frontiers in Marine Science

REVIEW
published: 21 November 2018
doi: 10.3389/fmars.2018.00382

Tailoring Microalgae for Efficient Biofuel Production

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Depleting fossil fuel, soaring prices, growing demand, and global climate change concerns have driven the research for finding an alternative source of sustainable fuel. Microalgae have emerged as a potential feedstock for biofuel production as many strains accumulate higher amounts of lipid, with faster biomass growth and higher photosynthetic yield than their land plant counterparts. In addition to this, microalgae can be cultured without needing agricultural land or ecological landscapes and offer opportunities for mitigating global climate change, allowing waste water treatment and carbon dioxide sequestration. Despite these benefits, microalgae pose many challenges, including low lipid yield under limiting growth conditions and slow growth in high lipid content strains. Biotechnological interventions can make major advances in strain improvement for the commercial scale production of biofuel. We discuss various strategies, including efficient transformation toolbox, to increase lipid accumulation and its quality through the regulation of key enzymes involved in lipid production, by blocking the competing pathways, pyramiding genes, enabling high cell biomass under nutrient-deprived conditions and other environmental stresses, and controlling the upstream regulators of targets, the transcription factors, and microRNAs. We highlight the opportunities emerging from the current progress in the application of genome editing in microalgae for accelerating the strain improvement program.

Keywords: microalgae, biofuel, lipid accumulation, biomass, abiotic stress, transcription factor, microRNA, genome editing

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Edited by:
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Specialty section:
This article was submitted to Marine Biotechnology, a section of the journal Frontiers in Marine Science

Received: *02 August 2018*
Accepted: *28 September 2018*
Published: *21 November 2018*

Citation:
Sharma PK, Saharia M, Srivstava R, Kumar S and Sahoo L (2018) Tailoring Microalgae for Efficient Biofuel Production. Front. Mar. Sci. 5:382. doi: 10.3389/fmars.2018.00382

INTRODUCTION

Depleting fossil fuels, soaring international crude oil prices, the energy crisis, and alarming global warming reports have upsurged global interest in alternative renewable energy sources (Behera et al., 2015). Biomass-derived liquid fuels have emerged as the most attractive source of renewable energy when compared with solar, tidal, and wind energies as they can be conveniently stored and easily transported and used directly in automobiles and other transport engines (Scott et al., 2010). Biofuels made from photosynthetic organism-based feedstocks, including land plants and aquatic microalgae, provide enormous opportunities to meet the global energy demand, satisfying carbon-neutral solutions and enabling carbon dioxide (CO_2) sequestration from the atmosphere (Stephenson et al., 2011; Ravindran et al., 2017). The cultivation of terrestrial crops for biofuel feedstock competes with food crops for arable land, compromises the price of edible oil if used as biofuel, and often meets less than the anticipated overall energy

to input energy demand of the biofuel required over the life cycle (Scott et al., 2010). Sustainable biofuel production using feedstocks, other than terrestrial crops is therefore very promising (Courchesne et al., 2009).

Oil rich microalgae have emerged as the most realistic feedstock for the large-scale production of biofuels as they do not require fresh water (Stephenson et al., 2011). Moreover, these photosynthetic organisms are known to fix solar energy into biomass at efficiencies exceedingly higher than terrestrial plants on a land area basis (Klok et al., 2014). Many oleaginous microalgal species accumulate very large amounts of lipids in the form of triacylglycerol (TAG), the convenient source of biofuel often exceeding 70% of dry cell weight in certain species (Scott et al., 2010). Apart from this, some microalgae produce valuable coproducts such as pigments, antioxidants, edible proteins, long-chain polyunsaturated fatty acids, and specialized bio-pharmaceuticals, favoring biorefineries to help to offset the biofuel production cost (Klok et al., 2014; Jagadevan et al., 2018). However, many challenges need to be overcome to realize this potential of large scale production of microalgal biofuels as a sustainable and cost-effective alternative for fuel. The major bottlenecks are the absence of two attributes, high lipid content and fast growth rate in existing microalgal species (Fan et al., 2014; Ghosh et al., 2016; Chen et al., 2017) and inefficient light harvesting capacity in naturalized growth conditions (Stephenson et al., 2011). Understanding the molecular intricacies of lipid metabolism, especially TAG biosynthesis, their genetic and metabolic regulations for triggered metabolic flux, target subcellular storage location, and possible secretion, could assist in strain improvement, thus, maximizing the cost effectiveness of biofuel production in microalgae.

In this review, we provide the current knowledge and potential biotechnological routes to enhance TAG accumulation, synchronized biomass production, improved light harvesting, maximize the utilization of available nutrients, and adapt to variable and fragile conditions prevalent in real-life conditions in aquatic habitats.

MICROALGAE AS A BIOFUEL SOURCE

Triacylglycerol is the primary form of energy storage in microalgal cells, which comprises 60–70% of the dry cell weight (Hu et al., 2008; Scott et al., 2010). Each TAG molecule consists of a glycerol backbone to which three fatty acid (FA) moieties are anchored. Each FA molecule is classified, depending on the degree of unsaturation, as either saturated FA (SFA), monosaturated FA (MUFA), or polyunsaturated FA (PUFA). Therefore, relative abundance of these diverse FAs in TAG decides the utility of TAG molecules for specific applications, including their use as transportation fuel, high-value nutrient supplements, emulsifiers, and industrial polymers. Microalgae-derived biomass can supply a wide range of biofuels such as biodiesel, bioethanol, biohydrogen, biomethane, and bioelectricity. Among all projected applications, microalgae-derived oils are the most promising for the production of biofuel (Shuba and Kifleb, 2018). Therefore, current microalgal studies worldwide primarily focus on enhancing lipid accumulation in microalgae under diverse growth conditions for higher oil production.

OVERVIEW OF OIL ACCUMULATION IN MICROALGAE

The biosynthesis of lipid molecules in microalgae, in particular that of the 'green algal' lineage, is an interconnected network of multiple metabolic pathways. It begins in the chloroplast of microalgal cells where the photosynthetic machinery utilizes atmospheric carbon to yield starch, which is later catabolized through glycolysis to form the building blocks of FAs and TAGs (**Figure 1**). Incorporation of these precursors in the form of acetyl-CoA to synthesize malonyl-CoA by acetyl-CoA carboxylase (ACC) initiates FA biosynthesis. Conversion of malonyl-CoA to malonyl-ACP marks the beginning of the elongation phase of FA biosynthesis, catalyzed by a prokaryotic type-II FA synthase (FAS II) localized in the stroma (Blatti et al., 2013; Shtaida et al., 2015). The process, however, is interrupted intermittently by fatty-ACP thioesterases (FATs) and as a consequence, the newly synthesized FAs escape from the acyl-ACP complex (Blatti et al., 2013). The generated free FA pool is assimilated while synthesizing various cellular lipids. The synthesis of storage lipids, in particular, follows a set of reactions as part of the Kennedy pathway, which involves the incorporation of FAs into a glycerol backbone to form TAG. In the Kennedy pathway, acyl-CoA or acyl-ACP acts as an acyl donor while microalgae can follow an alternate pathway for sourcing acyl groups for TAG synthesis, which uses phospholipids as acyl donors (Li-Beisson et al., 2015). The rapid stride in the manipulation of oil biosynthesis in plants for oil enhancement provides clues for engineering the microalgal lipid metabolism, provided the differences in the lipid metabolism process between microalgae and plants are understood.

