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Integrated biomolecular and bioprocess engineering strategies for enhancing the lipid yield from microalgae

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ABSTRACT

Algal biofuels have received wide attention in recent years for its potential to reduce the dependence on conventional fossil fuels. Despite the portrayed advantages of high growth rate, carbon sequestration and waste remediation; large scale application of microalgal biofuels is still lacking because of the lower percentage of extractable lipids obtained from the harvested biomass. Thus, there is a substantial impetus to analyse the strategies for enhancing the lipid profile and yield to improve the microalgal biofuel quality as well as to reduce the costs incurred at field scale. Several biochemical and molecular strategies to increase the algal lipid accumulation has gained huge scientific interest in recent years and have opened up new avenues for algal biorefinery. However, the time and cost involved as well as the ecological risks associated with real-time applications often restricts their utilization. The present review gathers a compendium of the key milestones associated with the recent approaches of biochemical, genetic and metabolic engineering for lipid quantity and quality enhancement. Biochemical and engineering aspects of coercing the cells to environmental stress and altering the mode of nutrition has been elucidated. The advancements in genetic and metabolic engineering, the associated risk factors and the future perspectives have been highlighted. Strategic integration of the bioprocess and biomolecular techniques to explore its synergistic impact to rationally engineer microalgae with improved triacylglycerols has been emphasized. Assessment of the long term risks associated herewith can be used to avert the challenges, making algal biofuels a commercial reality in future.

1. Introduction

Issues of climate change, environmental pollution along with the depleting reserves of fossil fuels have diverted our attention towards renewable alternative sources of energy. Renewable energy sources like solar energy, hydro-energy, geothermal energy etc., which have much lower environmental impacts are increasingly getting popular, but are apparently costly. However, to meet the growing energy demands without compromising the economy requires the need to exploit the resources at a lower capital cost and energy [1]. Over the past decades, the third generation biofuels from microalgae are increasingly capturing attention due to their diversified biochemical content [2]. Further, their ease of cultivation on arid lands using wastewater/brackish/marine water capturing industrial flue gases (especially carbon dioxide [CO_2]) implements additional advantages of curbing environmental pollution and facilitating carbon sequestration [3]. This integrated approach is postulated to provide resource recovery-based monetary benefits that can possibly assist in resolving the problems of energy security.

The desired yield or lipid productivity that is regarded as an essential criterion for the biofuel industry is largely influenced by the culture and operating conditions of microalgae [4]. Several studies have reviewed the factors influencing large-scale microalgae cultivation [1,5] harvesting and lipid extraction technologies [6] to make algal biofuels a commercially feasible option. Despite the advances, the commercialization of microalgae is lacking due to the low biomass as well as lipid productivity at the field scale. The reason being the lack of proper understanding of the biochemical pathways that influences the intracellular lipid accumulation [7]. As evident in Table 1, most reviews till date have discussed only a single manipulation method *i.e.* either the

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Abbreviation meaning

ACCase	Acetyl-CoA Carboxylase
ACP	Acyl Carrier Protein
ADP	Adenosine-5′ Diphosphate
ATP	Adenosine Triphosphate
AGPase	ADP Glucose Phosphorylase
BBM	Bold Basal Medium
CAT	Catalase
CO_2	Carbon Dioxide
COBRA	Constraint-Based Reconstruction and Analysis
COD	Chemical Oxygen Demand,
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DA-6	Diethyl-Aminoethylhexaonate
dFBA	Dynamic Flux Balance Analysis
DGAT	Diacylglycerol Acyltransferase
DHA	Docosahexaenoic acid
DHAP	Dihydroxy Acetone Phosphate
DNA	Deoxyribonucleic Acid
DRUM	Dynamic Reduction of Unbalanced Metabolism
EMP	Embden–Meyerhof–Parnas
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FAD	Flavin Adenine Dinucleotide
FAMEs	Fatty Acid Methyl Esters
FAS	Fatty Acid Synthases
FBA	Flux Balance Analysis
FBP	Fructose 1,6 Bisphosphate
FVA	Flux Variability Analysis
G3P	Glyceraldehyde-3-Phosphate
GFP	Green Fluorescence Protein
GM	Genetically Modified
GPAT	Glycerol-3-Phosphate-acyltransferase
GSMMs	Genome-Scale Metabolic Models
H_2O_2	Hydrogen Peroxide
HMP	Hexose Monophosphate Pathway
HRAP	High Rate Algal Ponds
IAA	Indole-3-Acetic Acid
LD	Lipid Droplet
LEDs	Light Emitting Diodes
LHC	Light Harvesting Complex
MCAT	Malonyl CoA-ACP Transacylase
ME	Malic Enzyme
MOMA	Minimization of Metabolic Adjustments
mRNA	Messenger Ribonucleic Acid
MUFAs	Monounsaturated Fatty Acids
N	Nitrogen
NAABB	National Alliance for Advanced Biofuels and Bio-products
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
$NaH_2PO_4{\cdot}2H_2O$	Sodium Phosphate Dibasic Dihydrate
$NaHCO_3$	Sodium Carbonate
$NaNO_3$	Sodium Nitrate
NH_4Cl	Ammonium Chloride
NO_3	Nitrate
O_2^-	Superoxide
$OH^\bullet$	Hydroxyl Radical
ORF	Open Reading Frame
P	Phosphorous
PBRs	Photobioreactors
PCR	Polymerase Chain Reaction
PDHC	Pyruvate Dehydrogenase Complex
PO_4	Phosphate
PtPDK	Putative Pyruvate Dehydrogenase Kinase
PSR	Phosphorus Starvation Response
PUFAs	Polyunsaturated Fatty Acids
RNAi	Ribonucleic Acid Interference
ROOM	Regulatory On/Off Minimization
ROS	Reactive Oxygen Species
RuBisCO	Ribulose-1, 5 Bisphosphate Carboxylase/Oxygenase
RuBP	Ribulose-1, 5 Bisphosphate
SOD	Superoxide Dismutase
SBPase	Sedoheptulose 1,7-bisphosphatase
TAGs	Triacylglycerols
TALENs	Transcription Activator-Like Effector Nucleases
TCA	Tricarboxylic Acid Cycle
TDS	Total Dissolve Solid
TEA	Techno Economic Analysis
TF	Transcription Factor
TPI	Triose Phosphate Isomerase
UV	Ultraviolet
ZFNs	Zinc-Finger Nuclease

biochemical approach of coercing the algal cells to environmental stress factors or the genetic/metabolic strategies to redirect the carbon flux towards lipid accumulation. **Sajjadi *et al.*** [16] have enlisted the manipulation of various environmental stress stimuli to achieve the desired lipid yield and fatty acid (FA) content, while the recent study by **Aziz *et al.*** [24] have emphasized the utilization of these strategies in combined manner to improvise the overall lipid productivity. Engineering strategies including reactor operation are seldom discussed except a brief outline provided in the review by **Chu, (2017)** [13], **Shin *et al.*** [17] and **Shokrayi *et al.*** [26]. **Aziz *et al.*** [24] has detailed the two-stage cultivation strategies and reactor operation providing insights on the economic feasibility, with no information over the energy and environmental impacts. Most reviews done till date to summarize the engineering approaches for enhancing algal lipids have seldom included the modelling and kinetic behaviour of microalgae, providing forecasts/predictive insights on the overall growth and productivity, except the brief outline provided by **Sajjadi *et al.*** [16] Further, these reviews have rarely discussed the lifecycle costs and energy impacts.

Several reviews (as shown in Table 1) have comprehensively elaborated the molecular biology techniques for triggering lipids, but a very few has focussed on the inclusion of system biology tools. **Lenka *et al.*** [27] have discussed the combinatorial integration of the genetic and computational approach for enhancing the microalgal lipid yields but have not much focussed on the engineering aspects of reactor operation and control. The critical reviews by **Singh *et al.*** [11], **Aratboni *et al.*** [7] and **Salama *et al.*** [28] were limited to the biochemical and genetic engineering techniques for enhancing lipid productivity. Recent reviews by **Banerjee *et al.*** [25] has made a commendable attempt to summarize the biochemical, genetic and metabolic strategies enhancing the yield of lipid and starch in microalgae. **Naghshbandi *et al.*** [23] has also provided insightful information on the genetic and metabolic engineering procedure to improve the biodiesel and biohydrogen yield in microalgae. Though limited information about the challenges faced during scale-up of genetically engineered algae is available, none of the reviews have critically discussed their techno-economic and environmental feasibility. Further, to the best of author's knowledge, none of the study till now has revisited these approaches all together for enhancing the accumulation of lipids with more focus on neutral lipids that could not only increase the yield, but also the biofuel quality, which could be used directly in the engine. Since, each of these strategies has pros and cons, it

is essential to thoroughly analyse their influence over the algal metabolism. Nevertheless, the commercial-scale application of microalgae for biofuels, requires consolidation of the underlying molecular principles influencing the lipid biogenesis with that of the engineering and synthetic biology approaches.

