorella vulgaris*. Gene Mol Res 2011;10:3427–34.

[103] Wang C, wang Y, Su Q, Gao X. Transient expression of the GUS gene in a unicellular marine green alga, *Chlorella* sp. MACC/C95, via electroporation. Biotechnol Bioproc Eng 2007;12:180–3.

[104] Niu Y-F, Yang Z-K, Zhang M-H, Zhu C-C, Yang W-D, Liu J-S, et al. Transformation of diatom *Phaeodactylum tricornutum* by electroporation and establishment of inducible selection marker. BioTechniques 2012.

[105] Leon-Banares R, Gonzalez-Ballester D, Galvan A, Fernandez E. Transgenic microalgae as green cell-factories. Trends Biotechnol 2004;22:45–52.

[106] Potvin G, Zhang Z. Strategies for high-level recombinant protein expression in transgenic microalgae: a review. Biotech Adv 2010;28:910–8.

[107] Ahmad I, Sharma AK, Daniell H, Kumar S. Altered lipid composition and enhanced lipid production in green microalga by introduction of brassica diacylglycerol acyltransferase 2. Plant Biotech J 2015;13:540–50.

[108] Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV. Genetic transformation of the green alga—*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant Sci 2004;166:731–8.

[109] Muto M, Fukuda Y, Nemoto M, Yoshino T, Matsunaga T, Tanaka T. Establishment of a genetic transformation system for the marine pennate diatom *Fistulifera* sp. strain JPCC DA0580–a high triglyceride producer. Mar Biotech 2013;15:48–55.

[110] Cheng R, Ma R, Li K, Rong H, Lin X, Wang Z, et al. *Agrobacterium tumefaciens* mediated transformation of marine microalgae *Schizochytrium*. Microbiol Res 2012;167:179–86.

[111] Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Curr Opin Biotechnol 2008;19:430–6.

[112] Guo SL, Zhao XQ, Tang Y, Wan C, Alam MA, Ho SH, et al. Establishment of an efficient genetic transformation system in *Scenedesmus obliquus*. J Biotech 2013;163:61–8.

[113] Vieler A, GuangxiWu C-HT, Bullard B, Cornish A, Reca CHICT, Rujira Achawanantakun, Christopher J Buehl, Michael S, Campbell DC, Kevin L. Childs, Clark Teresa J, Deshpande Rahul, Erickson Erika, Ferguson Ann Armenia, Handee Witawas, Kong1 Que, Li Xiaobo, Liu1 Bensheng, et al. Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. PLOS Genet 2012:8.

[114] Radakovits R, Eduafo PM, Posewitz MC. Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum*. Metabol Eng 2011;13:89–95.

[115] Purton S, Szaub JB, Wannathong T, Young R, Economou CK. Genetic engineering of algal chloroplasts: progress and prospects. Russ J Plant Physiol 2013;60:491–9.

[116] Gutiérrez CL, Gimpel J, Escobar C, Marshall SH, Henríquez V. Chloroplast genetic tool for the green microalgae *Haematococcus Pluvialis* (Chlorophyceae, Volvocales)1. J Phycol 2012;48:976–83.

[117] Day A, Goldschmidt-Clermont M. The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnol J 2011;9:540–53.

[118] Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, et al. Chloroplast proteomics highlights the subcellular compartmentation of lipid metabolism. Prog Lip Res 2010;49:128–58.

[119] Wang X, Ruan Z, Boileau D, Sears BB, Liu Y, Liao W. Transgenic expression of a bacterial thermophilic amylase in the *Chlamydomonas reinhardtii* chloroplast to facilitate algal biofuel production. BioEnerg Res 2014;8:527–36.

[120] Feschotte C, Wang D, Ning K, Li J, Hu J, Han D, et al. *Nannochloropsis* genomes reveal evolution of microalgal oleaginous traits. PLoS Genet 2014;10: e1004094.

[121] Misra N, Panda PK, Parida BK, Mishra BK. Phylogenomic study of lipid genes involved in microalgal biofuel production-candidate gene mining and metabolic pathway analyses. Evol. Bioinform. Online 2012;8:545–64.

[122] Zhang C, Hu H. High-efficiency nuclear transformation of the diatom *Phaeodactylum tricornutum* by electroporation. Mar Genom 2014;16:63–6.