A pioneering understanding of the model plant's lipid metabolism laid the foundation for the identification of key genes of TAG biosynthesis in microalgae. Microalgal omics has enabled the prediction and accurate annotation of lipid metabolism genes. Most of the predicted genes involved in FA biosynthesis are present as a single copy in the *Chlamydomonas* genome, suggesting that their encoded enzymes operate both in the chloroplast and mitochondria, in contrast to higher plants where compartment-specific enzymes increase the complexity of FA biosynthesis. Many copies of genes encoding acyl-CoA: diacylglycerol acyltransferases (DGATs) were predicted in the *Chlamydomonas* genome, compared with the fewer copies observed in higher plants, suggesting a crucial role of TAG in microalgal cell physiology (Liu and Benning, 2013). The model microalgae, *Chlamydomonas*, accumulates starch as the primary form for energy storage; however, stress redirects it to TAG formation. This carbon sifting is possibly due to adaptation for maintaining membrane integrity. Triacylglycerol molecules are catabolized back to release FAs upon stress reversal and used for membrane synthesis. Besides this, TAG molecules also act as a sink for channeling excess energy and reductive equivalents,

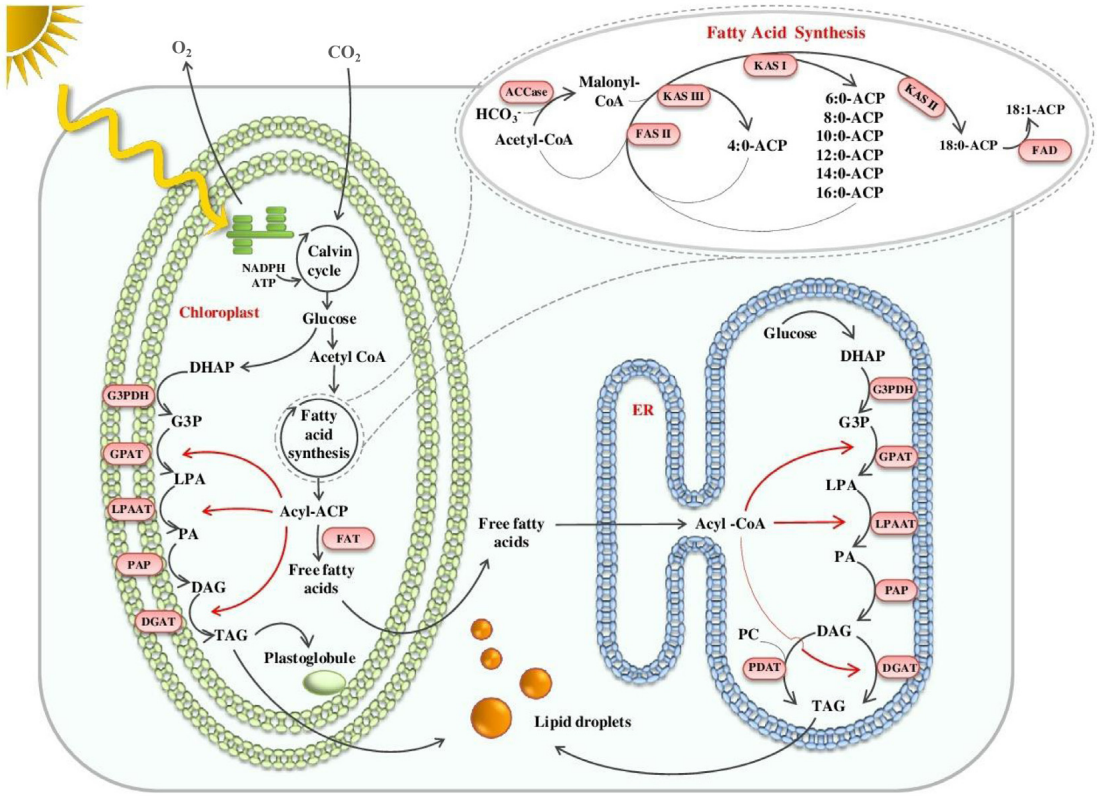

FIGURE 1 | Schematic illustration of TAG synthesis in microalgae. NADPH; Nicotinamide adenine dinucleotide phosphate; ATP, Adenosine Triphosphate; DHAP, dihydroxyacetone phosphate; G3P/G3pDH, Glyceraldehyde 3-phosphate / G3P dehydrogenase; GPAT, Glycerol 3-phosphate acyltransferase; PA/LPA/LPAAT/PAP, Phosphatidic acid/Lyso-PA/LPA acyltransferase/PA phosphatase; DAG/DGAT, di-Acylglycerol/ DAG acyltransferase; FAT, Fatty acyl-ACP thioesterase; ACP, Acyl-carrier protein; ER, Endoplasmic reticulum; PC, Phosphatidylcholine; PDAT, Phospholipid:DGAT; ACCase, Acetyl-CoA carboxylase; FAS, Fatty acid synthase; KAS, 3-ketoacyl-ACP synthase; FAD, Flavin adenine dinucleotide.

which otherwise risk cellular metabolisms of microalgae (Sharma et al., 2012; Shtaida et al., 2015). Another marked difference is the presence of unique lipids such as betaines, which offer an advantage to microalgae in adapting to nutrient-limiting conditions. On the contrary, higher plants exclusively use phosphate-associated lipids that play a central role in maintaining the membrane integrity (Van Mooy et al., 2009; Liu and Benning, 2013).

In contrast to plants, microalgae contain distinct acyl groups in storage lipids (Garay et al., 2014). In plants, a majority of TAGs are assembled in the endoplasmic reticulum (ER), whereas in microalgae, a major fraction of TAGs are assembled *de novo* in the chloroplast (Giroud et al., 1988; Fan et al., 2011). The key difference between these two organelle pathways is the presence of a 16-carbon acyl group in the sn-2 position of the glycerol backbone in lipids derived from the plastid pathway while an 18-carbon acyl group occupies the same position in ER-generated lipids (Xu et al., 2016). However, the recent identification of an ER-localized acyltransferase enzyme having specificity toward C-16 acyl-donor suggests that the difference in the prokaryotic and eukaryotic pathways may not be due to spatial separation but due to complex systemic control (Kim et al., 2018).

The triggers for TAG accumulation also appear to differ in plants and microalgae. In plants, TAGs are accumulated in the developing seeds controlled through developmental signaling, whereas stress conditions trigger lipid accumulation, but the underlying mechanism is not fully established (Liu and Benning, 2013; Garay et al., 2014), presumably because of the coordinated sequential consequence of cell cycle arrest. Cornell et al. (1977) found that, at least in some cell culture types, lipid synthesis is controlled at certain checkpoints in the cycle. As stressors often obstruct cell cycle progression, it led to a speculation that they effectively trigger lipid accumulation (Kwok and Wong, 2005). However, this type of induction process requires validation. Changes in buoyancy due to lipid accumulation, assisting in motility and protection of microalgae, are possibly an adaptation in aquatic environments. Although several external agents initiate lipid accumulation, metabolic regulators such as nitrogen response regulator 1 (NRR1) and phosphorus starvation response 1 (PSR1) are core to this process as they sense the changes in the cytoplasmic environment and activate various pathways associated with TAG biosynthesis (Gargouri et al., 2015).

CONVENTIONAL MEANS FOR IMPROVING OIL PRODUCTION IN MICROALGAE

Cultural manipulations such as subjecting cells to stressors, like nutrient depletion, variable light intensity, temperature, salinity, and pH, are conventionally used to enhance lipid accumulation within the cells' biological limits (Bartley et al., 2014; Chu et al., 2015; Suyono et al., 2015). Among these stressors, nitrogen starvation is the most potent for lipid enhancement (Belotti et al., 2013). Dual-stage cultivation (Doan and Obbard, 2014) and coculture techniques with chemical additives (Singh S.K. et al., 2016) facilitate the enhancement of lipid production in culture systems. However, the genetic modification of microalgae offers more avenues for the precise control of target mechanisms leading to enhanced cellular lipid accumulation under normal growth conditions (Xue et al., 2015; Lim and Schenk, 2017). Genome sequence databases and pathway databases (KEGG, dEMBF, and MetaCyc) are now valuable resources for implementing targeted genetic manipulation for higher lipid biosynthesis in microalgae (Ogata et al., 1999; Caspi et al., 2014; Misra et al., 2016). To date, successful nuclear transformation has been reported in more than 40 microalgal species, and considering the challenges posed by the enormous physiological and genetic diversities existing among these microalgal species, this number appears significant (Gangl et al., 2015; Gimpel et al., 2015; Doron et al., 2016). In recent years, different tools such as metabolic selection markers and techniques like 'CHYSEL' have been developed to target both plastids and nuclear genomes, allowing for the expression of target genes (Specht et al., 2010; Rasala et al., 2014). With these rapid strides in microalgal biotechnology, 'algomics' and integrated system-biology modeling have deepened the understandings of interconnections between genes, proteins, and metabolites (Jamers et al., 2009; Koussa et al., 2014; Reijnders et al., 2014; Benmoussa, 2016). Such integrated multidisciplinary studies can provide a clear picture of oil and high-value metabolite biosynthesis pathways, thereby accelerating strain improvement for the commercialization of microalgal biofuel (Lauersen et al., 2015; Patra et al., 2015; Barahimipour et al., 2016).

