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Metabolic engineering and genome editing strategies for enhanced lipid production in microalgae

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Abstract: Depleting global petroleum reserves and skyrocketing prices coupled with succinct supply have been a grave concern, which needs alternative sources to conventional fuels. Oleaginous microalgae have been explored for enhanced lipid production, leading towards biodiesel production. These microalgae have short life cycles, require less labor, and space, and are easy to scale up. Triacylglycerol, the primary source of lipids needed to produce biodiesel, is accumulated by most microalgae. The article focuses on different types of oleaginous microalgae, which can be used as a feedstock to produce biodiesel. Lipid biosynthesis in microalgae occurs through fatty acid synthesis and TAG synthesis approaches. In-depth discussions are held regarding other efficient methods for enhancing fatty acid and TAG synthesis, regulating TAG biosynthesis bypass methods, blocking competing pathways, multigene approach, and genome editing. The most potential targets for gene transformation are hypothesized to be a malic enzyme and diacylglycerol acyltransferase while lowering phosphoenolpyruvate carboxylase activity is reported to be advantageous for lipid synthesis.

Abbreviations

ACC	Acetyl-CoA carboxylase
Acyl-ACP thioesterases	Acyl–acyl carrier protein thioesterases
ADP-glucose pyrophosphorylase	Adenosine diphosphoglucose pyrophosphorylase
AGPase	ADP-glucose pyrophosphorylase
BE	Branching enzymes
cphA	Cyanophycin synthetase
CrDGAT2–4 gene	Diacylglycerol acyltransferase 2–4 gene
CRISPRs	Clustered regularly interspaced short palindromic repeats
DGAT	Diacylglycerol acyltransferase
FAME	Fatty acid methyl ester
FAS	Fatty acid synthetase
FAT	Acyl-ACP-thioesterase
G-1-P	Glucose 1-phosphate
G-6-P	Glucose 6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAP	Glyceraldehyde-3-phosphate
GPAT	Glycerol-3-phosphate acetyltransferases gene
LPAAT	Lysophosphatidic acetyltransferase gene
MAT	Malonyl CoA: ACP transacylase
ME	Malic enzyme
MLDP	Major lipid droplet protein
PAM	Protospacer adjacent motif
PEPC	Phosphoenolpyruvate carboxylase
PPi	Inorganic pyrophosphate
PK	Pyruvate kinase
PTA	Phosphotransacetylase
SS	Starch synthase
TAG	Triacylglycerol
TALENs	Transcription activator-like effector nuclease
tracrRNA	Trans-activating CRISPR RNA
ZFN	Zinc finger nucleases

Introduction

The cumulative effect of dwindling fossil fuels, the impact on the environment due to their excess consumption, the

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exponential rise in prices, infrastructure, storage, and supply issues in conventional fossil fuel-driven energy economy, etc., have triggered the exploration for clean, renewable, and bio-based fuels. The potential of biodiesel as an alternative fuel has attracted much attention among the extensively researched biofuels. Biodiesel is a mixture of fatty acid methyl esters and is typically made from vegetable oils or animal fats by transesterification of triacylglycerols (TAGs). Biodiesel has no/negligible net sulphur or carbon dioxide contribution to the atmosphere and a meagre gaseous pollutants emission compared to diesel. However, the production of biodiesel from plant oil is an expensive and tedious process of which 70%–85% of the total expense is due to the prices of the source which require enormous energy and land to produce adequate oilseed crops [1].

Conversely, animal fats are extremely viscous and generally in solid form at ambient temperature because of their high saturated fatty acids. Much emphasis has been laid on the production of microbial oils to reduce the cost of oil raw materials. It is reported that a variety of microorganisms, including microalgae, yeast, bacteria, and fungi, can accumulate oils under ideal cultivation conditions. The advantages of microbes include a rapid growth rate, cheap and abundant raw materials, less labour requirement, minimal susceptibility to variations in habitat/culture conditions, and easy scalability in the production process [2]. Among the aforementioned microorganisms, microalgae are considered candidates of great potential for biodiesel production owing to their negligible or no known adverse impact on the environment and food security.

The major obstacle to the commercialization of microalgal biofuel is its cost compared to other conventional sources; thus, various strategies to enhance biomass and lipid content are being implemented. Selection of suitable strains, improvement of these strains through mutagenesis and genetic engineering approaches, manipulation of nutritional and environmental factors, use of phytohormones, co-cultivation of microalgae with bacteria or yeasts, and genome editing are various strategies that are being implemented to enhance lipid content. This review includes detailed information about strategies reported for lipid production, improvement of fatty acid synthesis, and prospects of genome editing in oleaginous microalgae.

Oleaginous Microorganisms for Biodiesel Production

Despite many microbes being potent enough to deposit lipids in their membranous structure, emphasis has shifted towards oleaginous microbes, as they can accumulate lipids up to 20% of their biomass weight. These microbes can even accumulate lipids upto 70%–80% of their biomass under stress conditions [3]. The oils produced by oleaginous microbes such as fungi, yeast, microalgae, and bacteria are referred to as microbial oils [4]. Eukaryotic yeast, microalgae, and molds produce triacylglycerols (TAGs) that have compositional similarities with vegetable oils while bacteria produce specific lipids. During transesterification, these TAGs combine with alcohols in the presence of an acid or alkali catalyst to form fatty acid methyl ester (FAME, biodiesel), with glycerol as a by-product [5]. Microbial oils are gaining more importance than plant and animal oils because of their fast growth, non-reliance on seasonal and climatic changes, minimal labour, relatively low spatial footprint, and ease of scale-up [6]. Because of these features, microbial oils are emerging as a potential feedstock for biodiesel production.

Oleaginous microalgae

Microalgae are one of the efficient feedstocks for biodiesel production, as they can accumulate lipids up to 60%–77% of their dry weight under specific conditions [7]. Apart from biodiesel production, microalgae are also utilized in the generation of various valuable products. The residual biomass after lipid extraction can be further utilized as animal feed or as a source for the synthesis of biomethane [8]. In view of these advantages, microalgae are considered to be a sustainable feedstock in biodiesel production.