[123] Noor-Mohammadi S, Pourmir A, Johannes TW. Method for assembling and expressing multiple genes in the nucleus of microalgae. Biotechnol Lett 2013.

[124] Harwood JL, Guschina IA. The versatility of algae and their lipid metabolism. Biochimie 2009;91:679–84.

[125] Khozin-Goldberg I, Cohen Z. Unraveling algal lipid metabolism: recent advances in gene identification. Biochimie 2011;93:91–100.

[126] Liu B, Vieler A, Li C, Jones AD, Benning C. Triacylglycerol profiling of microalgae *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica*. Bioresour Technol 2013;146:310–6.

[127] Fan J, Cui Y, Wan M, Wang W, Li Y. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. Biotechnol Biofuel 2014.

[128] Wan M, Jin X, Xia J, Rosenberg JN, Yu G, Nie Z, et al. The effect of iron on growth, lipid accumulation, and gene expression profile of the freshwater microalga *Chlorella sorokiniana*. Appl Microbiol Biotechol; 2014.

[129] Shin H, Hong S-J, Kim H, Yoo C, Lee H, Choi H-K, et al. Elucidation of the growth delimitation of *Dunaliella tertiolecta* under nitrogen stress by integrating transcriptome and peptidome analysis. Bioresour Technol; 2015.

[130] Courchesne NMD, Parisien A, Wang B, Lan CQ. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. J Biotechnol 2009;141:31–41.

[131] Blatti JL, Michaud J, Burkart MD. Engineering fatty acid biosynthesis in microalgae for sustainable biodiesel. Curr Opin Chem Biol 2013;17:496–505.

[132] Liang MH, Jiang JG. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Prog Lipid Res 2013;52:395–408.

[133] Davis MS, Solbiati J, Cronan Jr. JE. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 2000;275:28593–8.

[134] Thelen JJ, Ohlrogge JB. Metabolic engineering of fatty acid biosynthesis in plants. Metabol Eng 2002;4:12–21.

[135] Dunahay T, Jarvis E, Dais S, Roessler P. Manipulation of microalgal lipid production using genetic engineering. Appl Biochem Biotechnol 1996;57–58:223–31.

[136] Wagner M, Hoppe K, Czabany T, Heilmann M, Daum G, Feussner I, et al. Identification and characterization of an acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) gene from the microalga *O. tauri*. Plant Physiol Biochem 2010;48:407–16.

[137] Baroukh C, Munoz-Tamayo R, Steyer JP, Bernard O. A state of the art of metabolic networks of unicellular microalgae and cyanobacteria for biofuel production. Metabolic Eng 2015;30:49–60.

[138] Xue J, Niu Y-F, Huang T, Yang W-D, Liu J-S, Li H-Y. Genetic improvement of the microalga *Phaeodactylum tricornutum* for boosting neutral lipid accumulation. Metabolic Eng 2015;27:1–9.

[139] Hsieh HJ, Su CH, Chien LJ. Accumulation of lipid production in *Chlorella minutissima* by triacylglycerol biosynthesis-related genes cloned from *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. J Microbiol 2012;50:526–34.

[140] Blatti JL, Beld J, Behnke CA, Mendez M, Mayfield SP, Burkart MD. Manipulating fatty acid biosynthesis in microalgae for biofuel through protein-protein interactions. PloS One 2012;7:e42949.

[141] Liu B, Benning C. Lipid metabolism in microalgae distinguishes itself. Curr Opin Botechnol 2013;24:300–9.

[142] Radakovits R, Jinkerson RE, Darzins A, Posewitz MC. Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 2010;9:486–501.

[143] Gonzalez-Fernandez C, Ballesteros M. Linking microalgae and cyanobacteria culture conditions and key-enzymes for carbohydrate accumulation. Biotech Adv 2012;30:1655–61.

[144] Ho SH, Huang SW, Chen CY, Hasunuma T, Kondo A, Chang JS. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresour Technol 2013;135:191–8.

[145] Breuer G, Jaeger L, Artus V, Martens D, Springe J, Draaisma R, et al. Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus*: (II) evaluation of TAG yield and productivity incontrolled photobioreactors. Biotechnol Biofuel 2014;7:70.