GENETIC ENGINEERING STRATEGIES FOR LIPID ENHANCEMENT IN MICROALGAE

Enhancing oil synthesis in microalgae primarily depends on the manipulation of enzymes involved in lipid biosynthesis or other competitive parallel pathways aimed to divert the carbon and reductive equivalents flux toward lipid biosynthesis (see **Figure 2**). The most widely used technique is the manipulation of individual genes encoding various steps of a metabolic pathway; however, owing to the multi-factorial regulation of lipid biosynthesis in microalgae, this strategy has seen mixed success (Bajhaiya et al., 2017). Recently, the transcriptional regulation of oil biosynthesis has brought widespread interests to control the activity or expression of multiple components of a metabolic pathway simultaneously (Courchesne et al., 2009). Additionally, attempts to manipulate various other targets, such as improving light use efficiency, controlling cell quiescence, and improving carbon sequestration, etc., which indirectly influence the lipid content by altering cell growth characteristics, have gained attention (**Figure 2**).

MANIPULATION OF THE OIL BIOSYNTHESIS PATHWAY

In contrast to subjecting microalgae to growth limiting stress conditions, efforts targeting enhanced lipid accumulation during the exponential growth phase are more practical means. According to Ohlrogge and Jaworski (1997), the FA supply regulates the lipid biosynthesis process, and therefore, some of the earliest attempts have been made to increase the expression of key enzymes involved in FA biosynthesis. Microalgal metabolic engineering aimed at increasing FA supply to lipid synthesis was first attempted by overexpressing the acetyl-CoA carboxylase gene (*ACCase*), which codes for the enzyme that carboxylates acetyl-CoA to malonyl-CoA, the first committed step in FA synthesis (Dunahay et al., 1996). Although the transformed microalgae showed a two- to three-fold increase in ACCase activity, it was not accompanied with an increase in FA content (Sheehan et al., 1998). This clearly indicated that the upregulation of ACCase had no direct impact on lipid biosynthesis. However, the simultaneous overexpression of a subunit of ACCase (accD) along with malic enzyme (ME), responsible for the conversion of malate to pyruvate, was successful in elevating the total lipid content in microalgae (Talebi et al., 2014). Therefore, it appears that ACC is not the sole rate-limiting step in lipid biosynthesis, indicating the existence of a secondary rate-limiting step apart from ACC. Limited availability of precursors for the whole lipid synthesis process (acetyl-CoA and glucose-6-phosphate) could be the secondary bottleneck in case ACC is overexpressed. Various studies attempted to elevate the intracellular concentration of lipogenic precursors, by tailoring the enzymes involved in the generation of reducing potential (NADH) and in carbon metabolism (Gimpel et al., 2015). Manipulation in the expression of several enzymes like pyruvate dehydrogenase, phosphoenolpyruvate carboxylase, acetyl-CoA synthase, NAD(H) kinase, and glycerol kinase has significantly enhanced the lipid content in different microalgal species without adversely affecting cell growth (**Table 1**).

Another possible strategy to increase the intracellular lipid content is by blocking the metabolic pathways that are competitive to lipogenesis, for example, starch synthesis and lipid catabolism. Some strains of microalgae use starch as the primary storage metabolite, and suppressing starch synthesis can funnel the carbon flow toward lipid biosynthesis (Ravindran et al., 2017). Knockdown of key genes involved in starch synthesis showed elevated lipid accumulation by redirecting carbon pool toward lipogenesis (**Table 1**). Since accumulation of starch as an energy storage molecule is not universal in microalgae (León-Saiki et al., 2017), the suppression of lipid catabolism is a more legitimate option to enhance the lipid content irrespective of

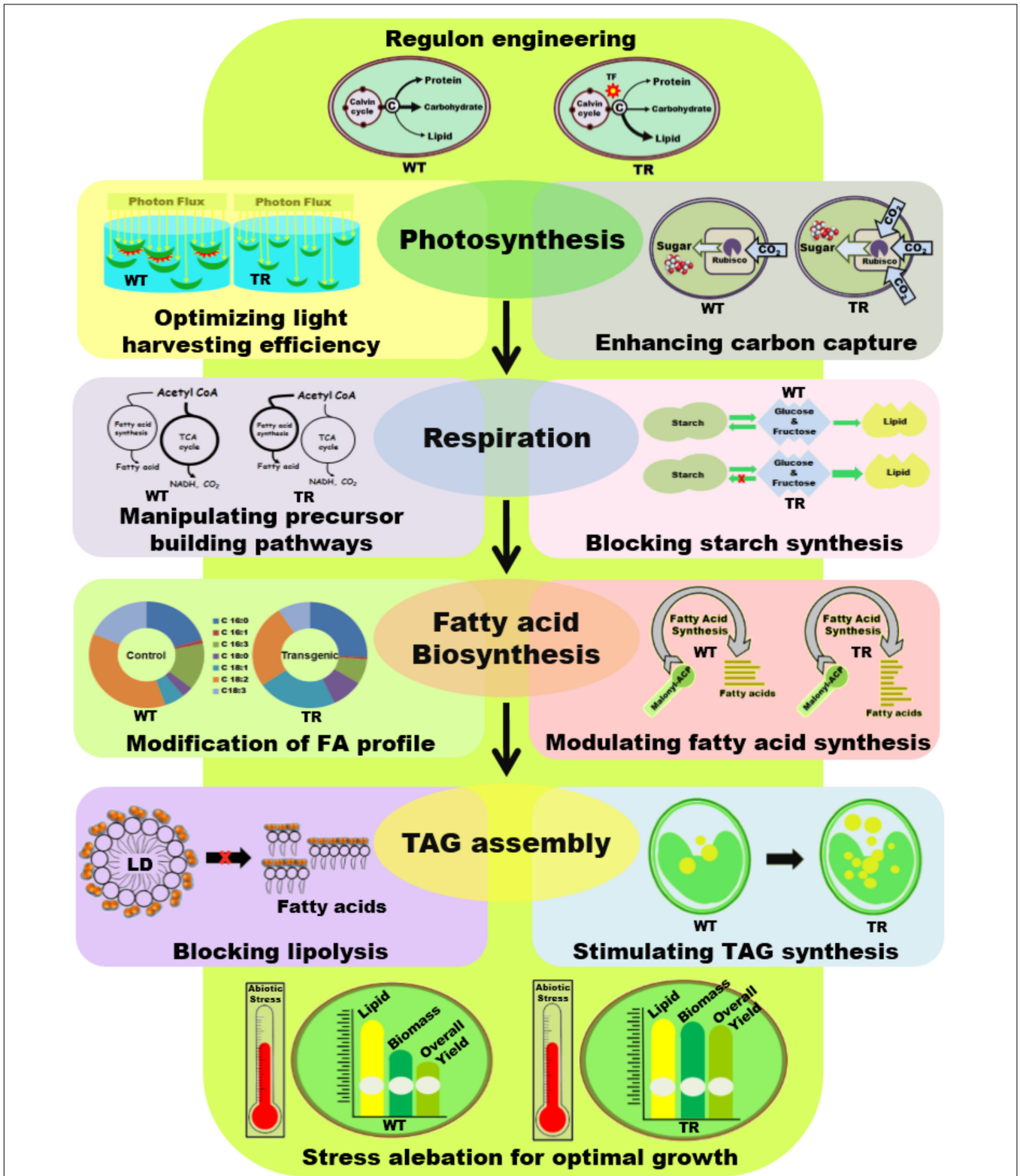

FIGURE 2 | Schematic illustration of different genetic engineering strategies applied in microalgae for biodiesel application. WT, Wild type cells; TR, Transgenic cells; TF, Transcription factor; TCA, Tricarboxylic acid cycle; NADH, Nicotinamide adenine dinucleotide; FA, Fatty acid; LD, Lipid droplet.