Galán et al. [9] reported 80% lipid content in *Schizochytrium* sp. which is 770 times higher than oleaginous plants such as sunflower, colza, etc. Microalgae belonging to Bacillariophyceae and Chlorophyta produce abundant amounts of oil rich in C16 and C18 fatty acids [10]. These oils are generally composed of polyunsaturated fatty acids with more than four double bonds, distinguishing them from vegetable oil. Chlorella is currently drawing the attention of international energy research institutions due to its suitability for large-scale breeding for biodiesel generation [11]. Furthermore, substantial research is underway addressing microalgae such as *Chlorella kessleri, Galdieria partita*, *Chlorococcum littorale*, *Nannochloropsis oculata*, *Synechococcus* PCC7942 and *Chlorella sorokiniana* BTA 9031 that can fix CO_2 from the gases and produce biodiesel [12].

Temperature, intensity of light, pH & salinity of the medium, availability of nitrogen, minerals, etc., are some of the parameters that affect the generation of oil in microalgae [13]. Besides, nutrient limitation, stress-induced conditions, and co-cultivation with other microorganisms also improve the lipid content in microalgae. Al-Lwayzy et al. [14] blended the biodiesel produced from *Chlorella protothecoides* with petroleum biodiesel and the efficiency of the blend was determined from the emission of a 25.8 kW agricultural tractor engine. Mostafa et al. [15] studied the physicochemical properties of blends (B2, B5, B10, and B20) prepared from *S. platensis* biodiesel and petro-diesel and found that the characteristics corroborated with petro-diesel that is being marketed in Egypt. An insight into various strains of oleaginous microalgae, their lipid content along with composition of saturated, mono, and polyunsaturated fatty acids under specific culture conditions have been shown in Table 1.

Autotrophic microalgae can be converted into heterotrophic organisms by changing growing conditions and employing genetic engineering approaches. The use of "engineered microalgae" with economic and ecological value/importance can improve oil accumulation.

Strategies for lipid production

Lipid enhancement in microalgae can be broadly classified into five approaches such as:

TABLE 1

Promising microalgae, its cultivation conditions, and lipid composition

Strain	Culture conditions	Lipid content (% dry weight)					Reference
		Total (%)	Saturated (%) of total	Monounsaturated (%) of total	Polyunsaturated (%) of total	Others (%) of total	
Chlorella vulgaris	Autotrophic	20–42	21	14	51	14	[16]
Phaeodactylum tricornutum	Autotrophic	18.7	24	25	31	20	[17]
Botryococcus braunii	Autotrophic	25–75	0	74	8	18	[18]
Neochloris oleoabundans	Mixotrophic	23	18	18	44	20	[19]
Isochrysis galbana	Mixotrophic	21.1	32	29	36	3	[20]
Nannochloropsis granulate	Autotrophic	23	21	29	32	18	[21]
Chaetoceros gracilis	Autotrophic	15.5–60.28	64.28	24.21	11.51	–	[22]
Chlorella protothecoides	Heterotrophic	49.0	29.6	6.5	63.9	–	[23]

1) Enhancement of FAS approach
2) Enhancement of TAG synthesis approach
3) Blockage of competitive pathways
4) Regulation of bypass approaches of TAG synthesis pathways
5) Multi-gene approach

Enhancement of fatty acid synthesis approach

Triacylglycerols (TAGs) are the lipids that are vital constituents in biodiesel production. Synthesis of TAG in microalgae can be accomplished by various approaches such as:

a. Overexpression of enzymes in fatty acid and TAG synthesis pathways
b. Multi-gene approaches

Fatty acid synthesis (FAS) approach

Microalgae use both organic (glucose, acetate) and inorganic (CO_2) carbon sources to produce lipids, which are further converted to acetyl-CoA that can produce other organic molecules [24]. In prokaryotes, TAG synthesis takes place in the cytosol, whereas in eukaryotes TAG synthesis occurs in endoplasmic reticulum and mitochondria. The predominant pathway for TAG biosynthesis is known as the Kennedy Pathway or Glycerol 3-phosphate pathway. Since FAS is one of the prominent pathways for lipid synthesis, exploring the pathway and its enzymes can certainly enhance lipid synthesis. Several research reports have illustrated that enzymes like acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS), and acyl-ACP-thioesterase (FAT) are vital for enhanced lipid synthesis in microalgae.

Among three enzymes, research data profoundly advocates the utilization of ACC for enhanced lipid synthesis. ACC facilitates the conversion of acetyl-CoA into malonyl-CoA, whereas malonyl-CoA to malonyl-ACP is catalyzed by malonyl CoA: ACP transacylase (MAT). In microalgae such as *Haematococcus pulvialis*, the overexpression of genes like ACP (acyl-carrier-protein), KAS (Beta-Ketoacyl-Acyl-carrier-protein Synthase), and FAT correlate with the synthesis of mono-and polyunsaturated fatty acids. Modification of these genes may lead to improved production of microalgal biofuels [25].

Acetyl-CoA carboxylase (ACC) approach

A study on the expression of *E. coli* ACC alone showed no significant improvement in fatty acid production. This implies that the later steps involved in the pathway could limit the flux, resulting in an insignificant increase in fatty acids [26]. However, co-expression of ACC and thioesterase I (*tesA* gene) resulted in enhanced fatty acid synthesis by six-fold. This indicates that the ACC catalyzing the committed step, is the rate-limiting step for fatty acid biosynthesis in *E. coli* [27].

Acyl-ACP feedback inhibition by the ACC enzyme may lead to enhanced fatty acid synthesis. However, the *tesA* gene may be critical in regulating this inhibition by generating free fatty acids, which enhances lipid production [28]. Another potential strategy for reducing feedback inhibition is the expression of a non-native gene (ACC), which is not recognized by the acyl-ACP of *E. coli*. Lazaro et al. [29] reported a three-fold increase in lipid content levels by heterologous expression of ACC in *E. coli* from *Acinetobacter calcoaceticus*.

In eukaryotes, total lipid accumulation has been hindered because of regulatory and metabolic control executed by fatty acids on the *ACC* gene. The role of the ACC1 enzyme is to provide malonyl-CoA, which is used in the fatty acid synthesis of cellular cytoplasm. Attempts have been made to

enhance the lipid content by heterologous expression of the *ACC1* gene derived from the oleaginous fungus *Mucor rouxii* into *Hansenula polymorpha* a non-oleaginous yeast, resulting in a 40% increase in fatty acid content [30]. Furthermore, overexpression of the *ACC* gene in *Aspergillus oryza* showed no significant increase in fatty acids or TAGs productivities compared to the parental strain [31]. An exception was found in *Yarrowia lipolytica*, where overexpression of the endogenous *ACC1* gene resulted in a two-fold rise in the overall lipid content [32].