The current review thus focuses on rational algae engineering strategies for achieving higher quantity and quality of lipids. The initial section deals with a brief overview of lipid synthesis. Various biochemical approaches like subjecting the cells to environmental stress, manipulating the nutrient regime, mode of cultivation for increasing algal lipid content have been thoroughly discussed. Furthermore, perspectives of system biology and molecular biology approach with genetic and metabolic engineering tools for strain improvement concerning lipid accumulation have also been comprehensively addressed. The lipid biogenesis pathways and the underlying mechanism of metabolic switch from starch to lipid transition is also highlighted. Engineering principles for operation and control strategies of photobioreactors (PBRs) has also been focussed for improving the lipid yield. Importance of kinetic and predictive mathematical model for simulating the algal growth behaviour along with techno-economic and environmental assessment of the conventional and advanced strategies for manoeuvring lipid accumulation are discussed. The challenges associated with each strategy, the recent advances, their implication for further research have been presented. The review is first of its kind to comprehensively and mechanistically discuss the biochemical, molecular and engineering strategies altogether. The systematic and synergistic coupling of these processes has been emphasized and is expected to provide the readers, a direction for future research to boost the algal biofuel industry in the recent future by enhancing lipid accumulation alleviating the negative externalities.

2. Microalgal lipid synthesis pathways

Fatty acids form the major component of acyl lipids occurring either as structural constituent of microalgae (glycerolipids) or in the form of inert storage component (triacylglycerols [TAGs]). The lipids in microalgae are synthesized mainly via *de novo* fatty acid biosynthesis pathway [29]. The insights of FA synthesis of microalgae have been derived from the basic framework of lipid metabolism pathway of *Arabidopsis* [30]. Though the algal lipid biogenesis is almost similar as that of plants, differences exist in the plastid based prokaryotic pathway of TAG synthesis and storage, as well as distinctive acyl groups linked with glycerolipids. Acetyl-CoA is the primary substrate of FA synthesis in chloroplast that is being carboxylated to Malonyl-CoA by Acetyl-CoA carboxylase (ACCase) forming palmitic and stearic acids, which undergo further saturation and elongation giving rise to oleic acids catalysed by fatty acid synthases (FAS). Elongation of chain is controlled by chloroplast acyl transferase and ACP (acyl carrier protein) thioesterase. TAG is formed when acyl groups are sequentially transferred from acyl-CoA to glycerol-3-phosphate backbone by acyl transferase in Kennedy pathway occurring in plastids and endoplasmic reticulum. The simplified process of lipid synthesis pathway in microalgae has been represented in Fig. 1. Since, starch and lipid synthesis share the common carbon precursor (glyceraldehyde-3-phosphate [G3P]) as shown in Fig. 1, understanding the starch metabolism also plays a crucial role in redirecting the carbon flux towards enhanced lipid accumulation.

ACCase, which is under redox regulatory control, is regarded as the key control point for *de novo* lipid synthesis [29]. Several intermediate enzymes acting in different compartments also regulates TAG synthesis. The *de novo* pathway (Kennedy pathway) and other alternative lipid biosynthesis routes have been discussed in detail by **Lenka *et al.*** [27] **and Sun *et al.*** [19]. **Beisson *et al.*** [31] have discussed the compartmentalization of different regulatory proteins and enzymes involved in lipid biogenesis with focus on their turn-over energetic aspects for enhancing the TAGs accumulation. Mainly, the concept of overflow hypothesis is involved in TAGs accumulation, where depending on the growth conditions and photosynthetic efficiency, carbon is being shunted either for growth and maintenance or towards storage compound synthesis. Many key enzymes involved in the central starch metabolism as demonstrated by **Ran *et al.*** [21] also governs the nutrient based metabolic switch or conversion of starch into lipids. Several researchers have prophesized variation in TAG levels under nutrient stress, owing to the above-mentioned concept [32,33]. Depending on this concept, oleaginous microalgae are said to accumulate TAGs mostly at an average of 20–70% (w/w) under different growth and culture conditions, with a few species accumulating >90% (w/w) lipids at

Table 1
List of reviews related to algal lipid enhancement strategies. [✓ - Discussed; × - Not discussed; * - Briefly discussed]

Year	References	Biochemical strategies		Molecular strategies			Engineering strategies		
		Nutrient manipulation	Physiochemical stress	System biology	Gene manipulation	Metabolic engineering	Reactor operation & control	Mathematical models	TEA/ LCA
2014	Ho et al. [8]	✓	✓	×	×	×	×	×	×
2015	Bhowmick et al. [9]	✓	✓	✓	✓	✓	×	×	×
2016	Sibi et al. [10]	✓	✓	×	×	×	×	×	×
2016	Singh et al. [11]	✓	✓	×	✓	✓	×	×	×
2017	Chen et al. [12]	✓	✓	×	✓	✓	*	×	×
2017	Chu, 2017 [13].	✓	✓	×	✓	✓	✓	×	×
2017	Ng et al. [14]	×	×	×	✓	✓	×	×	×
2017	Ravindran et al. [15]	✓	✓	×	✓	✓	×	×	×
2018	Sajjadi et al. [16]	✓	✓	×	*	×	×	✓	×
2018	Shin et al. [17]	✓	✓	×	×	×	✓	×	×
2018	Sharma et al. [18]	×	×	×	✓	✓	×	×	×
2019	Sun et al. [19]	×	×	×	✓	✓	×	×	×
2019	Park et al. [20]	×	×	×	✓	✓	×	×	×
2019	Aratboni et al. [7]	✓	✓	×	✓	✓	×	×	×
2019	Ran et al. [21]	✓	✓	×	×	×	×	×	×
2019	Zhao et al. [22]	×	✓	×	×	×	×	×	×
2019	Naghshbandi et al. [23]	×	×	×	✓	✓	×	×	×
2020	Aziz et al. [24]	✓	✓	×	×	×	✓	×	✓
2020	Banerjee et al. [25]	✓	✓	×	✓	✓	×	×	×
2020	Shokravi et al. [26]	✓	✓	×	✓	×	✓	×	×