TABLE 1 | Summary of genes manipulated for oil enhancement.

Gene	Mode of action	Species	Observation	Reference
Enhancing fatty acid biosynthesis:				
Acetyl CoA carboxylase (ACCase)	Overexpression	*C. cryptica*	∼2–3 X ACCase activity No changes in lipid content	Dunahay et al., 1996
	Overexpression	*D. salina*	1.14 X Total lipid content	Talebi et al., 2014
Acetyl CoA synthase (ACS)	Overexpression	*Schizochytrium* sp.	1.3 X Biomass productivity and improved FA profile	Yan et al., 2013
	Overexpression	*C. reinhardtii*	2.4 X TAG content	Rengel et al., 2018
Acyl-ACP reductase	Overexpression	*C. merolae*	3 X TAG accumulation	Sumiya et al., 2015
Manipulation of carbon partitioning:				
Malic enzyme (ME)	Overexpression	*P. tricornutum*	2.5–2.7 X Lipid content	Xue et al., 2015
ADP-glucose pyrophosphorylase (*sta6*)	Suppression	*C. reinhardtii*	10 X TAG content	Li et al., 2010
	Suppression	*Coccomyxa* sp.	Higher lipid content	Takahashi et al., 2018
Isoamylase (*sta7-10*)	Suppression	*C. reinhardtii*	∼1.8 X total lipid	Work et al., 2010
Pyruvate dehydrogenase kinase (PDK)	Suppression	*P. tricornutum*	+82% Neutral lipid	Ma et al., 2014
Phosphofructo-2-kinase (PFK2)	Overexpression	*T. pseudonana*	Increased glycolytic activity	Abbriano et al., 2018
Citrate synthase (CIS)	Suppression	*C. reinhardtii*	∼3 X TAG productivity	Deng et al., 2013a
Phosphoenolpyruvate carboxylase (PEPC 1)	Suppression	*C. reinhardtii*	+20% TAG level	Deng et al., 2014
Phosphoenolpyruvate carboxylase 2	Suppression	*C. reinhardtii*	+14–28% Oil content	Deng et al., 2011
UDP-Glucose pyrophosphorylase (UGPase)	Suppression	*P. tricornutum*	45 X TAG accumulation	Daboussi et al., 2014
	Suppression	*P. tricornutum*	+25% Total lipid content	Zhu et al., 2016
Glycerol kinase	Overexpression	*F. solaris*	+12% Lipid productivity	Muto et al., 2015
	Overexpression	*S. quadricauda*	1.6 X G3P content	Gomma et al., 2015
Glycerol-3-phosphate dehydrogenase (G3PDH)	Overexpression	*S. quadricauda*	1.9 X intracellular G3P level	Gomma et al., 2015
	Overexpression	*P. tricornutum*	1.9 X Neutral lipid content	Yao et al., 2014
	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012
Increasing intracellular reducing equivalents:				
NAD(H) kinase	Overexpression	*C. pyrenoidosa*	∼1.6 X lipid content	Fan et al., 2015
Ferrodoxins (FDX)	Overexpression	*C. reinhardtii*	2.5 X lipid level	Huang et al., 2015
Blocking TAG hydrolysis:				
TGL1 (triglyceride lipase 1)	Suppression	*P. tricornutum*	Increased TAG level in lipid extracts	Barka et al., 2016
PNPLA3	Overexpression	*P. tricornutum*	1.7 X Neutral lipid content	Wang et al., 2015
	Overexpression	*P. tricornutum*	1.55 X TAG content	Wang et al., 2018
Thaps3_264297	Suppression	*T. pseudonana*	∼3.3–4.1 X lipid content	Trentacoste et al., 2013
LIP (diacylglycerol lipase)	Suppression	*C. reinhartdii*	Delay in TAG hydrolysis	Li et al., 2012
cht7 (TAG lipase)	Suppression	*C. reinhartdii*	10 X TAG level	Tsai et al., 2014
Lipid droplet protein (StLDP)	Overexpression	*P. tricornutum*	Increased lipid droplet accumulation	Yoneda et al., 2018
Oleosin protein 3 (AtOLEO3)	Overexpression	*P. tricornutum*	1.4 X TAG content	Zulu et al., 2018
Increasing TAG content (single gene):				
GPAT	Overexpression	*C. reinhardtii*	∼1.5 X TAG content	Boyle et al., 2012
	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012
LPAAT	Overexpression	*C. reinhardtii*	+20% TAG	Yamaoka et al., 2016
	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012
PAP	Overexpression	*C. reinhardtii*	+7.5 to 21.8% lipid content	Deng et al., 2013b
	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012

(Continued)

TABLE 1 | Continued

Gene	Mode of action	Species	Observation	Reference
DGAT	Overexpression	*N. oceanica*	+69% lipid content	Li et al., 2016
	Overexpression	*C. reinhardtii*	2 X TAG content	Ahmad et al., 2015
	Overexpression	*T. pseudonana*	1.9 X TAG content	Manandhar-Shrestha and Hildebrand, 2015
	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012
	Overexpression	*S. obliquus*	∼2 X lipid content	Chen et al., 2016
	Overexpression	*C. reinhardtii*	Unchanged lipid content	La Russa et al., 2012
	Overexpression	*C. reinhardtii*	2.5 X TAG content	Iwai et al., 2014
	Overexpression	*P. tricornutum*	Increased neutral lipid content	Niu et al., 2013
	Overexpression	*T. chui*	+40–115% TAG content	Úbeda-Mínguez et al., 2017
PDAT	Overexpression	*C. minutissima*	+30–40% TAG content	Hsieh et al., 2012
	Overexpression	*C. reinhardtii*	32% increase in TAG content	Zhu et al., 2018
Increasing TAG content (multiple gene):				
Quintuple construct containing G3PDH, GPAT, LPAAT, PAP, DGAT, and PADAT	Overexpression	*C. minutissima*	∼1.8 X TAG content	Hsieh et al., 2012
Co-expression of GPAT and DGAT	Overexpression	*P. tricornutum*	2.6 X Total lipids	Zou et al., 2018
Co-expression of DGAT and oleosin	Overexpression	*P. tricornutum*	3.6 X TAG content	Zulu et al., 2018
Manipulation of transcription regulators:				
CONSTANS like	Suppression	*C. reinhartdii*	+25% TAG level	Deng et al., 2015
P-II Like	Suppression	*C. reinhartdii*	∼1.3 X TAG content	Zalutskaya et al., 2015
bHLH2	Overexpression	*N. salina*	+33% FAME productivity	Kang et al., 2015
Wrinkled1 (WRI1)	Overexpression	*N. salina*	+64% FAME yield	Kang et al., 2017
basic leucine zipper (bZIP)	Overexpression	*N. salina*	+203% Neutral lipids	Kwon et al., 2017
PSR1	Overexpression	*C. reinhartdii*	7 X lipid content	Ngan et al., 2015
ZnCys	Suppression	*N. gaditana*	+103% Lipid productivity	Ajjawi et al., 2017
Dof	Overexpression	*C. ellipsoidea*	∼1.5 X lipid content	Zhang et al., 2014
	Overexpression	*C. reinhardtii*	∼2 X Total lipid	Ibanez-Salazar et al., 2014
Modifying fatty acid profile:				
Acyl-ACP thioesterase	Overexpression	*Nannochloropsis*	∼1.5 X C 14:0 and C 16:1 fatty acids	Ozaki, 2016
C 12 thioesterase	Overexpression	*P. tricornutum*	∼1.2 X C 12:0 FA accumulation	Radakovits et al., 2011
C 14 thioesterase	Overexpression	*P. tricornutum*	2 X C 14:0 Fatty acid	Radakovits et al., 2011
	Overexpression	*D. tertiolecta*	2 X C 14:0 & C 12:0 FA content	Lin et al., 2018
Stearoyl-ACP desaturase (SAD)	Overexpression	*C. reinhardtii*	2.7 X C 18:1 FA content	Hwangbo et al., 2013
	Suppression	*C. reinhardtii*	2 X C 18:0 FA in TAG	de Jaeger et al., 2017
Delta-12 desaturase (Δ12D)	Overexpression	*N. oceanica*	4 X C 18:2 FA level	Kaye et al., 2015
Delta-5 desaturase (Δ5D)	Overexpression	*P. tricornutum*	+64–75% PUFA and MUFA	Peng et al., 2014
Delta-6 FA desaturase (Δ6D)	Overexpression	*P. tricornutum*	+47.66% in EPA (C 20:5) content	Zhu et al., 2017
Omega-3 fatty acid desaturase (ω-3 FAD)	Overexpression	*C. vulgaris*	Enhanced C18:3, n3 FA accumulation	Norashikin et al., 2018
Delta-5 elongase (Δ5E)	Overexpression	*P. tricornutum*	8 X DHA (22:6, n-3) content	Hamilton et al., 2014