Acyl-ACP-thioesterase (FAT)

Acyl-ACP-thioesterase is a group of enzymes that hydrolyze acyl-ACPs into ACP and free fatty acids, synthesized as a result of the *FAT* gene insertion. FAT breaks the cycle of fatty acid elongation and releases the free fatty acids. Azimov et al. [33] reported that the introduction of *Umbellularia californica* FAT enzyme into *E. coli* deficient in fatty acid degradation resulted in increased hydrolytic activity, leading to the accumulation of medium-chain fatty acids. This highlights the feedback inhibition of the acyl-ACP intermediate for fatty acid synthesis. Insertion of *FAT* genes obtained from various plants into *E. coli* strain ML103 resulted in fatty acid production that varied in both quantity and composition. The strains carrying the FAT genes from *Ricinus communis* and *Jatropha* produced C14, C16:1, and C16 fatty acids at levels of about 40%, 35%, and 20%, respectively [34]. The results infer that the FAT has a profound influence on the accumulation of free fatty acid.

Enhancement of TAG synthesis approach

Acyl-CoA: glycerol-sn-3-phosphate acyl-transferase (GPAT)

The first reaction of TAG synthesis where lysophosphatidate (LPA) is formed via the Kennedy pathway catalyzed by GPAT. Additionally, LPA is also formed by the acylation of dihydroxyacetone phosphate (DHAP) by acyl-CoA: DHAP acyltransferase (DHAPAT) followed by reduction. Major GPAT activities in *S. cerevisiae* are coded by *GAT1* and *GAT2* genes. Among these, the *GAT1* gene product uses both glycerol-3-phosphate (G3P) and DHAP with similar efficiencies, whereas the *GAT2* gene product prefers G3P to DHAP with a higher preference for C16 fatty acyl chains. Metabolic studies revealed a 50% decrease in TAG synthesis in GAT2 yeast, demonstrating that GAT2 initiates the crucial route for TAG [35]. It was also reported that the expression of the *GPAT* gene of plastidial safflower and *E. coli* raised oil content from 8% to 29% in *Arabidopsis* seeds [36]. The *GPD1* gene of yeast, which codes for cytosolic glycerol-3-phosphate dehydrogenase (Gly3PDH) has been expressed in oilseed rape under the influence of a napin promoter. The resulting transgenic plants showed two-fold improvement in Gly3PDH activity, 3–4 fold enhanced Gly3P levels, along with a 40% increase in total seed oil content [36].

Lysophosphatidate acyl-transferase (LPAT)

LPAT acylates lysophosphatidate (LPA) to phosphatidate (PA). The incorporation of the yeast *SLC1-1* gene, which encodes a variant LPAT, resulted in a remarkable shift in the volume and composition of the fatty acids. Improved expression of glycerolipid acyltransferases in seeds leads to a greater flux of intermediates and better accumulation of TAG, as evidenced by the reported increase in oil content from 8% to 48% in rapeseed and *Arabidopsis* [36].

Acyl-CoA: diacylglycerol acyl-transferase (DGAT)

The last step of TAG synthesis is catalyzed by the enzyme DGAT. In yeasts and plants, DGAT1/DGAT2 and phospholipid: diacylglycerol acyltransferase (PDAT) are responsible for TAG formation. LRO1 encodes PDAT in yeast, rendering it incapable of synthesizing sterols. Deletion of LRO1 in *Y. lipolytica* resulted in a 40% loss of TAG [37]. Gao et al. [38] reported that upon incorporation of *Arabidopsis* DGAT, the yeast showed a 3- to 9-fold enhanced TAG accumulation. Upon overexpression of DGAT, it was observed that rather than phospholipid formation, TAG synthesis increased significantly. Guo et al. [39] inferred that DGAT might be instrumental in the synthesis of TAG, as a strong relation between DGAR activity and TAG content is prominent.

Glycerol 3-phosphate dehydrogenase (GPDH)

GPD1 is an isoform of GPDH that reduces DHAP to G3P, facilitating glycerol for TAG. Upon expressing the gene responsible for GPD1 into *Brassica napus* L., with the specific promoter, a 40% enhancement in lipid content has been observed [36]. The *GUT2* gene codes for GPDH isomer that catalyses the formation of DHAP from G3P, was deleted in *Y. lipolytica*. This deletion boosted the availability of G3P and increased lipid accumulation 3-folds [40].

Regulation of bypass approaches of TAG synthesis pathways

Besides the mentioned enzymes, there are a few more enzymes that are not directly involved in lipid metabolism but enhance the essential metabolites for lipid biosynthesis. The enzymes that fall under this category are acetyl-CoA synthase (ACS), malic enzyme (ME), and ATP: citrate lyase (ACL). Increased ACS levels enhance activation of acetyl-CoA, which further increases the rate of fatty acid synthesis.

Malic enzyme (ME)

ME is involved in the irreversible decarboxylation reaction where malate is converted back to pyruvate. Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), which is required for the biosynthesis of fatty acids, is synthesized during this reaction. Enhanced NADPH synthesis from overexpression of ME is utilized by TAG synthesis-related enzymes ACC, FAS, and ATP: citrate lyase (ACL) to produce more lipids [41]. Overexpression of genes encoding for ME and its isoforms in *Mucor circinelloides* resulted in increased lipid accumulation by 2.5 folds and biomass from 12% to 30% when compared with leucine auxotrophic strain [41].

ATP citrate lyase

ACL is involved in the conversion of citrate to acetyl-CoA and oxaloacetate. It is regarded as one of the key enzymes for lipid production in mammals, fungi, and oleaginous yeasts. This enzyme catalyzes a rate-limiting reaction for lipid

biosynthesis. Enhanced expression of ACL in *Aspergillus oryzae* resulted in a 1.7-fold increase in fatty acid productivity and a 1.9-fold improvement in TAG compared to the parental strain [42].