[146] Li Y, Han D, Hu G, Sommerfeld M, Hu Q. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotechnol Bioeng 2010;107:258–68.

[147] Parsaeimehr A, Sun Z, Dou X, Chen YF. Simultaneous improvement in production of microalgal biodiesel and high-value alpha-linolenic acid by a single regulator acetylcholine. Biotechnol Biofuels 2015;8:11.

[148] Nascimento IA, Marques SSI, Cabanelas ITD, Pereira SA, Druzian JI, COd Souza, et al. Screening microalgae strains for biodiesel production – lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. BioEnerg Res 2012.

[149] Guo F, Wang H, Wang J, Zhou W, Gao L, Chen L, et al. Special biochemical responses to nitrogen deprivation of filamentous oleaginous microalgae *Tribonema* sp. Bioresour Technol 2014;158:19–24.

[150] Liua X, Shengb J, Curtiss R. Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci USA 2011;108:6899–904.

[151] La Russa M, Bogen C, Uhmeyer A, Doebbe A, Filippone E, Kruse O, et al. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. J Biotech 2012;162:13–20.

[152] Henley WJ, Litaker RW, Novoveská L, Duke CS, Quemada HD, Sayre RT. Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. Algal Res 2013;2:66–77.

[153] Andow DA, Zwahlen C. Assessing environmental risks of transgenic plants. Ecol Lett 2006;9:196–214.

[154] Hlavova M, Turoczy Z, Bisova K. Improving microalgae for biotechnology-From genetics to synthetic biology. Biotech Adv 2015.

[155] Qin S, Lin H, Jiang P. Advances in genetic engineering of marine algae. Biotechnol Adv 2012;30:1602–13.

[156] Guldhe A, Singh P, Kumari S, Rawat I, Permaul K, Bux F. Biodiesel synthesis from microalgae using immobilized *Aspergillus niger* whole cell lipase biocatalyst. Renew Energ 2016;85:1002–10.
[157] Song M, Pei H, Hu W, Ma G. Evaluation of the potential of 10 microalgal strains for biodiesel production. Bioresour Technol 2013;141:245–51.
[158] Xu H, Miao X, Wu Q. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J Biotechnol 2006;126:499–507.
[159] Vijayaraghavan K, Hemanathan K. Biodiesel production from freshwater algae. Energy Fuels 2009;23:5448–53.
[160] Miao X, Wu Q. Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 2006;97:841–6.
[161] Guldhe A, Singh B, Rawat I, Permaul K, Bux F. Biocatalytic conversion of lipids from microalgae Scenedesmus obliquus to biodiesel using Pseudomonas fluorescens lipase. Fuel 2015;147:117–24.
[162] Chen Y-H, Huang B-Y, Chiang T-H, Tang T-C. Fuel properties of microalgae (*Chlorella protothecoides*) oil biodiesel and its blends with petroleum diesel. Fuel 2012;94:270–3.
[163] Mostafa SSM, El-Gendy NS. Evaluation of fuel properties for microalgae *Spirulina platensis* bio-diesel and its blends with Egyptian petro-diesel. Arab J Chem 2013.
[164] Talebi AF, Mohtashami SK, Tabatabaei M, Tohidfar M, Bagheri A, Zeinalabedini M, et al. Fatty acids profiling: A selective criterion for screening microalgae strains for biodiesel production. Algal Res 2013;2:258–67.
[165] Halim R, Gladman B, Danquah MK, Webley PA. Oil extraction from microalgae for biodiesel production. Bioresour Technol 2011;102:178–85.
[166] Yu X, Zhao P, He C, Li J, Tang X, Zhou J, et al. Isolation of a novel strain of *Monoraphidium* sp. and characterization of its potential application as biodiesel feedstock. Bioresour Technol 2012;121:256–62.
[167] Levine RB, Costanza-Robinson MS, Spatafora GA. Neochloris oleoabundans grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. Biomass Bioenerg 2011;35:40–9.
[168] Jazzar S, Quesada-Medina J, Olivares-Carrillo P, Marzouki MN, Acién-Fernández FG, Fernández-Sevilla JM, et al. A whole biodiesel conversion process combining isolation, cultivation and in situ supercritical methanol transesterification of native microalgae. Bioresour Technol 2015;190:281–8.