'+' increase in quantity percentile; 'X' fold change; FAME, fatty acid methyl ester; FA, fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; TAG, triacylglycerol; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

the microalgal strains. For instance, inhibiting the expression of a multifunctional lipase/phospholipase/acyltransferase enzyme in *Thalassiosira pseudonana* resulted in increased lipid yields without affecting the growth (Trentacoste et al., 2013). The mutant strain showed a 2.4- to 3.3-fold higher lipid accumulation in comparison with the control, when subjected to silicon starvation. However, blocking these vital metabolic pathways (lipid catabolism and starch synthesis) may result in reduced microalgal growth and lipid yield (Radakovits et al., 2010; Chu, 2017). One way to overcome this is by using RNAi mediated gene silencing under the control of inducible promoters. Upon attaining high cell-density, the mechanism can be activated to suppress the expression of key genes involved in starch synthesis and lipid catabolism. Many such promoters have been identified in microalgae, including one with light-responsive elements in *Dunaliella* (Park et al., 2013; Baek et al., 2016).

Besides the manipulation of carbohydrate metabolism- and lipid metabolism-related genes for increasing cellular neutral lipids (TAG), the overexpression of acyltransferases has also yielded interesting outcomes (see **Table 1**). The Kennedy pathway for TAG assembly includes several steps catalyzed by different acyltransferases, including acyl-CoA: glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA: lysophosphatidic acyltransferase (LPAAT), and acyl-CoA: DGAT. These TAG assembly genes were found to be worthy targets in lipid pathway engineering (Bhowmick et al., 2015; Maravi et al., 2016). In the green microalgae *Chlorella minutissima*, simultaneous expression of five acyltransferases (phosphatidic acid phosphatase, LPAAT, glycerol-3-phosphate dehydrogenase, GPAT, and DGAT) resulted in a two-fold increase in lipid content (Hsieh et al., 2012). These instances of co-expressing multiple enzymes exemplify the effectiveness of system level control of metabolic flux toward lipid overproduction. Transcriptional regulation can have a similar effect on the systemic metabolomic flux as transcription factors can target multiple regulatory points in a metabolic pathway. Overexpression of genes encoding transcription factors targeting the upregulation of downstream lipid biosynthesis genes can result in increased oil content. In this realm, higher plants have been in the spotlight with numerous literature highlighting the benefit of transcription factor engineering for enhanced lipogenesis (Cernac and Benning, 2004; Mendoza et al., 2005). However, in microalgae, the major focus of transcriptional regulation studies is limited to a select microalgal species (see **Table 1**). In this context, the identification of endogenous transcription factors and their subsequent manipulation in their host would be more viable to trigger lipid accumulation (Tsai et al., 2014; Ngan et al., 2015; Kwon et al., 2017). Although the manipulation of transcription factors like PSR1 and compromised hydrolysis of triglycerols 7 (CHT7) have led to enhanced lipid accumulation without compromising biomass production, weak carbon partitioning for lipid synthesis still remains a bottleneck which may be overcome by finding other potential lipid-triggers (Chen et al., 2018). In one such groundbreaking effort, the knockdown of a single transcription regulator ZnCys in *Nannochloropsis gaditana* resulted in a 103% increase in lipid content, indicating a lipid yield to the tune of $\sim$5 g/m^2/day (Ajjawi et al., 2017). Even though the overexpression of endogenous transcription factors for increasing oil content in microalgae is very promising, the lengthy functional characterization process greatly limits its applications. A more direct approach is to consider the heterologous expression of a transcription factor of plant origin to regulate the microalgal lipid biosynthetic pathway. Several lipogenesis promoting transcription factors from higher plants were overexpressed in microalgae and showed to have a remarkable impact on the lipid accumulation pattern (see Table1).

In addition to engineering for enhanced oil content, it is also important to improve the quality of oil for better biodiesel fuel properties. The carbon chain length and degree of unsaturation of the FAs present in oil affect the cold flow and oxidative stability properties of the fuel. Oils derived from microalgal feedstocks commonly contain FAs of chain length between 14 and 20, mostly C16:0, C16:1, and C18:1, while the ideal should be C12:0 and C14:0 (Radakovits et al., 2010). The key factor that determines FA chain length is the thioesterase enzyme, which catalyzes the release of the FA chain from the FA synthase complex. Several acyl-ACP thioesterases specific to short FA chain length have been identified, and engineering the expression of these enzymes can successfully modify the fuel properties. Transgenic microalgae containing exogenous short-chain length biased FA acyl-ACP thioesterases have directed an increase in percent composition of myristic (C14:0) and lauric (C12:0) acids in the overall FA profile (see **Table 1**). A seamless biodiesel fuel also requires a balanced coalescence of MUFAs, SFAs, and PUFAs in oil (Durrett et al., 2008). The scant presence of MUFAs in the microalgal lipid profile (Patil et al., 2007) requires biotechnological interventions to modify the degree of unsaturation. The desaturase enzyme, which catalyzes the formation of unsaturated FAs, was targeted to manipulate the FA profile primarily in MUFA and PUFA contents (see **Table 1**). Enhancements in PUFA entities like linoleic acid and eicosapentaenoic acid are particularly noteworthy considering their high nutritional value.

Apart from biodiesel, microalgal oils can also be used to produce gasoline and jet fuel, which requires FAs with even shorter chain lengths. Even though it is possible to chemically synthesize suitable feedstocks for gasoline or jet fuel by breaking down the long chains into shorter hydrocarbon chains, the genetic engineering of microalgae to synthesize short-chain FAs will significantly reduce the production cost (Radakovits et al., 2010). For instance, the overexpression of 8:0- and 10:0-biased thioesterases from *Cuphea hookeriana* in Canola has reportedly enhanced the synthesis of short-chain FAs (Dehesh et al., 1996). Replication of this achievement of raised short-chain FA profile in different oleaginous microalgal species would have a high impact.