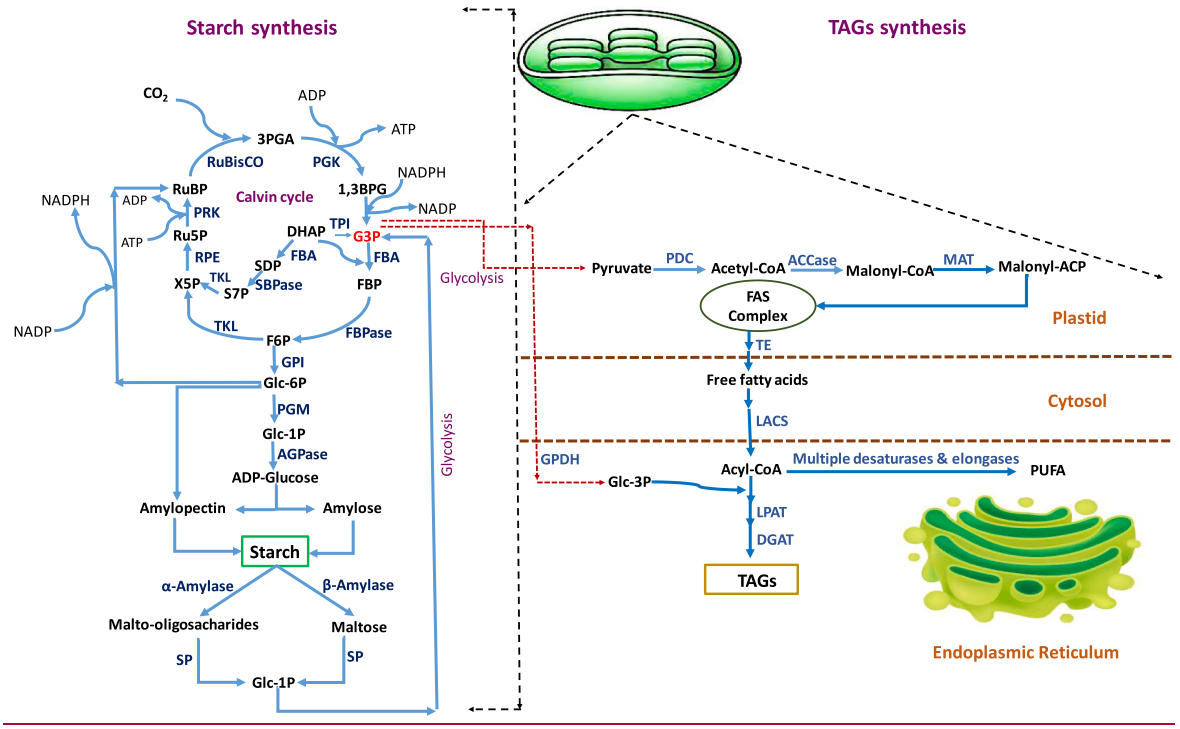

Fig. 1. ***De novo pathway*** **for lipid synthesis in microalgae along with the intracellular enzymes,** [Red coloured dotted arrow represents the cross-talk and common precursors and pathways between the starch and lipid synthesis], {Abbreviations PDC: Pyruvate decarboxylase complex; AACase: Acetyl-CoA carboxylase; MAT: Malonyl-CoA/ACP transacylase; ACP: Acyl carrier protein; FAS: Fatty acid synthase; TE: Thioesterase; LACS: Long chain acyl-CoA synthetase; GPAT: Glycerol-3-phosphate acyltransferase; LPAT: Lyso-phosphatidylcholine acyltransferase; DGAT: Diacylglycerol acyltransferase; PUFA: Polyunsaturated FAs; TAGs: Triacylglycerols; ADP: Adenosine 5′-phosphate; ATP: Adenosine triphosphate; NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen; NADP: nicotinamide adenine dinucleotide phosphate; CO_2: Carbon dioxide; 3-PGA: 3-Phosphoglyceric acid; 1,3-BPG: 1,3-Biphosphoglycerate; G3P: Glyceraldehyde-3-phosphate; FBP: Fructose 1,6-bisphosphate; DHAP: Dihydroxyacetone phosphate; F6P: Fructose 6-phosphate; X5P: Xylulose 5-phosphate; SDP: Sedoheptulose 1,7-bisphosphate; S7P: Sedoheptulose-7-phosphate; Ru5P: Ribulose-5-phosphate; RuBP: Ribulose 1,5-bisphosphate; Glc-6-P: Glucose-6-phosphate; Glc-1-P: Glucose-1-phosphate; RuBisCO: Ribulose 1,5-bisphosphate carboxylase; PGK: 3-Phosphoglycerate kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GPDH: Glycerol-3-phosphate dehydrogenase; FBA: Fructose-bisphosphate aldolase; FBPase: Fructose 1,6-bisphosphatase; TPI: Triose phosphate isomerase; SBPase: Sedoheptulose-1,7-bisphosphatase; TKL: Transketolase; RPE: Ribulose-5-phosphate-3-epimerase; PRK: Phosphoribulokinase; GPI: Phosphoglucose isomerase; PGM: Phosphoglucomutase; AGPase: ADP-glucose pyrophosphorylase; SP: Starch phosphorylase}.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

specific operational conditions [34,35]. The subsequent sections highlight in detail the methodical strategies on biochemical, molecular and bioprocess engineering principles to modulate the overflow hypothesis for enhancing the lipid yield in microalgae.

3. Biochemical approaches for enhancing lipid accumulation

3.1. Environmental stress to improve the microalgal lipid productivity

Under favourable conditions of growth, microalgae assimilate only a limited amount of lipids and carbohydrates [17]. Microalgae often accumulate more lipids under extreme physical environmental conditions [36] or variation of nutrients [37].

Irradiance plays an essential role in determining the CO_2 biofixation, thereby the growth rate, and composition of algal biomass especially under photoautotrophic conditions [4]. Phototrophic cultivation results in accumulation of significant quantities of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), glyceraldehyde-3-phosphate (G3P), which serves as essential cellular metabolites governing the algal growth. Optimization studies by **Moreno-Garcia *et al.*** [38] with *Chlorella* sp., and **Wahidin *et al.*** [39] for *Nannochloropsis* sp., have projected a decline in lipid content following an increase in light irradiance and photoperiod. Possible reason being an increase in light intensity often directs the energy and carbon flux towards growth accumulating more biomass than TAGs. Contrary to the above-mentioned trend, **Nzayisenga *et al.*** [40] reported enhanced biomass and FA content with maximum accumulation of 11.6% (w/w) lipids in *Scenedesmus* sp with increase in irradiance. However, authors have postulated the change to be mainly attributed to nitrogen starvation. Thus, along with the operational conditions, interactive effect of media constituents also influences algal lipids. **Ra *et al.*** [41] have postulated that the correlation between the light intensity and lipid accumulation potential is strain specific, depending on the inherent cellular metabolism. The study reported 52%, 53% and 56%(w/w) lipids in *N. salina*, *N. oceanica* and *N. oculata* respectively, cultured in f/2 medium on exposure to green light emitting diodes [LEDs] (100 μmol/m^2/s) with 12 h light regime after 2 days. Since, algal reproduction and TAG accumulation is dependent over the photo-assimilation, the quality of light and photoperiod also influences the quantity and quality of lipids [39]. **Ra *et al.*** [41] reported better growth of *Nannochloropsis* sp., (0.037 h^{-1}) in blue LED lights, whereas higher lipid accumulation (56%) with green LED lights, both with 100 μmol/m^2/s intensity, showing the inherent metabolic algal efficiency to be also dependent over the photo-spectrum of light absorbed. Intermittent exposure of *C. sorokiniana* cells to ultraviolet (UV) radiation has been also reported to increase the C16 and C18 FAs and decrease the polyunsaturated FAs (PUFAs), as the mechanism by which ATP is assimilated in *de novo* FA synthesis varies under the conditions of photo-oxidation [42].

Temperature variation influences the microalgal growth and lipid productivity by affecting the activity of enzymes involved during lipid biogenesis [43]. Recent study by **Chaisutyakorn *et al.*** [44] reported

Table 2
Effect of various environmental stress and growth conditions on the lipid content of microalgae.