Overexpression of enzymes of lipid biosynthesis

Fatty acid and TAG biosynthesis involve different steps catalyzed by various enzymes, among which acetyl-CoA carboxylase (ACCase) plays a key role. ACCase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA thereby directing carbon flux towards fatty acid synthesis. A number of studies have emphasized that overexpression of ACCase increases the availability of malonyl CoA, resulting in an increase in fatty acid biosynthesis in chloroplasts [43,44]. Its role is significantly explored in lipid enhancement studies of both plants and microalgae.

Rawat et al. [45] reported that by integrating and expressing PtACC2 in the chloroplast of *Phaeodactylum tricornutum* strain ACCase increased 3.3-fold, significantly enhancing lipid content up to 1.77-fold, reaching 40.8% of dry weight. Similarly, Chen et al. [46] cloned two strains of *Chlamydomonas reinhardtii* for overexpression of ACCase, resulting in 55.45% and 56.15% unsaturated fatty acids content, which is 1.16 fold higher than the wild strain. However, in a few species like *Navicula saprophila* and *Cyclotella cryptic*, expression of the *ACCase* gene only increased the activity of ACCase, not the synthesis of fatty acids [47]. These reports confirmed that random integration of target genes or non-specific expression of ACCase will not enhance fatty acid biosynthesis, even though strains are genetically modified. Therefore, identifying and employing a potential genetic engineering tool will improve the overproduction of fatty acids in oleaginous microalgae [47].

The additional enzymes targeted for increasing fatty acid and TAG synthesis in microalgae are fatty acid synthatase (FAS), malic enzyme (ME), and acetyl-CoA synthase (ACS). Out of these enzymes, Xue et al. [48] reported a 2.5-fold enhancement of lipid content in *Phaeodactylum tricornutum* by over-expressing ME without tampering with the cell growth. Besides, a couple of proteins also play a key role in enhancing fatty acid and TAG synthesis. For instance, in *Cyanidioschyzon merolae*, even under normal growth conditions, the overexpression of CmGPAT1 led to a noticeable accumulation of TAG, with a maximum TAG productivity that was 56.1 times higher than that of the control strain [49].

Blocking competing pathways

Lipid synthesis can be enhanced using metabolic engineering approaches by blocking competitive pathways. These approaches are mainly aimed at channeling the metabolic flux towards TAG biosynthesis, thereby enhancing lipid production. The main competitive pathways to be repressed are the β-oxidation pathway, phospholipid biosynthesis, phosphoenolpyruvate carboxylase (PEPC), and starch biosynthesis. Among these pathways, repression of PEPC and starch biosynthesis are widely explored, through which enhanced lipid content has been reported.

β-oxidation repression

Lipid accumulation can be enhanced by reducing the lipid breakdown and downregulating the genes responsible for the synthesis of fatty acids and TAG, as well as genes involved in β-oxidation. Dysfunction of acyl-CoA oxidase (AOX) in *S. cerevisiae* leads to inactivation of the β-oxidation process, resulting in the accumulation of cellular fatty acids. Feedback regulation of the oxidation process affects hydrophobic substrate transporters and controls the diffusion process. Hence, non-oleaginous yeast such as *S. cerevisiae* cannot accumulate lipids on par with the oleaginous microbes [50]. On the other hand, *Yarrowia lipolytica*, an oleaginous yeast, accumulates lipids up to 50% of its dry weight [51]. This organism contains several AOX proteins encoded by (plant homeobox) POX1–POX6 genes, which catalyze the limiting step of peroxisomal β-oxidation. Alterations in the POX genotype prevent lipid degradation and enhance lipid accumulation. Additionally, phospholipid biosynthesis is an alternative competitive pathway for TAG formation as it competes against TAG biosynthesis for a common substrate, phosphatidate (PA) [52]. PA enters the pathway leading to the synthesis of phospholipids if it is transformed into CDP-diacylglycerol. The production of long fatty acids is caused by inhibition of phospholipid synthesis.

Phosphoenolpyruvate carboxylase repression

The substrate used in the production of proteins and lipids is phosphoenolpyruvate (PEP). Animals and fungi do not synthesize PEPC, an enzyme that changes pyruvate into oxaloacetate. It is primarily produced by plants and photosynthetic microalgae such as cyanobacteria. In TAG biosynthesis, PEP is produced through a series of reactions that result in pyruvate, acetyl-CoA, malonyl CoA, and fatty acids [53]. PEPC is a strategic enzyme that readily converts the substrate phosphoenol pyruvate into oxaloacetate and directly plays a role in protein anabolism (Fig. 1). In contrast, in the lipid synthesis process, PEP is converted into pyruvate by pyruvate kinase (PK). This pyruvate is converted into acetyl-CoA using the pyruvate dehydrogenase enzyme, ultimately leading to the formation of fatty acid. To enhance lipid content, inhibition of PEPC activity is desired. In *Chlamydomonas reinhardtii*, knocking down PEPC and overexpression of GroELS chaperon resulted in enhanced biomass and lipid content of 2.56 g/L and 23.5 mg/L, respectively [54].

Research studies on antisense PEPC expressed in *B. napus* resulted in a 6.4%–18% increase in oil content [53]. Moreover, significant increase in lipid contents have been reported in the transgenic soybean lines containing the construct of an anti-*PEPC* gene [55]. These studies substantiate that lipid enhancement can be achieved by repressing the *PEPC* gene in plants.

However, in microalgae, thorough investigations must be conducted to validate the significance of PEPC in fatty acid synthesis regulation. A recent study in *Anabaena* sp. has reported that an increase in lipid content (46.9%) was observed by repression of PEPC activity, which implies the engineering prospects for enhanced lipid content [56].

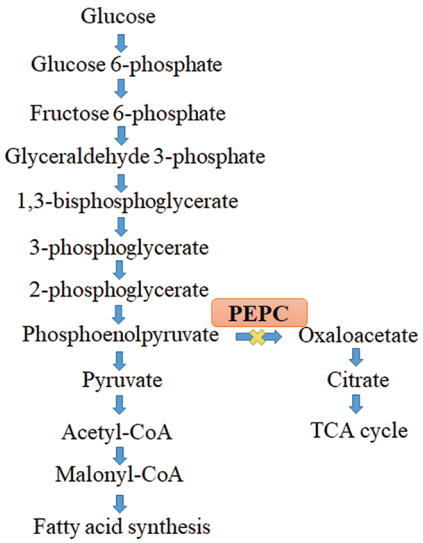

FIGURE 1. Role of strategic enzyme phosphoenol pyruvate carboxylase (PEPC) in the fatty acid biosynthesis pathway.