ENHANCING THE BIOMASS YIELD

Enhancing the biomass yield is very important as the total energy output relies on both energy density and the total biomass content (Barry et al., 2015). In photosynthetic organisms including microalgae, abiotic stress, CO_2 fixation rate, and light

utilization efficiency are the primary factors that govern biomass productivity (Chu, 2017). Engineering microalgal strains for stress tolerance and higher photosynthetic efficiency can ensure the cost-effective production of biofuel. A number of studies report transgenic microalgal strains tolerant to abiotic stress through enhanced reactive oxygen species (ROS) scavenging, hypertolerance to DNA damage, and polyploidization (see **Table 2**). Kotchoni et al. (2016) reported the manipulation of intracellular steady-state ATP levels for cold adaptation of microalgal cells. The identification of key transcription regulators and enzymes, as well as stress-responsive promoters through omics analysis, can serve as the toolbox for future genetic engineering designs.

Manipulation of carbon fixation is vital to improving the photosynthesis rate. The Calvin cycle is the initial pathway for carbon fixation in all photosynthetic organisms, and strategies seeking improvement in the photosynthetic efficiency require a breakthrough in the regulation of this pathway. Carboxylation of ribulose 1,5-bisphosphate (RuBP) and its subsequent regeneration are the checkpoints in the Calvin cycle. The enzymes that catalyze these regulatory steps are ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), fructose 1,6-bisphosphate aldolase (aldolase), and sedoheptulose 1,7-bisphosphatase (SBPase), and these three enzymes are prime targets for manipulating the Calvin cycle owing to their high flux control coefficient values (Raines, 2003; Yang et al., 2017). Among the three enzymes, Rubisco is the primary target as the carboxylation capacity of Rubisco majorly influences the rate of carbon assimilation. However, efforts to directly manipulate this enzyme have been met with limited success, owing to the complex enzyme kinetics of Rubisco, which challenges the operational understanding and makes it difficult to spot a change in the phenotype upon manipulation (Tcherkez et al., 2006). Therefore, efforts are being made to target factors that regulate Rubisco activity instead of the direct manipulation of the enzyme itself. One such target is the Rubisco activase (RCA) enzyme, which regulates the activity of Rubisco by regenerating the catalytic sites (Hazra et al., 2015). Ultimately, RCA determines the rate of carbon fixation by maintaining a high proportion of catalytically competent active sites of Rubisco. An attempt to overexpress RCA in an oleaginous microalga, *Nannochloropsis oceanica*, resulted in elevated photosynthetic activity accompanied by enhanced biomass and lipid accumulation (Wei et al., 2017). Apart from Rubisco, aldolase and SBPase are also crucial to improve carbon fixation as these enzymes are involved in the regeneration of precursor substrates for Rubisco. As a proof of concept, the overexpression of genes encoding aldolase and SBPase in different microalgal strains resulted in enhanced photosynthetic efficiency and biomass production (**Table 2**).

Abiotic factors such as availability of photon energy also affect the efficiency of carbon fixation. Photosynthetic microalgae have developed large photosynthetic antenna systems to maximize the photon absorption and conversion efficiency as an adaptation to its habitat where low light intensity is a growth limiting factor. However, the sustainability of microalgal biomass production requires large-scale cultures with high cell density. In such dense cultures, the high pigment density due to the large antenna systems limits the penetration of light into the deeper layers of the culture. Under such conditions, cells at the surface receive an excess amount of photon energy, which quickly saturates the photosynthesis process, and dissipate the excess energy through non-photochemical quenching (NPQ). At the same time, cells in the deeper layers are exposed to a low-light intensity, which compels the cells to perform respiration instead of photosynthesis (Formighieri et al., 2012). This uneven distribution of light energy leads to suboptimal photosynthetic efficiency, which in turn reduces the overall biomass productivity of the culture. One approach to enhance the photosynthetic yield is by reducing the size of light capturing antenna systems in microalgae to minimize the energy loss due to NPQ. In biological terms, antennas, or light-harvesting complex (LHC), are pigment-binding proteins, which capture the light energy and relay it to photosynthetic reaction centers. In green microalgae, they bind the majority of pigments and, therefore, are mainly responsible for the optical density of the culture. Wild type photosynthetic microalgae harbor a vast number of chlorophyll molecules associated with both photosystems I and II; however, only a few of these are essential to carry out the vital functions of photosynthesis (Simionato et al., 2013). Therefore, it is possible to improve light transmission and light absorption capacities by reducing the number of chlorophyll molecules from the LHC in microalgal cells. Mutants have been developed with truncated antenna systems in different microalgal strains through downregulating the genes encoding LHC pigment binding-proteins, which showed a marked reduction in energy losses by NPQ and increased biomass production under laboratory scale culture conditions (see **Table 2**). Mass culture of these truncated antenna mutants is expected to fine-tune light absorption characteristics influencing higher biomass productivity and the eventual reduction in the production cost. However, susceptibility of such strains with the shrunk antenna system to photodamage by intense solar radiation is a great limitation. de Mooij et al. (2015) found a smaller antenna size that made the mutants vulnerable to high light intensity while tailoring the antenna size in *Chlamydomonas reinhardtii*. The reduced fitness due to impaired photoprotection mechanisms triggered by altered antenna size lead to insignificant changes in the biomass productivity of mutants. Therefore, future strategies for antenna size reduction in microalgae should address the unintended side effects of antenna size on mutants. Interestingly, transgenic *C. reinhardtii* strains generated by Perrine et al. (2012), having variation in antenna sizes and reduced chlorophyll (Chl) *b* content, showed a higher growth rate in mutants with intermediate antenna size. The results conform to the hypothesis that reduction but not the elimination of Chl *b* content would result in the optimal photosynthesis process.

Among the abiotic factors that influence rate of carbon fixation, the most critical is the availability of inorganic carbon, as the concentration of CO_2 in the vicinity of Rubisco affects its carboxylase property. Microalgae have developed a CO_2 concentrating mechanism (CCM) to alleviate the stress caused by limited CO_2 in aquatic ecosystems (Wang et al., 2011).

TABLE 2 | Summary of genes manipulated for increasing the biomass yield.

Gene	Mode of action	Species	Observation	Reference
Abiotic stress tolerance:				
Superoxide dismutase (SOD1)	Overexpression	*Schizochytrium* sp.	Enhanced PUFA production without compromising growth rate	Zhang et al., 2018
E3 ubiquitin ligases (PUB5 and PUB14)	Suppression	*C. reinhardtii*	Induced lipid accumulation 9.8–61. 8%	Luo et al., 2015
Ubiquitin (UBC2)	Overexpression	*C. reinhardtii*	Increased cell lipid and growth rate	Fei et al., 2017
Diploid *C. reinhardtii* (CMD ex1 and ex4)	Polyploidization	*C. reinhardtii*	Accumulated two times more biomass and FAME yield	Kwak et al., 2017
AMP deaminase (AMPD)	Suppression	*C. reinhardtii*	Displayed higher growth rate and lipid productivity	Kotchoni et al., 2016
Manipulation of Calvin cycle:				
Rubisco (rbcL and rbcS)	Overexpression	*S. elongatus*	1.4-fold increase in Rubisco activity	Atsumi et al., 2009
	Overexpression	*Synechocystis*	Increased growth rate and photosynthesis	Liang and Lindblad, 2017
Rubisco type-I	Overexpression	*Synechococcus* sp.	Rubisco activity was improved by fourfold	Iwaki et al., 2006
RuBisCO activase (RCA)	Overexpression	*N. oceanica*	Biomass and lipid productivity increased by 46 and 41% respectively	Wei et al., 2017
Sedoheptulose 1,7−bisphosphatase (SBPase)	Overexpression	*D. bardawil*	Improved photosynthetic performance	Fang et al., 2012a
Fructose 1,6-bisphosphate aldolase (aldolase)	Overexpression	*C. vulgaris*	Increased photosynthetic capacity by 1.2-fold and enhanced cell growth	Yang et al., 2017
Optimizing light use efficiency:				
tla3 (CpSRP43)	Suppression	*C. reinhartdii*	Improved solar energy conversion efficiency and photosynthetic productivity	Kirst et al., 2012a
tla1	Suppression	*C. reinhartdii*	Higher photosynthetic productivity	Polle et al., 2003
tla2 (CpFTSY)	Suppression	*C. reinhartdii*	Improved solar energy conversion efficiency	Kirst et al., 2012b
tla4 (CpSRP54)	Suppression	*C. reinhartdii*	Higher photosynthetic productivity	Jeong et al., 2017
Stm3LR3 (NAB 1)	Suppression	*C. reinhartdii*	Higher photosynthetic quantum yield	Mussgnug et al., 2007
Chlorophyllide a oxygenase (CAO)	Suppression	*C. reinhartdii*	Two-fold increase in photosynthetic rate	Perrine et al., 2012
LHCP translocation defect (LTD)	Suppression	*C. reinhartdii*	Culture accumulated higher cell density	Jeong et al., 2018
Knockout of seven LHC genes	Suppression	*N. gladitana*	Exhibit up to ∼50% reduction in photosynthetic antennae size	Verruto et al., 2018
Enhancing carbon concentrating mechanism:				
Pyr-decarboxylase	Overexpression	Not mentioned	Aims to improve carbon fixation	Allen and Dupont, 2014
Bicarbonate transporter (ictB)	Overexpression	Not mentioned	Enhancing photosynthetic rate is the target	Wang et al., 2014
ATP-dependent bicarbonate anion transporter (HLA3)	Overexpression	Not mentioned	Target is to enhance CO_2 fixation	Sayre et al., 2017