Environmental stress and culture conditions	Microalgae	Effect on lipid content	References
Temperature stress (17–35 °C) with nitrogen deprived TAP medium; 100 μmol/m^2/s continuous illumination; standard CO_2 levels over 4 days	*C. reinhardtii* starchless mutant BAF-J5	Shifting the temperature towards 32°C resulted in maximum 76% w/w of total FAs, which remained fairly constant by further temperature increase	[49]
Temperature stress (20–30 °C)applied with Modified Tamiya medium with nitrogen deprivation (1–10%); light intensity of 130 μmol/m^2/s (14:6 L:D); 1.5% CO_2 at 20 L/h during illumination over 3 days	*Chlorella* sp.	Lipid content increased from 9-22% to 37–46% w/w under nitrogen stress of 1–3% N, irrespective of temperature	[50]
Modified BG 11 media with variation in light intensity (3000–6000 lux); pH(5–9); 20–40 mg/L K_2HPO_4 at 27 °C, with photoperiod of 14:10 light dark cycle for 15 days	*Scenedesmus abundans*	Though biomass productivity increased in all cases, lipid content declined from 24% to 22% w/w and from 26% to 21% w/w with increase in K_2HPO_4 conc. and pH respectively, while 21–32% w/w of increase in light intensity was evident	[51]
Modified BG 11 media with variation in light intensity (260–1000 μmol/m^2/s) supplied continuously; pH (6.5–10.5) at 25 °Cat 0.78 d^{-1} dilution rate in chemostat over 143 days	*Ettlia* sp. *YC001*	Lipid content increased from 17% to 23% with increase in light intensity and was not much influenced by pH fluctuations, though biomass productivity declined with increase in pH	[52]
Modified Chu medium; 60 μmol/m^2/s; 24 h illumination; 10–30% CO_2; 0.4–0.9 vvm flow rate; 10^5-10^7 initial cell density; 7 days incubation time	*Nannochloropsis* sp.	Max. lipid content (45% dry weight) was obtained at 20% v/v CO_2; 0.4 vvm with 10^6 cell density, gradually declined with increase in CO_2 levels	[53]
Domestic sewage having 400 mg/L COD; 75 mg/L TDS; 115 mg/L nitrate supplemented with 500 mg/L each of glucose, sodium nitrate and sodium phosphate and 0.5–2.0 g/L NaCl, pH: 8.2; 8 days incubation time	Native microalgae from lentic water body	Max. lipid content of 23.4% w/w with biomass content of 6.12 g/L was obtained at 1 g/L NaCl, with further increase in salt concentration both biomass and lipid levels declined	[37]
Chicken waste compost fertilizer aerated at 0.4 L/min; pH:3–3.5; light intensity 60 μmol/m^2/s; 12:12 h photoperiod, 4 days incubation time	*C. vulgaris*	Nutrient starvation increased lipid content from 16% to 17% w/w in one day but declined to 13% w/w after 4 days in combination with salinity stress (6 g/L)	[54]
Community wastewater, 50–300 μmol/m^2/s; 16 h photoperiod, 25 °C, aerated at 0.1 L/min; 15 days incubation	***Desmodesmus* sp.**	Max. lipid content of 6.8% w/w with biomass productivity of 1.1 g/L was obtained with increase in light intensity from 50 to 300 μmol/m^2/s	[24]
PE-001A medium; 200 μmol/m^2/s for 12 h; pH (6–9); 25 °C; 1 L/min aeration	***C. sorokiniana***	Maximal biomass productivity of 0.14 g/L/d at pH of 6 which declined to 0.07 g/L/d at pH 9, but no significant variation in lipid content (29.5% w/w on an average)	[30]
Biomass grown in Jaworski's Medium (JM) resuspended in 17 g/L salts in sea water; with gradual increase in irradiance from 66–360 μmol/m^2/s, 25 °C, 12 days	***Golekinia* sp.**	37.2% w/w lipids (48% higher than control) with 1.02 g/L biomass content	[33]
Modified Detmer's medium; light intensity (180–540 μmol/m^2/s), 28 °C, bubbled with 2.5% (v/v) CO_2 at 0.4 vvm, 9 days incubation	***S. obliquus* CNW–N**	22.40% (w/w) lipids after 5 days of N starvation (2-fold more than control) with 626.6 mg/L/d biomass productivity No effect of light intensity on lipid content	[40]
Walne medium having 1176 μM NO_3^- and 128 μM PO_4^{3-} exposed to 300 μmol/m^2/s light at 27 °C, aerated with ambient CO_2 for 7 days	***T. lutea***	Comparable growth rate as that of control was achieved with P-limited (500 μM NO_3^- and 4 μM PO_4^{3-}) and in N-limited (125 μM NO_3^- and 125 μM PO_4^{3-}) medium, but increased TAG levels were witnessed in both cases	[17]
1–150% phosphorous in modified f/2 medium; 110 μmol/m^2/s with 10:14 h light: dark cycle at 23 °C for 14 days	***I. galbana U4***	Maximum lipid accumulation upto 50% w/w with phosphorus limited conditions 1–12.5% P	[42]
BG11 without $NaNO_3$ with light intensity of 10 W/m^2 at 30 °C, aerated with sterile air at 0.5 vvm	***C. pyrenoidosa***	47.10% (w/w) lipids with 0.87 g/L biomass content after 120 h compared to 25.14% (w/w) with total nutrients	[22]
40% wastewater, 60–120 μmol/m^2/s light intensity, 20 °C for 9 days	**Microalgae-bacteria for *Chlorella* sp.,**	Higher lipid content (17.2% w/w) was obtained at low light intensity and high organic carbon	[38]

20% (w/w) lipids in *Chaetoceros* sp., at 25 °C which was found to decline by 2-fold and 2.5-fold with increase to 35 °C and 45 °C respectively. High light intensities above 400 μmol/m^2/s combined with temperature under 35 °C causes physiological changes facilitating degradation of proteins and carbohydrates, as well as restructuring of membrane lipids, which act as precursors thereby, promoting accumulation of neutral lipids up to 57% (w/w) with higher amount of C16–C18 FAs in *Monoraphidium dybowskii* Y2 grown in BG11 medium [43].

pH of the growth media affects the algal metabolism and productivity [45]. **Qiu *et al.*** [45] reported that pH influences the biomass content as it controls the availability of bicarbonate ions but had no effect over the lipid accumulation, even-though biodiesel with desirable properties are preferably obtained at low pH. Contrary to this, **Wang *et al.*** [46] postulated that pH and CO_2 supply often jointly influence the algal specific growth rate and lipid content via the availability of bicarbonate ions. Maximum lipid content of 53% (w/w) in *C. vulgaris* was achieved at a pH of 7.5, beyond which no significant effect on lipid accumulation was observed by **Sakarika *et al.*** [47]. Optimum pH influences the available form of ions, which in turn might influence the key enzymes and precursors, thereby affecting lipid yield.

Similar to other environmental factors, media salinity also affects the degree of FA saturation [37]. Hyper-salinity provides a favourable FA profile with increased concentration of saturated and monounsaturated FAs (MUFAs) compared to PUFAs in a native algal consortium grown in wastewater [37]. **Rearte *et al.*** [48] also projected 37.2% (w/w) in *Golekinia* sp., with balanced quantity of MUFA/PUFAs from biodiesel with palmitic, linoleic and oleic acid comprising 90% of total fatty acid methyl esters (FAMEs). Several environmental stress stimuli as individual and in combination along with their influence over the lipid content are also illustrated in Table 2, thus projecting the antagonistic and synergistic effects over algal biochemical characteristics.

3.2. Manipulating microalgal lipid accumulation by nutrient stress

One of the most common strategies for increasing lipid concentration involves subjecting the cells to nitrogen (N) and phosphorous (P) limitations [11]. These elements reorient the carbon flux and redirects the cellular energy, thereby changing the physiochemical response associated with lipid metabolism and accumulation [32]. **Ho *et al.*** [55] reported that *Scenedesmus obliquus* CNW–N under N starvation accumulates two fold higher lipids with C16 and C18 constituting 76–84% of total FAs. Nitrogen depleted conditions (HS medium without NH_4Cl) have been reported to cause 64% increase in total energy accumulating 20-fold higher TAGs in *C. sorokiniana*, over one-month

period due to enhanced photosynthetic energy capture with less impacts over biomass content [56]. **Lai *et al.*** [33] projected that decline in nitrogen in the medium often results in conversion of accumulated starch to lipid, thereby triggering TAGs accumulation. Study by **Roopnarain *et al.*** [57] has also projected phosphorus as the limiting nutrient influencing algal biomass and lipid content. Recent study by **Huang *et al.*** [32] with *T. lutea* combined the biochemical and proteomic analysis to provide better insights into the lipid biosynthesis metabolism under nutrient limitations. Comparable growth rate as that of the control was achieved with P and N-limited medium, but increased TAG levels were witnessed in both cases. The authors reported an increase in neutral lipid accumulation was due to the degradation of membrane lipids into storage forms under N starvation and with upregulation of genes associated with *de novo* lipid synthesis at late stationary phase during P limitation. More such studies are essential for better understanding of the influence of these environmental stress factors at the cellular or genetic levels to facilitate increased lipid accumulation in microalgae.

3.3. Role of reactive oxygen species in enhancing lipids in microalgae

Cellular aerobic metabolism is tightly regulated by antioxidant enzymes and non-enzymatic antioxidant molecules which governs the formation of reactive oxygen species (ROS) like the superoxide (O_2^-) ions, hydroxyl radical ($OH^\bullet$) ions, and hydrogen peroxide (H_2O_2) [58]. Oxidative stress occurs, when the dynamic stable equilibrium between the ROS generation and elimination is disturbed thereby, increasing the ROS levels. Recent reports have suggested, ROS to act as the general decisive and quantitative reference marker during stress and other adverse conditions that influence lipid yield. **Menon *et al.*** [59] reported that the intracellular ROS levels can be linked by an inverse power law to the lipid accumulation in *C. vulgaris*. **Chokshi *et al.*** [60] reported increased lipid content from 24.31% to 29.92% (w/w) in *Acutodesmus* sp. with lower ROS levels due to increased activity of superoxide dismutase (SOD) and catalase (CAT) under nitrogen depletion. However, in contradiction to the above study, very recent study on *C. pyrenoidosa* by **Zhang *et al.*** [36] reported the maximal increase in ROS level under N limitation. Transcriptome analysis revealed upregulation of genes associated with FA and glycerol biosynthesis pathways on increase in OH^{-1} concentration owing to ROS generation. The study also reported a decline in biomass content from 1.43 to 0.87 g/L because of cytotoxic effects of ROS at cellular level. More insights into the ROS generation, antioxidant based defence system on lipid synthesis can be obtained in the study by **Sun *et al.*** [61] and **Zhang *et al.*** [62]. Due to inconclusive results reported so far, there is a necessity to understand the cross-talk occurring between the secondary metabolism associated with the application of environmental stress and intracellular species-specific ROS generations to effectively manipulate the lipid biogenesis.