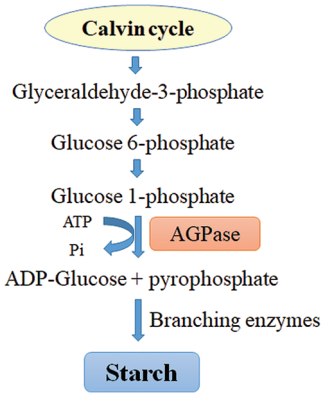

FIGURE 2. Starch biosynthesis pathway in the chloroplast of microalgae. ADP-glucose pyrophosphorylase (AGPase) catalyzes the rate-limiting step of the pathway.

Starch biosynthesis repression

A homopolymer of α-D glucose units, starch acts as a storehouse for carbon and energy, especially during times of stress. As carbon dioxide is assimilated during starch biosynthesis, glyceraldehyde-3-phosphate, glucose-6-phosphate, and glucose-1-phosphate are produced. Conversion of glucose-1-phosphate to ADP-glucose and Pi catalyzed by ADP-glucose pyrophosphorylase (AGPase) is the rate-limiting step of starch synthesis in plastids (Fig. 2). Consequently, ADP-glucose is utilized by starch synthases (SS) and branching enzymes (BE) to elongate glucan chains of starch polymer. To increase the lipid content, blocking starch synthesis is one of the strategic approaches in the green microalga *Chlamydomonas reinhardtii* rather than manipulation of the fatty acid pathway.

Li et al. [57] reported a 10-fold increase in TAG synthesis by the inactivation of AGPase in a starchless mutant (*Chlamydomonas reinhardtii)*, illustrating the streamlining of photosynthetic carbon from starch synthesis to TAG formation [57]. In a separate study, the lipid content of one of the *C. reinhardtii* mutants (BAFJ5), defective in the small subunit of AGPase, was 46.4% higher than that of the wild type.

Single/multiple gene approach

The essential genetic alterations of microalgae have recently been possible due to advancements in the field of genetic engineering and improved understanding of biochemical processes [58]. Numerous investigations have revealed that TAG productivity could be increased by genetic engineering by expressing key GAP pathway enzymes [59,60] (Table 2).

TAG synthesis starts with the acylation of glycerol-3-phosphate (G3P) and undergoes a five step process as shown in the Fig. 3 which is coordinated via four enzymes *viz*., glycerol 3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase

TABLE 2

Metabolic engineering and genome editing studies for improved lipid content in microalgae

Microalgae	Modification	Variation in microalgal lipid profile	Reference
Chlamydomonas reinhardtii	Repression of *MLDP* gene expression	Increase in lipid droplet diameter up to 40%	[61]
	Knockout of citrate synthase gene	TAG increases upto 169.5%	[62]
	Artificial silencing of *CrDGAT2–4 gene*	Lipid content increases by 24%–34%	[63]
	Inactivation of ADP-glucose pyrophosphorylase	TAG increases by 10 folds	[57]
Chlorella minutissima	Overexpression of GPAT, LPAAT and DGAT	Lipid content increases by 2-folds	[64]
Phaeodactylum tricornutum	Disruption of the gene encoding a Hotdog-fold thioesterase involved in acyl-CoA hydrolysis	Enhanced TAG synthesis	[65]
	Suppression of gene expression of TAG lipase	Increased accumulation of shorter chain length fatty acids	[58]
Synechocystis sp.	Deletion of *cphA* gene	Fatty acids secretion into the medium	[66]
	PTA gene deletion	Increases fatty acid production	[67]
C. reinhardtii	CRISPRi based gene regulation	Increases lipid production up to 94%	[68]

Table 2 (continued).

Microalgae	Modification	Variation in microalgal lipid profile	Reference
P. tricornutum	Expression of ME	Enhances lipid productivity by 2.5	[69]
	Overexpression of G6PD	Increases lipid production upto 55.7%	[48]
T. pseudonana	Knock-down of a multifunctional lipase or phospholipase or acetyltransferase enzyme	Mutant strains produce 2.4–3.3 fold increased amount of lipids	[45]

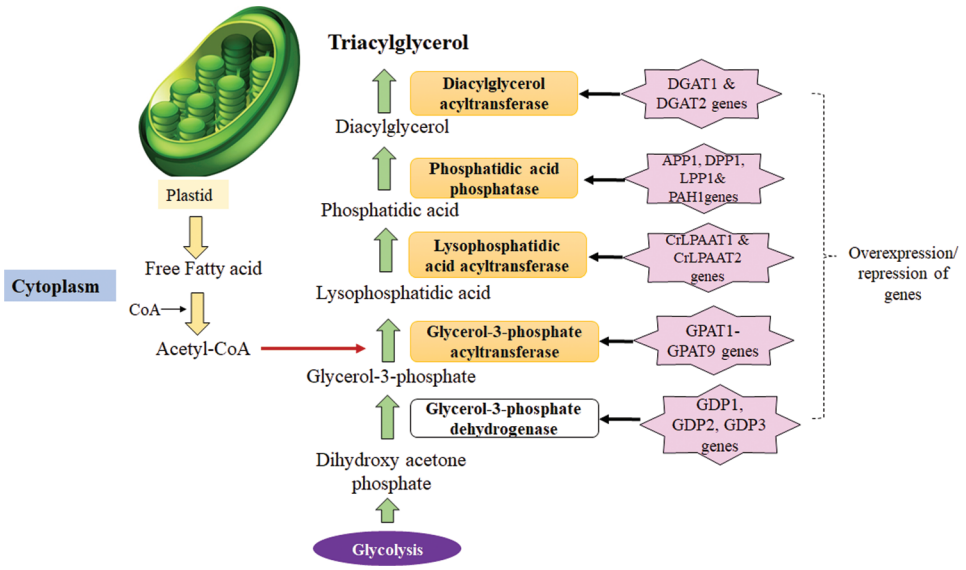

FIGURE 3. Triacylglycerol biosynthesis in microalgae (Key enzymes in the TAG synthesis have been highlighted).