Microalgae elevate the CO_2 concentration at the site of Rubisco through the operation of CCM. Since Rubisco also has the inherent capacity to divert the carbon pool toward unimportant photorespiratory pathways, an elevated CO_2 concentration favors carboxylation, thereby increasing the rate of carbon fixation (Singh P. et al., 2016). Inorganic carbon (Ci) import, enzymatic catalysis of the imported carbon to form CO_2, and compartmentalized Rubisco systems are the functional components of microalgal CCM. Environmental conditions such as the Ci content, ratio of $[CO_2]/[O_2]$, and dissolved CO_2 concentration are ascribed for the regulation of microalgal CCM (Morales et al., 2018). Several studies in *C. reinhardtii* have identified various factors such as CIA5, which acts at the cellular level and regulates the transcription of multiple genes having a role in CCM (Fukuzawa et al., 2001; Yoshioka et al., 2004; Fang et al., 2012b). Tapping these regulatory factors in photosynthetic microalgae has assumed significance in enhancing fitness toward naturally occurring low CO_2 conditions (Price et al., 2013). Besides these, modulations in other functional components of CCM such as Ci transporters and carbonic anhydrases can also facilitate enhancements in the carboxylation reaction, which in turn increases the photosynthetic performance and biomass yield. However, except a few filed patents (see **Table 2**), to date, there are no reports of successful CCM engineering in microalgae. Thus, suppression of the oxygenase activity of Rubisco through tailoring CO_2 capture mechanism in microalgae remains a challenge to be addressed for improving the carbon fixation process.

GENOME EDITING IN MICROALGAE FOR STRAIN IMPROVEMENT

Metabolic pathway engineering is crucial for enhancing the productivity of a microalgal strain, and for this purpose, gene editing offers a powerful and easy mechanism to overcome the genetic inadequacies (Ng et al., 2017). Until recently, the RNAi technology was frequently used as a tool for gene silencing and proved efficient in pathway engineering and gene function alteration. However, RNAi has its limitations, which include incomplete suppression, silencing of the RNAi transgene, and inconsistent suppression in different transformants (Banerjee et al., 2018). In contrast, the emergence of genome editing bypasses the limitations of RNAi, offering new avenues to modify and edit the genome of cells. The genome editing techniques based on engineered nucleases like clustered regularly interspaced palindromic sequences/CRISPR-associated protein 9 (CRISPR/Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) provide the means for dissecting the operational organization of genes, gene families, and protein networks. These genome editing tools induce double-strand breaks at a specific locus in the genome, which get repaired through the non-homologous end joining machinery of the DNA repair process and introduce insertions or deletions at sites creating frameshift mutations (Gan and Maggs, 2017). Among the genome editing tools, CRISPR/Cas9 has gained much focus because of its simple, accurate, and efficient nature of operation (Jeon et al., 2017). In the CRISPR/Cas9 system, the Cas9 nuclease is directed by a single guide RNA (sgRNA) molecule, which binds to the target site in the genome following simple base-pairing rules. Steady progress in research on the CRISPR/Cas9 system has resulted in the development of many different variants of this technology. A mutated form of the Cas9 protein (dCas9) lacking the nuclease activity can be used with the CRISPR system to modulate the expression of specific target genes. Depending on the type of the effector molecule fused with dCas9/sgRNA, it is possible to precisely both stimulate and repress the activity of a target gene (Gilbert et al., 2013; Piatek et al., 2015). In addition to the expression modulation of a single gene, multiple genes can be simultaneously activated or silenced by the simple addition of guide RNAs for each of the targets into the dCas9/sgRNA variant of the CRISPR/Cas9 system (Kim and Kim, 2014). The versatility in the application of the CRISPR/Cas9 system makes the technique a remarkable and powerful tool in metabolic pathway engineering.

In microalgae, the utility of CRISPR is on the rise as it has considerable scope in microalgal trait improvement for biofuel and nutraceutical applications. Various advances in CRISPR/Cas and other genome editing tools have led to several successful attempts in many microalgae species (see **Table 3**), which endorse this technology for its effectiveness in generating targeted mutants. One prominent advantage of applying the CRISPR technology in microalgae is the ease of multiplexing, which, unlike the conventional mutagenesis and RNAi mediated knockout and knockdown approaches, facilitates a less complicated and more programmable approach for manipulating metabolic pathways. In case of lipid engineering in oleaginous microalgal strains, this technique can improve the lipid profile of the microalgal strain by simultaneously blocking the metabolic routes competitive to lipid production such as starch generation, lipid degradation, and β-oxidation. Other than gene silencing, a dCas9 variant can be recruited to activate stress responsive elements of the lipid synthesis pathway under nonstress conditions, thus bypassing the inhibitory effects on the biomass yield. An activator molecule fused with dCas9 can be used to stimulate supportive pathways such as FA synthesis, which facilitates the production of precursors for lipogenesis. Functional characterization of a novel gene is another aspect of utilizing the CRISPR technology in addition to gene editing. Annotating novel genes encoding proteins significant for lipid production can broaden the spectrum of target selection for superior biofuel production.

Indigenous microalgal strains promoted for biofuel production have some limitations for commercial scale production, which include suboptimal lipid profile and light harvesting efficiency, among others. However, adjusting these limiting attributes is not recommended as it interferes with the normal physiology of microalgae. For example, generating truncated LHC in microalgae is associated with susceptibility to photodamage (de Mooij et al., 2015). Therefore, it is advantageous to have a system that can detect a trigger such as the presence of a chemical or a variation in light intensity; therefore, once the culture is grown for some time, the trigger can be activated resulting in improved productivity. Development of a dCas9 variant that can be activated by light or chemicals can facilitate a tool for the conditional modulation of molecular intricacies, bypassing the physiological interference of the change in cell metabolism (Polstein and Gersbach, 2015; Zetsche et al., 2015). Despite several advantages, this system has its share of challenges in the form of cytotoxic effects of the Cas9 nuclease in some of the microalgae species, which have limited the full-scale utilization of this system. Off-target effects of the Cas9 protein have been linked with cytotoxicity in cells transformed with the Cas9 gene construct. However, modifications in the Cas9 protein delivery through the ribonucleoprotein (RNP) complex has been reported to reduce the off-target problems associated with the Cas9 protein. Replacing the Cas9 protein with a Cas12a variant is also an alternative to consider as it has been reported to solve the cytotoxicity in cyanobacteria (Naduthodi et al., 2018). Apart from these, the recent characterization of several other variants of the CRISPR system has extended the prospect of a genetic toolbox for microalgal genome engineering. Utilization of these precise genome editing tools along with microalgal system biology can create an optimized platform customized for biofuel application and high-value product generation.