3.4. Manipulation of cultivation and the nutritional regime of microalgae

The mode of cultivation significantly affects the growth rate, biomass productivity and lipid accumulation. Microalgae usually have three basic modes of nutrition namely phototrophic (uses sunlight as energy and CO_2 as carbon source), heterotrophic (uses sugars and organic compounds as carbon source and occurs in the absence of light) and mixotrophic (uses both CO_2 and organic compounds as carbon source, with simultaneous occurrence of both respiration and photosynthesis) [63]. The recent study by **Mondal *et al.*** [64] projected that *C. sorokiniana* grows best with 80 μmol/m^2/s light intensity for 12 h in BG11 supplemented with molasses (5 g/L) resulting in 1.55 g/L biomass and 30% w/w lipids under mixotrophic condition, followed by 26% (w/w) lipids during autotrophic growth with same light intensity having 5 g/L $NaHCO_3$. Mixotrophic and autotrophic conditions results in increased activity of metabolic enzymes concerning FA biosynthesis, thus accumulates higher TAGs with more C16–C18 FA favourable for biofuel production. Heterotrophic conditions in the absence of light energy provided lower amount of ATP from fructose as carbon source which was mostly utilized for growth thereby, accumulating only 15% (w/w) lipids. Similar conclusions can also be drawn from the lipid content of different algal species grown under various modes of cultivation as represented in Table 3. **Adesanya *et al.*** [69] modelled and experimentally validated the growth and accumulation of lipids by *Chlorella* sp., under mixotrophic and phototrophic modes, thereby projecting increased biomass productivity under mixotrophic mode with nitrogen sufficient conditions and enhanced accumulation of TAGs with nitrogen starvation. Nevertheless, the mode of nutrition must be optimized and synergistically combined in a gradient manner during the two stage cultivation to maximize biofuel production efficiency.

4. Enhancing lipid yield via molecular engineering and synthetic biology

4.1. Developments in system biology approach and in-silico analysis

With the availability of complete genome sequences and the advent of high data content measurement techniques for transcripts, proteins, metabolites and their interactions, a new level of understanding of cells and organisms has become possible. System biology approach uses a comprehensive and consistent system to integrate the individual cellular components and predict the directed outcome and underlying dynamics through modelling and flux analysis. The framework involves several steps and knowledge of different interdisciplinary subjects like molecular biology, computer science, control systems and modelling aspects. **Behera *et al.*** [70] utilized *in-silico* computational approach to redesign the 3D structure (94% accuracy as obtained by Ramachandran plot) of ACCase (key limiting enzyme of FA synthesis) with *C. reinhardtii* as model, and proposed that binding with biotin can improve the enzyme activity and inherent lipid metabolism.

With the knowledge of more cellular compartments and genetic networks in microalgae, computational modelling is increasingly being applied to integrate the biological data to predict the yield. Initial steps involved the use of fundamental information of genes, their networks, underlying interactions and functions, to construct the model, which is then perturbed based on environmental and genetic stimuli for refinement, adapted to make realistic predictions [71]. Different omics tools to identify the potential enzymes, underlying cellular metabolic pathways to be remodelled via metabolic and genetic engineering approaches for enhancing lipid accumulation in microalgae has been elucidated in the study by **Arora *et al.*** [72]. **Banerjee *et al.*** [73] and **Aratboni *et al.*** [7] have very recently reviewed the strategies of system biology tools concerning increased lipid biogenesis. Pathway tools and Constraint-based reconstruction and analysis (COBRA) has been used to study, analyse, simulate and remodel the flux distribution during lipid synthesis [74]. To identify the effect of gene alteration (insertion/deletion/substitution) on the targeted product before going for genome editing, strain optimization tools like EDGE, OptORF and OptKNOCK are commonly used [74]. A bevel programing framework Biolog phenotype microarray was used to validate the reactions and pathways to comprehensively remodel and reconstruct *C. vulgaris* UTEX 395, with 843 genes, 2994 reactions and 1770 metabolites [75]. **Chaiboonchoe *et al.*** [76] used phenotype microarray technology to refine the existing COBRA-based metabolic model of *C. reinhardtii* iRC1080, via the addition of 284 reactions. This model would also serve as a novel prototype for functional profiling of other algal species with desired phenotypes. In spite of the advances, still there exists several challenges to be resolved via more intensive research for better utilizing the omics-based system biology approach for driving the algal biotechnology.

4.1.1. Genome-scale metabolic models

The complexity of microalgae and large genome size makes interpretation of the inherent metabolism difficult. Genome-scale metabolic

Table 3
Microalgal biomass concentration and lipid content concerning the strategies adopted in the mode of cultivation.

Microalgae	Mode of cultivation and growth conditions	Biomass Conc. (g/L)	Lipid content (%)	References
Chlorella sp. Y8-1	*Photoautotrophic*: White fluorescent light (4300 lux); 24: 0 L:D cycle; 30 °*C*; 10% CO_2 at 2 vvm; 15 days incubation in 1 L bottles having modified Walne medium	0.22	16.5	[65]
Chlorella sp. Y8-1	*Heterotrophic*: Absence of light; 30 °*C*; 10% CO_2 at 2 vvm, with 0.5 g/L urea and 0.75 g/L sucrose; 15 days incubation in 1 L bottles having modified Walne medium	0.17	5.9	[65]
Chlorella sp. Y8-1	*Mixotrophic*: White fluorescent light (4300 lux); 24: 0 L:D cycle; 30 °*C*; 10% CO_2 at 2 vvm, with 0.5 g/L urea and 1 g/L sucrose; 15 days incubation in 1 L bottles having modified Walne medium	0.45	35.5	[65]
Native microalgae from lentic water body	*Mixotrophic*: 0.23 m^2 surface area open ponds with domestic sewage having 400 mg/L COD; 75 mg/L TDS; 115 mg/L nitrate with 500 mg/L each of glucose, sodium nitrate and sodium phosphate and 1 g/L NaCl, pH: 8.2; 8 days incubation time	6.12	23.4	[37]
Chlorella sp.	*Phototrophic*: 14 W/m^2 fluorescent light intensity; BBM with 2% CO_2 at 10 °*C*, 23 days incubation time	0.20	20.7	[66]
Chlorella sp.	*Mixotrophic*: 14 W/m^2 fluorescent light intensity; BBM having 25 mM glycerol with 2% CO_2 at 10 °*C*, 23 days incubation time	0.20	28.2	[66]
D. salina	*Photoautotrophic*: Modified D medium; 100 μmol/m^2/s light intensity; 12:12 h light: dark cycle with 10 days incubation time	0.59	24.6	[67]
D. salina	*Mixotrophic*: Modified D medium having 0.05 M glucose; 150 μmol/m^2/s light intensity; 12:12 h light: dark cycle with 10 days incubation time	1.16	31.3	[67]
N. oculata CCAP849/1	*Photoautotrophic*: Modified f/2 medium; 80 μmol/m^2/s light intensity; 12:12 h light: dark cycle with 10 days incubation time	0.54	26.5	[67]
N. oculata CCAP849/1	*Mixotrophic*: Modified D medium having 0.02 M glucose; 150 μmol/m^2/s light intensity; 12:12 h light: dark cycle with 10 days incubation time	1.69	37.3	[67]
Graesiella sp. WBG 1	*Mixotrophic:* Modified BG11 medium with 1.3 mM Nitrate supplemented with 1.12 mM sodium acetate 300 μmol/m^2/s light intensity; 42:6 h light: dark cycle; 1% v/v CO_2; 8 days incubation time	1.12	37.6	[68]

model (GSMMs) constructed via the use of the biochemical data, computational frameworks and automation tools provide a comprehensive description of the cellular metabolic reactive pathways with the use of stoichiometric coefficients and simple mathematical formulations [77]. GSMMs have revolutionized the field of metabolic engineering and are being widely used to study the genotype-phenotype association, identification of gene manipulation targets and other metabolic strategies for the production of biofuel [71].