(DGAT) [70]. Over-expression of genes encoding these enzymes individually or collectively in microalgae has been experimentally proven to increase in lipid content by various researchers. For instance, over-expression of GPAT in *Phaeodactylum tricornutum* showed 2-fold rise in fatty acid content compared to wild strain which accounted for 42.6% lipid content per dry weight [71]. TAG levels were increased by 13% and 20% in *P. tricornutum* and *Chlamydomonas reinhardtii* by over-expression of LPAT and DGAT, respectively [72].

Muñoz et al. [72] found that overexpression of single and multiple encoding genes for LPAT, GPAT, and DGAT in *N. oleoabundans* produced 45% and 39% of triacylglycerols which is almost 1.3 folds higher than that of wild strain. Above all over-expressing multiple genes-LPAT, GPAT, and DGAT improved lipid production even during undernourishment with no significant effect on growth [72]. Similarly, main regulatory genes for fatty acid biosynthesis pathway in *H. pluvialis* was linked to the enzymes: acyl carrier protein, 3-ketoacyl-ACP-synthase, and acyl-ACP thioesterase because higher expression resulted in increased synthesis of mono unsaturated and polyunsaturated fatty acids [73]. Because acyl-CoA intermediates function as both product and feedback inhibitors in the fatty acid (upstream) pathway and as important precursors in the TAG (downstream) pathway, many gene approaches may be promising options for improving quality and increasing fatty acid production. So, by depleting acyl-CoA intermediates and increasing the rate of TAG accumulation in lipid bodies, up-regulation of the downstream pathway produces a driving force by properly regulating the necessary genes simultaneously.

Altering fatty acid chain length for improved lipid quality

Composition of the microalgal lipids determines the properties of biodiesel. Various strategies must be followed to increase the quality of the lipids so as to meet the specific standards of the biodiesel [74]. Saturated and monounsaturated fatty acids with carbon length of 12:0, 14:0, 16:0, 16:1, 18:0 and 18:1 are suitable for biodiesel production. Acyl-ACP thioesterases control the release of fatty acid chains of various lengths from fatty acid synthase [75]. As per the metabolic needs, various organisms synthesize thioesterases that are specific for particular fatty acids chain. Hence, transformation of thioesterases genes into microalgae could enrich the fatty acid composition desirable for standard fuel properties. Upon transforming the fatty acid acyl-ACP thioesterases from *Cinnamomum camphora* and *Umbellularia californic* into *Phaeodactylum tricornutum*, the diatom produced lipids with increased percentage of lauric and myristic acids [58]. From previously reported studies, it is evident that alteration in the length of fatty acid chain has influence on the composition of the lipids.

Effect of Environmental Factors on Metabolic Engineering of Microalgae

Environmental factors play a significant role in metabolic engineering strategies aimed at enhancing lipid production in microalgae. These factors can influence various aspects of

microalgal physiology and metabolism, ultimately affecting lipid biosynthesis. Among various environmental factors-light, temperature, nutrients, CO_2, stress conditions, pH and salinity are recognized as crucial parameters to impact during metabolic engineering in microalgae [13].

- Light intensity and quality: Light is a crucial environmental factor that directly affects photosynthesis, biomass growth, and lipid accumulation in microalgae. Optimizing light intensity, photoperiod, and spectral quality can enhance lipid productivity [17,76]. Low light intensity stimulates the formation of TAGs, which make up the majority of non-polar lipids found in biodiesel. Conversely, low light levels cause polar lipid concentration to decrease in order to safeguard the photosystem. A higher concentration of light stimulates the production of oxidizing agents that break down polyunsaturated fatty acids, lowering the amount of polar lipids and raising the amount of non-polar lipids. Most of the microalgae species show optimal growth at a light intensity of 200–400 µmol photons $m^{-2}s^{-1}$ [77]. According to Nzayisenga et al. [78], when *Scenedesmus obliquus* was exposed to 300 µmol photons $m^{-2}s^{-1}$ of light, its fatty acid content doubled from 5.8% to 11.6%. Research conducted on *Chlorella* sp. and *Monoraphidium* sp. revealed that their neutral lipid content rose when they were grown in light intensities lower than 400 µmol photons $m^{-2}s^{-1}$ [79].
- Temperature: Temperature influences the metabolic activity and growth rate of microalgae, thereby impacting lipid production. Lower temperatures often promote lipid accumulation, while higher temperatures can enhance biomass productivity. According to Wu et al. [80], *Monoraphidium* sp. thrived well at temperatures between 25°C and 35°C, reaching its maximum lipid output of 29 mg $L^{-1}d^{-1}$ at 30°C. At 40°C, the cells began to degrade, producing very little biomass and no lipids as compared to cells that were under 25°C–35°C. An additional investigation into *Chaetoceros* sp. revealed that it grows best at 25°C and has a lipid content of roughly 20.42% [81]. Lipid buildup in *Chlorella vulgaris* and *Nannochloropsis oculata* rose from 7.90% to 15.31% and 5.90%–16.41% respectively at 25°C–30°C [82].
- Nutrient availability: Nutrients such as nitrogen, phosphorus, and micronutrients are essential for microalgal growth and lipid biosynthesis. Nutrient limitation, particularly nitrogen deprivation, induces lipid accumulation in many microalgae. A two-fold increase in TAG yield (from 23.47 to 46.19 mg $L^{-1}d^{-1}$) has been observed in *Neochloris oleoabundans* when cultivated under nitrogen-deprived conditions [83]. Similarly, Yang et al. [84] reported that *Chlamydomonas reinhardtii* cultivated in a medium without nitrogen and with various concentrations of phosphorous showed the highest total fatty acid (TFA) concentration of 105 µg/mg (i.e., 104.7% higher than the control) in the media deprived of both nitrogen and phosphorous.
- CO_2 concentration: Elevated CO_2 levels can stimulate photosynthesis and redirect carbon flux towards enhanced lipid biosynthesis pathways. Furthermore, coupling microalgal cultivation with CO_2 capture technologies can mitigate greenhouse gas emissions while promoting lipid production. Carbon content influences fatty acid composition and saturation [85]. Certain strains produce elevated levels of long-chain fatty acids (C18:0, C18:1, and C20:0) when exposed to a 15% increase in CO_2 concentration [86]. For instance, *Chorella vulgaris* and *Gloeothece membranacea* produced lipid contents of 25.6% and 36%, respectively, when the CO_2 concentration was raised to 15% in a photobioreactor under blue light [86]. Different CO_2 concentrations and nitrogen: phosphorus (N:P) ratios were used in another study to grow *Scenedesmus dimorphus*, where a lipid content of 31.6% dry weight was found at 6% CO_2 [87].
- pH and salinity: pH and salinity affect cellular physiology and metabolic processes in microalgae. Optimal pH and salinity conditions vary among microalgal species and can influence lipid accumulation. The most suitable pH range for microalgal growth is 6 to 8.76. *Chlorella* sp., *Nannochloropsis salina*, and *Pavlova lutheri* exhibited optimal growth at pH 8, with lipid content of 23%, 24.75%, and 35%, respectively [88,89]. Under optimum conditions, lipid content increased from 150 to 190 g lipid kg^{-1} biomass in *Amphora subtropica*, and from 190 to 280 g lipid kg^{-1} biomass in *Dunaliella* sp. [90]. However, the ideal pH and salinity ranges vary depending on the microalgae strain and their individual requirements for lipid productivity.
- Stress conditions: Environmental stress conditions *viz.*, drought, high light intensity, and oxidative stress, can induce lipid accumulation as a protective mechanism in microalgae. Under salinity stress condition, the lipid content of *Chlorella vulgaris* increased from 60% to 70% by increasing the NaCl concentration from 0.5 to 1.0 M [91]. This is due to an increase in ACCase and a decrease in AGPase [92].