CHALLENGES AND FUTURE PROSPECTS

Microalgae as an alternative energy source hold immense potential to revolutionize the biofuel production system without putting much pressure on agriculture and the forest ecosystem. Despite the promises, commercialization of the

TABLE 3 | Overview of the application of different genome editing tools in photosynthetic microalgae.

Species	Genome editing tool	Mode of action (efficiency)	Target gene	Reference
Chlamydomonas reinhardtii	CRISPR/Cas9	Knockout, low efficiency	*Hygro*, *mGFP*,*FKB12*, and *Gluc*	Jiang et al., 2014
	CRISPR/Cas9	Knockdown and knock-in	*MAA7*, *CpSRP43*, and *chlM*	Shin et al., 2016
	CRISPR/Cas9	Double knockout	*ZEP* and *CpFTSY*	Baek et al., 2016
	CRISPR/Cas9	Knockout	Zeaxanthin epoxidase gene	Baek et al., 2018
	CRISPRi	Knockdown	*PEPC1* and *RFP*	Kao and Ng, 2017
	CRISPR/Cpf1	Knockout (0.1–10%)	*CpFTSY*, *CpSRP43*, and *PHT7*	Ferenczi et al., 2018
	Zinc-finger nuclease (ZNF) and CRISPR/Cas9	Knockout (5–15%)	*COP1/2*, *COP3* (encoding channelrhodopsin 1 [ChR1]), *COP4* (encoding ChR2), *COP5*, *PHOT*, *UVR8*, *VGCC*, *MAT3*, and *aCRY*	Greiner et al., 2017
	ZNF-mediated	Gene repair and gene knockout	*aphVIII*, *COP3*	Sizova et al., 2013
Phaeodactylum tricornutum	Meganucleases and TALEN-mediated	Knockout	*UGPase/NAT* gene	Daboussi et al., 2014
	TALEN-mediated	Knockout (50%)	Blue-light dependent transcription factor Aureochrome1a (*PtAureo1a*)	Serif et al., 2017
	TALEN-mediated	Knockout (24%)	Urease gene	Weyman et al., 2015
	CRISPR/Cas9	Knockout (31%)	Chloroplast signal recognition particle 54 (*CPSRP54*)	Nymark et al., 2016
	CRISPR/Cas9	Knockout (60%)	Urease gene, and eight genes involved in vanillin biosynthesis	Slattery et al., 2018
	CRISPR/Cas9	Knockout	A vacuolar protein, *Vtc2*, and a putative phosphate transporter, *Pho4*	Stukenberg et al., 2018
Thalassiosira pseudonana	CRISPR/Cas9	Knockout	Urease gene	Hopes et al., 2016
	CRISPR/Cas9	Knockout, highly efficient	Silacidin gene	Belshaw et al., 2017
Nannochloropsis oceanica	CRISPR/Cas9	Knockout (1%)	Nitrate reductase	Wang et al., 2016
	CRISPR/Cas9	Knockout	Nitrate reductase	Poliner et al., 2018
	CRISPR/Cas9	Knockout	Homolog of fungal Zn(ii)2Cys6 encoding gene (*ZnCys*)	Ajjawi et al., 2017
Nannochloropsis gaditana	CRISPR/Cas9	Knockout (∼80%)	*Ble*, *GFP*, *Aco1*, *ZnCys*, and Seven LHC genes	Verruto et al., 2018
Pseudochoricystis ellipsoidea	TALEN-mediated	Knockout	Uridine monophosphate synthetase (*UMPS*)	Kasai et al., 2015

microalgal biofuel technology is far from real, owing to its high production cost. Development of economically feasible technologies, such as microalgal strain improvement for improved oil production, holds the future for commercial scale production of algal biofuel. As summarized by Chung et al. (2017), biotechnological interventions could reduce the microalgal biofuel production cost by 15–20% in comparison with traditional approaches. Accordingly, implementation of key molecular schemes targeting pivotal cost-contributing attributes comprising superior feedstocks, oil extraction procedures, and quality of biodiesel can ease the financial burden imparted by these factors. The successful realization of these approaches can make microalgal biofuel production competitive with fossil fuel. To materialize the goal of gaining economic parity with fossil fuel, recent progress in microalgal biotechnology particularly in the field of biocatalyst engineering, synthetic biology, and genome editing has facilitated the necessary tools to design novel microalgal strains as per the culture condition. Furthermore, merging the primary goal of biofuel production with the intended coproduction of value-added products, such as antioxidants, nutraceuticals, and pharmaceuticals, could help in generating returns for financial investments (Jagadevan et al., 2018). Additionally, the adaptation of a consolidated biorefinery and phycoremediation approaches are also projected to diversify the utility of microalgal biomass (Rizwan et al., 2018). However, the sustainability of these approaches largely depends on the cost incurred during the culturing process of microalgal strains. As large-scale open pond culture is the most economic method for microalgae biomass production, the majority of the commercial microalgae are cultured by this system (Kumar et al., 2018). Thus, open pond culturing of genetically modified (GM) microalgae appears more promising in cutting down the cost; however, the impact on human health and environmental risks form the major concerns with transgenic microalgae if exposed to natural ecosystems (Rastogi et al., 2018). Being one of the primary producers in aquatic ecosystems, any involuntary introduction of GM microalgae could result in an ecological calamity (Singh S.K. et al., 2016).

Strict monitoring and risk assessment analysis are, therefore, necessary to design the biosafety regulations for GM microalgae. Apart from these, techniques for the bio-containment of transgenes with codon reassignment and mutagenesis might be helpful in mitigating environmental risks through the deletion of genes crucial for survival in the wild but lack importance for culture (Henley et al., 2013; Gressel et al., 2014; Young and Purton, 2016). Additionally, a long-term comprehensive evaluation of the impact of non-indigenous and engineered microalgal strains on the native ecosystem could be helpful in eliminating the ambiguities around regulations on the cultivation of GM algae. In one notable case, Szyjka et al. (2017) reported that when a microalgal species, *Acutodesmus dimorphus*, was cultured in an open pond, neither the transgenic nor the wild type counterpart of the microalge species were successful in outcompeting the native strains. The study concluded that the outdoor culturing of GM microalgae fails to affect the microalgal diversity in the native ecosystem. However, before drawing any conclusion, extensive studies should be conducted as it is evident that regulatory certainty would be critical in the development of economically viable processes for algae-based biofuel production (Glass, 2015; Randhawa et al., 2017). Recent success in technology demonstration of biojet fuel is a sign of emerging prospects of microalgal biofuel for commercial ignition (Siobhan, 2010; Gyekye, 2017; Chandra, 2018).

CONCLUSION

Microalgae-based biofuels are projected as the suitable alternative to fossil fuel because of their promising yield in nature besides their sustainable advantages over traditional terrestrial feedstocks. They possess novel metabolic features, which can be tuned for the commercial scale production of renewable biofuels. However, genetic abilities of identified microlagal strains are far from optimum to serve as feedstocks for sustainable production. Genetic improvement of inherent capacities such as high photosynthetic conversion rates, rapid biomass production, alteration to their core structures for the generation of suitable biofuel feedstocks, and adaptation to diverse climatic conditions envisage to bring new opportunities for sustainable biofuel production. The rapid stride in genome biology studies and high throughput genome sequencing and transcriptome mapping in diverse oleaginous organisms have ensured a means to analyze and manipulate metabolic pathways by triggering the expression of candidate genes for enhanced lipid production in microalgae.

AUTHOR CONTRIBUTIONS

LS conceived the structure and focus of the review. PS wrote the basic framework of the review. MS and SK assisted in writing. RS organized the critical components. LS analyzed and edited the write-up.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.