GSMM reconstruction involves the following steps: i). Identification of metabolic transport reaction and pathways through the use of genomic annotations, experimental reactions and enzyme homology via the use of databases like Plant Metabolic Network, KEGG, Model SEED, BioCyc and TCD databases; ii). Formulating equations for biomass generation based on the physiochemical features of different algal species; iii). Utilization of semi-automated curation methods like *GapFind* and *GapFilling* to identify incomplete transport reactions [78]. GSMMs for several microalgae and cyanobacteria has been listed in the study by **Tibocha-Bonilla *et al.*** [78], *Synechocystis* PCC 6803 is the most well studied strain having about 12 GSMMs like *i*HK677 [79]; and *im*Syn716 [80]. **Joshi *et al.*** [81] recently reconstructed the existing GSMM of *Synechocystis* PCC 6803 (*i*SynCJ816) with 816 genes, 1045 reactions and 925 metabolites, which could predict gene deletions with 77% accuracy, therefore, can be used during qualitative experiment based growth studies using online databases. GSMMs for *A. plantensis* (*i*Ak692) [82], *Cyanothece* sp., [*i*Cyc792, *i*Cyn731, *i*Cyj826, *i*Cyp752, *i*Cyh755] [83] and *Synechococcus* sp. [*i*Syp811 [84]; *i*Syu683 [85] are also available. Based on the nuclear genome (CCAP19/18) of *D. salina*, carbon core metabolic flux model has been reconstructed encompassing 221 reactions and 212 metabolites involving cytosol, chloroplast and mitochondria, that projected a more favourable flux for carotenoid synthesis rather than lipid accumulation with increase in NADPH levels [86]. Reconstructed algal GSMMs having multi-omics dataset are often combined with high-throughput phenotyping experiments to study the intracellular fluxes and evaluate the biochemical energy utilized in carbon fixation and other assimilatory pathways via flux balance analysis.

4.1.2. Flux balance analysis

With the increase in the number of biochemical reconstructions, the system based approaches to validate the metabolic models *in-silico* has been developed. Flux balance analysis (FBA) is being used as a constrain based systemic approach to model and predict the phenotypes underlying the metabolic pathways [87]. FBA integrates the biochemical databases with the interconnected metabolic network reactions, using the necessary stoichiometry, energy and redox balance modelling via linear programming [9]. Mathematically, the mass balance equation depicting the relationship between reactive metabolites and the products is represented as a stoichiometric matrix **(Eq. (1))**,

$$S.v = 0 \quad \text{Eq. (1)}$$

S represents an (*m* x *n*) matrix, where *m* represents the number of metabolites and *n* represents the number of flux or reactions. The matrix also hypothetically includes a null space for accounting the integration of different biochemical flux in the design network to balance the model. FBA calculates the internal reaction rate under steady-state assumptions, keeping the concentration of metabolites constant. The change in flux with response to the gene insertion and knock-out or changes in growth conditions can be studied using improved versions of FBA like dynamic FBA (dFBA), flux variability analysis (FVA), regulatory on/off minimization (ROOM) and minimization of metabolic adjustments (MOMA) [88]. Dynamic reduction of unbalanced metabolism (DRUM), a variant of FBA utilizes quasi steady state assumptions to predict the accumulation of metabolites without genome information [89]. The complexity and solution space during FBA was reduced from 90,000 to 3079 distributions via incorporation of the thermodynamic and energy constraints illustrating the connection between respiration and photosynthesis with variation in photon flux in *C. reinhardtii* by **Cogne *et al.*** [90]. Though, the study did not provide information on lipids, this technique could be extended for its prediction by adding constraints to lipid synthesis reactions.

FBA of algal metabolism for maximizing TAG production is challenging compared to other microbial counterparts due to their phototrophic nature, and dependency over the light dark cycle, which does not assume a steady state [9]. **De Bhowmick *et al.*** [9] have summarized the flux analysis studies on different microalgal strains. Dynamic FBA was used by **Muthuraj *et al.*** [91] to predict the metabolism of *Chlorella* sp. FC2 IITG under light-dark cycle, and it was observed that neutral lipid synthesis (29.7% content) was upregulated due to elevated glycolytic and acetyl-CoA flux under light cycle with heterotrophic conditions grown in BG11 (supplemented with 15 g/L glucose) with 20

μmol photons/m^2/s with 8 h photoperiod. **Zhu and Huang** [92] proposed the FBA for *C. sorokiniana* under heterotrophic conditions (supplemented with 35 g/L glucose) having 34 metabolic reactions including Embden–Meyerhof–Parnas (EMP), hexose monophosphate pathway (HMP), tricarboxylic acid cycle (TCA), and FA biosynthesis pathway. The study reported an increased carbon flux towards lipid biosynthesis with the decrease in the concentration of $NaNO_3$ (0.125 g/L). Thus, system biology tools of GSMMs and FBA can be used to metabolically model microalgae to obtain insights into the lipid synthesis.

4.2. Genetic engineering techniques to enhance algal lipid accumulation

Genetic engineering tools are increasingly becoming popular for harnessing microalgae as possible cell factories for biofuel production. Key milestones related to the progress in genetic engineering of microalgae have been shown in Fig. 2. Genetic engineering of microalgae to enhance the lipid content and quality mainly focusses on i). Increasing the production of FA precursors; ii). Inhibition of β oxidation pathway in peroxisomes to inhibit lipid catabolism; iii). Overexpression of Kennedy pathway genes for regulating TAG synthesis; and iv). Regulation of thioesterases and desaturases to optimize FA chain length for improving the biodiesel profile. **Levering *et al.*** [93] and **Sharma *et al.*** [18] have summarized the genetic engineering approaches for increasing algal lipid accumulation. Some of the outcomes of lipid accumulation witnessed via algal genetic engineering with detailed description of culture conditions has been summarized in Table 4. Industrial application of microalgae requires utilization of random mutagenesis through UV irradiation or chemical mutagens and transformation via electroporation, silicon carbide whiskers and biolistic nanoparticles [102]. A shuttle vector transformation method for integrating circular and linear DNA fragments having *ble* cassette and *pds* selection marker to efficiently engineer *H. pluvialis* to act as a safe and industrially relevant strain for biofuel production has been demonstrated by **Sharon-Gojman *et al.*** [103]. Starchless mutants of *S. obliquus* produced through UV mutagenesis by **de Jaeger *et al.*** [104] is one of the successful strategy till date, that limited carbohydrate formation redirecting the energy and carbon flux towards lipid synthesis, resulting in 41% increase of FA productivity compared to wild strains under phototrophic nitrogen limited condition.

Genetic modification can also be induced via the utilization of bioengineering strategies like Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR associated with the protein 9 (CRISPR–Cas9), Transcription Activator-Like Effector Nucleases (TALENs) and Zinc-Finger Nucleases (ZFNs) as detailed by **Fajardo *et al.*** [105]. Over the past two decades, genome editing has been revolutionized with the use of ZFNs and TALENs that has been successfully used in insects and plants. However, there are very few reports of successful gene editing using the above-mentioned approach in microalgae mainly with *P. tricornutum* [14]. Multi-gene engineering through regulation of expression of numerous target genes is often done with CRISPR-Cas 9 system [105]. Especially from 2014, this tool has brought a significant revolution in genome editing for achieving desirable yield with high specificity compared to RNA interference (RNAi) method, which has low efficacy due to non-specific targeting [7]. CRISPR has been successfully implemented to increase the oil yield in *Nannochloropsis* sp. [106], *C. reinhardtii* [107] and *Synechocystis* sp [108].

Algal genetic engineering strategies to manipulate lipid content broadly involving gene overexpression, shuffling and repression linked to FA synthesis enzymes along with the approach to increase the photosynthetic efficiency have been detailed in following subsections.

4.2.1. Insertion and overexpression of gene of interest

Understanding the FA synthesis pathways for increasing the expression of key enzymes is considered an essential approach to engineer microalgae with high lipid content. Overexpression of gene encoding for the malic enzyme (ME) involved in pyruvate metabolism and CO_2 fixation in *P. tricornutum* resulted in 2.5-fold increase in total lipid content and 31% increase in neutral lipids which was 66% higher than wild strains [109]. Overexpression of malic enzyme (PtME) and 5-desaturase (PtD5b) by promoter Pt 202 and Pt 667 respectively in *P. tricornutum* resulted in a 2.4-fold increase in neutral lipid content with no detrimental influence over other physiological traits [110]. **Dinamaraca *et al.*** [111] reported that overexpression of diacylglycerol O-acyl transferase [DGAT2D] (50-100-fold increase in mRNA level with 30-50-fold increase in enzyme) in *P. tricornutum* increased the carbon flux towards lipid synthesis, and led to 2-fold higher lipid accumulation than wild strain under nitrogen depletion, growing only 15% slower under light saturation conditions (120 μmol/m^2/s).