Lipid Secretion

The cost-intensive steps in microalgal biodiesel production are harvesting biomass and extracting lipids from the biomass. Genetic modification is an economic strategy that enables the secretion of lipids from microalgae into the medium. Singh et al. [93] introduced acyl-acyl carrier thioeseterase gene into wild type *Synechocystis* sp., enabling the secretion of fatty acids up to 1.8 ± 0.06 mg/L. The transformed cyanobacteria secreted 197 ± 14 mg/L of fatty acids, which is 109 times higher than that secreted by the wild strain. Furthermore, by deleting the competitive genes responsible for fatty acid synthesis and removing the genes specific for surface proteins, fatty acid synthesis was enhanced in microalgae. These genetic modification strategies are not only energy-efficient but also non-toxic, as they do not involve the use of toxic chemicals for lipid extraction. Despite these advantages, the drawbacks of this technique includes cell fragility and reduced CO_2 aeration, which affect cell photosynthesis. Therefore, this strategy required substantial modification for improved lipid yield at industrial scale.

Prospects of transcription factors and genome editing for enhanced lipid production

Metabolic engineering has showed potential for enhanced lipid content, but several drawbacks still hinder maximizing the technique's potential. For the past two decades, zinc finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALENs) have been used as promising genome editing tools [94]. The zinc-finger domain comprises of 3–6 nucleotide triplets and DNA cleavage domain (nucleases) that function as dimers. Some successful studies on genome editing using ZFN in *Chlamydomonas* have been reported [95]. Although ZFNs offer few off-target effects, identifying 3 and 6 triple base targets for cleavage may not be possible in some instances, making the method laborious and challenging to apply for each editing study [95]. Unlike ZFNs, TALENs recognize single nucleotide and can amend any sequence [96]. However, the TALENs process is susceptible to cytosine methylation, requires a separate design for each nuclease, and has limited for off-target effects [97].

Transcription factor-mediated strategies, such as using GmDof4 from soybean, LEAFY COTYLEDON2 (LEC2) and WRINKLED1 (WRI1) from Arabidopsis, have been observed to enhance lipid content in several microalgae. For instance, in *C. reinhardtii*, over-expression of stress-induced transcription factors was observed to produce a 50% increase in lipid yield [98]. Over-expression of LEC2 transcription factor in tobacco under the control of acetaldehyde inducible promoter (Alc) led to an increase in lipid content from 2.9% to 6.8% [99].

Genome editing approach

In comparison to ZFNs and TALENs, recent investigations have shown that clustered regularly interspaced short palindromic repeats (CRISPRs) have greater potential for making desirable changes in the DNA for enhanced lipid content. CRISPRs are loci of DNA with tandem repeats of base sequences and are mostly associated with Cas genes, which code for CRISPRs related proteins. This system is a prokaryotic immune system that provide resistance to phages and plasmids [100].

CRISPR functions by identifying the spacer sequences recognizable and excising these exogenous genetic elements, similar to the RNAi mechanism [99]. Among eleven types of CRISPRs, type II CRISPR/Cas systems are widely adapted as a genome editing (GE) tools and differ from others by the presence of protospacer adjacent motif (PAM) sequence and a second RNA called trans-acting CRISPR RNA (tracrRNA), which together act in the maturation and redirecting of the Cas9 nuclease to DNA [101]. Furthermore, crRNA and tracrRNA are fused to form a 'guide' RNA (sgRNA or gRNA), which has huge potential in genome editing [102]. In genome editing process, the CRISPR/Cas9 system use gRNA to target specific nucleic acid for degradation through nuclease. Unlike ZFN and TALEN, gRNA synthesis and designare easier and more cost-effective than the other protein domains of CRISPR [103]. Besides, the CRISPR/Cas system has shown improved efficiency over ZFNs and TALENs with desirable mutations and can also be employed to introduce multiple genes by using multiple gRNAs (multiplexed mutations). Although the CRISPR/Cas system has great advantages over ZFNs and TALENs, it has the drawback of causing mutations at non-specific loci (off-target) [103].

Cas9 expression in cyanobacteria *S. elongatus* UTEX2973 and PCC7942 and in the eukaryotic microalgae *C. reinhardtii* showed toxicity due to consistent off-target expression. Off-site targeting often results in cell toxicity and hampers the technology's potential [104]. A recent study comparing chromosomal and episomal expression of CRISPR/Cas9 in *P. trocornutum* showed that in both methods, mutation ratio is similar; however, episomal mutants required more growth time, while chromosomal expression of Cas9 showed re-editing genes with small indels [105]. This implies that the design for efficient Cas protein coupled with sgRNA expression is important to consider. To overcome this problem, fusing codon optimized Cas gene with nuclear localization signals could be a viable strategy for efficient nuclear DNA targeting [106]. Additionally, to increase the specificity, sgRNA can be constructed with flanking self-cleaving ribozyme ends or engineered hairpin structures of sgRNA spacer, or highly efficient sgRNA sequences can be selected using web tools. Besides designing constructs, delivery method and regulatory sequences could significantly enhance genome editing efficiency [107].

In a recent study, a genome editing efficiency of 30% was achieved for the adenine phosphoribosyl transferase (Apt9) gene in *C. reinhardtii* through cell agitation with glass bead before particle bombardment [108]. Furthermore, Tanwar et al. [109] reported 60% genome editing efficiency in *P. tricornutum* using sgRNA, Cas9, antibiotic-marker elements expressed within a plasmid containing endogenous promoters, terminators and introns. In addition to CRISPR/Cas9, other strategies such as Cas12a variant and Cas9 ribonucleoprotein complexes (RNP) could be deployed for higher genome editing efficiency [110].

Integrating Omics with Gene Editing Tools

Integrating data generated through various omics tools during genome-scale metabolic modelling has significant implications for enhancing lipid production in microalgae. Rapid advances in high throughput analytical techniques for sequencing genome of organisms and measuring gene expression, protein, lipid and other metabolites have fundamentally altered metabolic modelling paradigms. Above all, the cost of data creation and analysis has significantly decreased, enabling exponential growth in omics data generation for an organism within a very short period [111]. The effect of omics data in genome-scale metabolic modelling of microalgae can be useful in the following contexts.

- Identification of Lipid Biosynthesis Pathways: Transcriptomics and proteomics data are particularly useful in identifying genes and enzymes involved in biosynthesis pathways. This approach has already been successful in fungal strains [112] and similar approach can be extended to microalgae. Analyzing gene expression and protein abundance data helps researchers pinpoint the key enzymes responsible for

lipid synthesis during fatty acid biosynthesis and triacylglycerol assembly.

- Quantitative Assessment of Metabolic Fluxes: Omics data provide quantitative information about the expression levels of genes, proteins, and metabolites involved in lipid metabolism. Integrating this data into metabolic models allows for the estimation of metabolic fluxes through lipid biosynthesis pathways under different growth conditions. This information is crucial for predicting lipid production rates and optimizing metabolic engineering strategies [113].
- Optimization of Growth Conditions: Omics data provide researchers with necessary understanding of the impact of various culture conditions (e.g., nutrient availability, light intensity, temperature) on lipid metabolism in microalgae. Such information helps optimize growth conditions to enhance lipid production [114].
- Dynamic Modelling of Lipid Production: Omics data enable the development of dynamic models that can simulate lipid production over time. As reported by Wang et al. [106], integrating time-resolved omics data into dynamic modeling approaches helps predict lipid metabolism based on dynamic changes in environmental conditions or genetic perturbations for sustained lipid production in microalgae cultures.
- Flux Balance Analysis (FBA) is a computational method used to analyse the flow of metabolites and predict the behaviour of biochemical pathways in various organisms, including microalgae. TAG biosynthesis is a vital pathway for lipid production, which has applications in the biofuel and nutraceutical industries. To perform FBA for TAG synthesis, a metabolic network model comprising all relevant biochemical reactions (such as glycolysis, carbon fixation, pentose phosphate pathway, fatty acid synthesis and TAG assembly) need to be constructed [115]. This should be followed by defining certain constraints (like nutrient and light availability, rate of CO_2 uptake) and objectives (maximizing TAG and biomass production) for the FBA simulation [116]. Through mathematical optimization techniques, FBA calculates the fluxes/rates of all biochemical reactions that satisfies the defined constraints and optimizes the objective function. The output provides insights into the functioning of metabolic network under optimized conditions and identify strategies for enhancing TAG production. Several software tools like COBRA Toolbox in MATLAB, COBRApy in Python are available to perform flux balance analysis.

Challenges and Future Perspective

Metabolic engineering of microalgae for lipid production presents several challenges, but with ongoing research and technological advancements, promising future perspectives are emerging. Many microalgae species have inherently low lipid productivity, making it challenging to achieve economically viable lipid yields for industrial applications. Additionally, microalgal metabolism is highly complex, with interconnected metabolic pathways. Identifying and engineering key metabolic pathways for increased lipid production while minimizing undesirable side effects is another significant challenge.

Lipid production in microalgae mostly depends upon the availability of nutrients and light intensity. Deviation from the required concentration may lead to a drastic reduction in the lipid production, so optimizing nutrient utilization and developing strategies for nutrient recycling are important for sustainable lipid production. Furthermore, many microalgal species lack robust genetic tools and efficient transformation methods, hindering the ability to engineer them for improved lipid production.

Developing genetic engineering tools, optimizing transformation protocols, utilizing bioinformatics tools and machine learning algorithms for data analysis and model prediction for a wide range of microalgae species are essential. Additionally, screening natural biodiversity and selecting promising strains for further engineering can accelerate the development of high-yielding lipid-producing microalgae strains. Overall, overcoming the challenges associated with metabolic engineering of microalgae for lipid production requires interdisciplinary collaboration and continued innovation across multiple fields.

Conclusion

The exploitation of microalgae as a source for quality lipids is highly desirable due to advantages like shorter incubation time, higher lipid content. Traditionally biochemical approaches have resulted in meager lipid content. To improve lipid content, metabolic engineering approaches coupled with genome editing technique could be useful. Targeting and overexpression of genes responsible for FAS and TAG biosynthesis could result in higher lipid content, particularly through the co-expression of *ACC* and *tesA* genes. Additionally, repression of competitive pathways, such as AGP-ase and PEPC genes, leads to enhanced TAG formation. This process is important for streamlining photosynthetic carbon towards lipid synthesis rather than direct manipulation of lipid synthesis.

Moreover, a multigene approach using CRISPR-Cas9 technique could be employed for an efficient genome editing process. Genome editing and metabolic engineering techniques are further envisioned to improve the designed strains. Embracing advanced techniques coupled with fermentation strategies could increase the lipid content in microalgae. Unprecedentedly, the energy demand could be satiated with microalgae to bolster sustainable energy. Furthermore, biorefinery development could alleviate the cost incurred in biodiesel production, thus creating a circular economy, which is inevitable for the commercial viability of the technology.

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