A new generation technique of tailoring microalgae with high TAGs also involves modulating genes not directly associated with FA synthesis or insertion of transcription factor (TF) to overproduce lipids. Overexpression of gene encoding lipid droplet (LD)-associated protein (PtLDP1), in *Phaeodactylum tricornutum* increased total lipid content by 1.5-fold with larger lipid droplets [112], thus highlighting the role of these protein in lipid synthesis and mobilization. A recent study by **Kang *et al.*** [113] reported 36.5% increase in lipid accumulation of *N. salina* by inserting a gene corresponding to Wrinkled1 TF type AP2 (known to regulate lipid synthesis in plants) present in *Arabidopsis thaliana* (AtWRI). Several studies have also shown contradictory results associated with overexpression of FA synthesis genes. For instances, increase in Lauryl ACP and FatB1 theoesterase [114] and, DGAT2 [115] in *C. reinhardtii* showed no change in TAG levels under standard growth or N starvation, even though transcript levels increased. This suggests the future research to be directed in understanding the influence of multiple genes and enzymes linked to FA synthesis, which might dominate and act antagonistically influencing the lipid biosynthesis.

4.2.2. Gene shuffling

Genome shuffling is a strain improvement approach utilized over decades, often in combination with random mutagenesis and synthetic biology techniques for bioprospecting of potential algal strains. Sexual recombination, protoplast formation, and fusion are often utilized for genome shuffling to produce industrially relevant microbes. Strategies like homologous recombination for activation of gene expression and *Agrobacterium tumefaciens* mediated gene transfer can be utilized to replace the gene of interest in microalgae [116].

Microalgae grown under photoautotrophic conditions derive their biomass and lipids via the conversion of CO_2 into ribulose-1,5 bisphosphate (RuBP) and 3-phosphoglycerate using RuBP carboxylase/oxygenase (RuBisCO), which often acts as limiting agent under conditions of depleted CO_2 or with high light intensity/temperature. One of the initial studies by **Zhu *et al.*** [117] reported that three rounds of polymerase chain reaction (PCR) based shuffling of *Chlamydomonas rbcl* gene with oligonucleotides, followed by strain selection lead to 20% and 56% increase in RuBisCO specificity and activity respectively. Though the mutant strains showed affinity for CO_2, no significant increase in biomass content was observed and no implications on lipids were provided. Nevertheless, similar studies with the aim to increase the activity of key limiting enzymes of lipid biogenesis holds a probability for increasing the lipid yields in microalgae. **Takouridis *et al.*** [118] reported that unique progeny generated via genome shuffling through sexual recombination in *C. reinhardtii* resulted in increased tolerance towards salinity from 300 to 600 mM with cell density enhanced to 0.49 g/L from 0.29 g/L. This study has demonstrated the potential of developing sexually competent strains with desirable traits facilitating resilience to outdoor culture conditions for reducing costs during biofuel production. Recent study by **Fields *et al.*** [119] engineered *C. reinhardtii* strain (originally grown in TAP medium with 100 μE light intensity) via UV mutagenesis of nuclear genome followed by genome shuffling through sexual recombination with wild type, resulting in 15-fold enhancement of Green Fluorescence Protein (GFP) expression. Though the technique was just developed over a period of 3 months, and was

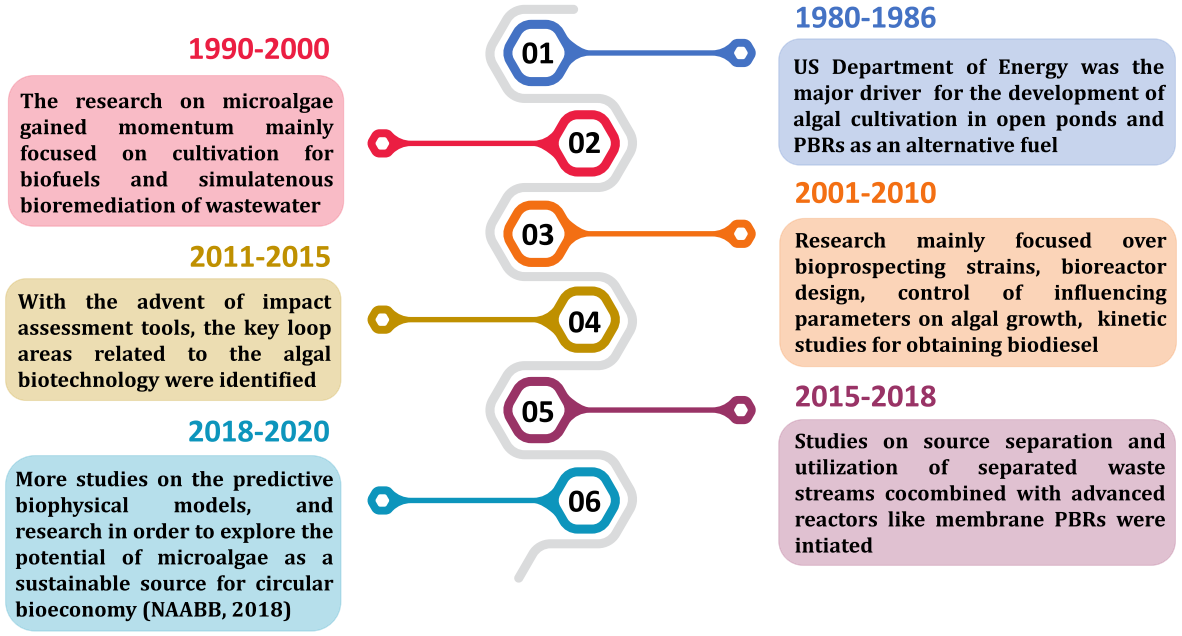

Fig. 2. Key milestones highlighting the progress in molecular approaches for engineering microalgae.

Table 4
Variation in lipid content of microalgae with different genetic modification methods.

Host organism	Growth conditions	Genetic modification	Effects observed	References
P. tricornutum CCMP 2561	F/2 medium at 21 °*C*, 200 μmol/m^2/s with 15:9 h light: dark photoperiod	Overexpression of isoform of diacylglycerol acyltransferase (DGAT 2)	Neutral lipid content increased by 35%, with more oil bodies	[94]
P. tricornutum LAMB014	Sterile seawater F/2 medium at 22 °*C*, under 100 μmol/m^2/s with 12 h light	Overexpression of gene encoding endogenous D6 desaturase	Increase in eicosapentaenoic acid (EPA) by 16.4–18.64%	[95]
N. oceanica CCMP1779	F/2 medium in shake flasks, 120 rpm mixing, with 100 μmol/m^2/s illumination for 12 h at 22 °*C*	Overexpression of genes encoding Δ12 and Δ5 fatty acid desaturase	25% increase in EPA and 35% increase in other long chains PUFAs	[96]
C. reinhardtii TKAC1017	TAP medium with 23–39 μE, at 25 °*C*, for 4 days	Insertion of medium chain specific thioesterase gene from *C.lanceolata*	No increase in free FAs; 1.5–1.8-fold increase in TAGs	[97]
D. tertiolecta LB-999	Sterile ATCC- 1174 liquid medium with 0.5 M NaCl under 50 μmol/m^2/s with 14h light regime	Simultaneous expression of lauric acid-biased TE (C12TE) and MCFA-specific ketoacyl-ACP synthase (KASIV)	7-fold increase in lauric acid content and 4-fold increase in myristic acid	[98]
Synechococcus sp. PCC 7002	F/2 medium at 29 °*C*, illuminated with 40–120 μmol/m^2/s with 16 h light/8 h dark regime	Substitution of gene encoding the native enzyme FabH with a *Chaetoceros* ketoacyl-ACP synthase III	5-fold increase in medium-chain FA synthesis	[99]
N. oceanica CCAP 849/10	Modified f/2 liquid medium at 25 °*C*, 200 μmol/m^2/s with 12 h light Regime	Overexpression of novel bZIP1 transcription factor	Induced lipogenesis and 16.2-fold increase of secreted lipids	[100]
N. oceanica CCAP 849/10	Artificial seawater supplemented with f/2 nutrients illuminated with 130 μmol/m^2/s for 24 h at 25 °*C*	Insertion of transposon complex Tn5	Accumulation of 73% intracellular lipids	[101]

projected to be easily deployed at commercial level, yet, there was no information regarding the exact position of the mutation in the genome. Gene or ortholog domain shuffling in microalgae are limited to acclimatize the selected strain in growth medium with efficient nutrient removal [120] and are not much explored for lipid enhancement. It could be reasonably assumed that, such principles could be combined with high-throughput screening for selecting better lineages of oleaginous algae.

4.2.3. Gene repression and silencing

One of the efficient technique to enhance lipid accumulation is by repressing the genes associated with its catabolism. Antisense mediated knockdown of gene coding multi-functional acyltransferase/lipase/phospholipase declined the lipid degradation, leading to 3.3 times increase in lipid content under both nutrient depleted and repleted conditions, without inhibiting the biomass productivity in *T. pseudonana* [121]. Disruption of gene encoding Hotdog-fold theosterase believed to catalyse hydrolysis of Acyl-CoA, by TALENs increased the precursor pools and resulted in 1.7-fold increase in TAG levels in nitrogen replete medium after 8 days [122]. Studies have also reported repression of genes related to carbohydrate metabolism can redirect the carbon flux towards lipid synthesis. Knockdown of gene encoding phosphoenol pyruvate carboxykinase in *P. tricornutum* downregulated the decarboxylation of pyruvate in C4 pathway resulting in lipid accumulation [123]. Antisense knock down of 1261-bp putative pyruvate dehydrogenase kinase gene (PtPDK) from *Phaeodactylum tricornutum*, resulted in 82% neutral lipid content, and produced cells smaller in size but with similar biomass productivity as the wild strain, under standard growth conditions (f/2 medium; 200 μmol/m^2/s white light for 12 h; 21 °*C*) [124]. Modulation of $Zn(II)_2Cys_6$ (ZnCys) regulator through CRISPR-Cas 9 mediated attenuation of 5′UTR and RNAi doubled lipid productivity under N replete (0.8 mM nitrate) growth medium in semi-continuous mode, without inhibiting the biomass content [125]. This was attributed to the downregulation of N assimilatory genes restricting flux

towards protein production, redirecting it towards TAG accumulation. Gene repression has also been utilized to understand the role of putative genes on lipid synthesis that act as hub for regulatory and other developmental processes. **Luo *et al.*** [126] reported that silencing the gene encoding flagella derived protein 89 (CrFAP89) (kind of WD40 repeat protein) in *C. reinhardtii* led to better growth under nutrient starvation but 22% decline in lipid content was observed after 5 days. The study reported a decline in DGAT levels by 16–78% under the influence of CrFAP89 thereby, decreased lipid accumulation. More such studies are essential to unravel the role of interconnected networks in lipid biogenesis, to better modulate them for achieving desired productivity.

4.2.4. Strategies to trigger lipids via increase in photosynthetic efficiency

Increasing the photosynthetic efficiency of microalgae is regarded as an essential strategy to attain enhanced lipid productivity, which is ultimately determined by the biomass content and energy density of microalgae. Often, RuBisCO causing carboxylation and influencing rate of carbon flux is primary target to manipulate the calvin cycle, thereby the photosynthetic capacity. **Hazra *et al.*** [127] reported that overexpressed RuBisCO activase increases the catalytic active sites of RuBisCO, thereby increasing the algal carbon fixation capacity. Overexpression of the enzyme in *N. oceanica* has also been reported to increase its photosynthetic activity by several folds by **Wei *et al.*** [128]. The review by **Sharma *et al.*** [18] has enlisted strategies overexpressing aldolase and sedoheptulose 1,7-bisphosphatase (SBPase) linked with the regeneration of precursors of RuBisCO in different algal strains, thereby increasing their photosynthetic efficiency.

Maximizing algal biofuel production capacities without inhibiting biomass productivity requires genetic manipulation of microalgae to reduce their inherent photo-protective mechanisms, which limits light absorption beyond a threshold limit. One of the gene manipulation mechanism involves reduction in the content of photosynthetic pigments or the size of light harvesting complex (LHC), causing them to absorb less light per cell, thus resulting in saturation at higher light intensities compared to the wild strains. Also, the conversion of solar to chemical energy is much more efficient owing to decline in heat and energy loss in the mutant cells leading to higher biomass accumulation [129]. Truncation of LHC in *C. reinhardtii* and *Dunaliella salina* by insertional and chemical mutagenesis generated strain with higher light utilization efficiency during mass cultivation. **Mussgnug *et al.*** [130] reported that the energy losses due to photochemical quenching was much reduced with the down regulation of LHC genes through RNAi technology. Another approach to increase the photosynthetic efficiency involves extending the adsorption efficiency of photosynthetic pigments [131]. The photosynthetic efficiency of microalgae could be enhanced by 19% with just 50 nm increase in wavelength of light adsorption [132]. Co-expression of genes encoding *Chl d* and *Chl f* from *Acaryochloris marina* which absorb light in infrared regions with *Chl a* has been proposed as the possible strategy for expanding photosynthetic adsorption spectrum, thereby provide more quantum of energy increasing the photosynthetic efficiency [133]. **Fu *et al.*** [134] genetically modified *P. tricornutum* via the enhanced expression of green fluorescent protein that functioned as a chromophore thereby absorbing light in spectral regions, which were otherwise restricted in wild strains. **Verruto *et al.*** [135] combined CRISPR-Cas 9 with *Cre recombinase* to engineer *N. gaditana* as a model system for multiple gene editing. The above-mentioned system with markerless knockout of 7 genes related to light harvesting complex and Acyl-CoA oxidase along with 2 kb insert with ZnCys gene had doubled lipid productivity with no compromise in biomass under N replete semi-continuous system. **Panahi *et al.*** [136] have precisely summarized the genetic engineering strategies to reduce the photoinhibition effects and redirect the photosynthetic energy towards algal lipid accumulation. More insights related to the mutagenesis and genetic engineering strategies to produce truncated antennae based mutants can be obtained from the recent review by **Kumar *et al.*** [137]. Nevertheless, further research and counteracting the challenges like photo-damage and unwanted side-effects of mutation in photosynthetic algal mutants can successfully translate these technologies for producing microalgae with high biomass and lipid productivity.

4.3. Advances in metabolic engineering strategies for increasing the lipid yields

Metabolic engineering involves intentional modulation of cellular pathways in microalgae to study the influence of growth conditions on the phenotypic profile. Classical metabolic engineering for increasing lipid yield involves gene modulation techniques based on the prior knowledge of the rate limiting steps in FA synthesis. With a desire to increase the efficiency at decreased cost and time, metabolic engineering is often driven by the system biology approach [78]. This approach involves understanding the effect of operational parameters over the intracellular metabolic networks concerning lipids through rational model linked experiments and simulations using GSMMs. Both the above-mentioned strategies are detailed in the subsequent sections. Detailed step-by-step procedure of the metabolic engineering starting from lab based experiments can be referred from the review by **Naghshbandi *et al.*** [23].

4.3.1. Metabolically engineering strains for enhanced lipid accumulation by gene modulation

Lipid biogenesis and accumulation is often complicated due to the phylogenetic diversity with numerous metabolic networks in microalgae. Metabolic engineering involves redirecting the carbon flux towards lipid biogenesis as well as alteration of trophic conversion to promote lipid accumulation. **Rai *et al.*** [138] systematically integrated metabolomics and proteomics data to reveal that regulated time-dependent nitrogen limitation process can be correlated with the availability of substrates like phosphoenolpyruvate and acetyl CoA redirecting the carbon flux and reduced power towards lipid accumulation. The schematic representation of the pathways to be engineered along with the techniques for modulating the gene expression, directing increased TAG synthesis has been elucidated in Fig. 3. One of the commonest strategies for metabolically increasing FA synthesis is to regulate the activity of ACCase, which is considered rate limiting. This strategy has been demonstrated to increase FAs by 1.6-fold in *S. quadricauda* by overexpression of ACCase, whereas, 1.45-fold increase was observed in combination with over-expressed genes for glycerol kinase and glycerol-3-phosphate dehydrogenase [139]. Though a higher level of increase was expected, an interplay of several metabolic networks can influence the activity of each other, thereby affecting lipid yield. Another possible strategy involves increasing the transcript abundance of Malonyl CoA-ACP transacylase (MCAT) enzyme that catalyzes the formation of malonyl-ACP. **Chen et al.** [140] reported 36% higher lipids, with enhanced levels of myristic, palmitic and palmitoleic acids in transformants with overexpression of NoMCAT gene compared to wild strains of *N. oceanica* cultured in natural seawater supplemented with f/2 medium exposed to 150 μmol/m^2/s (12 h illumination). **Mao et al.** [141] reported that overexpression of DGAT genes (*CzDGAT1A and CzDGTT5*) in endoplasmic reticulum regulated by the transcriptional factors bZIP3 and MYB1 respectively, increased acetyl-CoA pools, enhanced TAG yields with higher C16-18 FA content in C. *zofingienis*, grown under nitrogen deficit condition in Kuhl medium with 1.5% (v/v) CO_2 aeration, 30 μmol/m^2/s continuous light regime at 25 °*C*. This study further reported the heterologous expression of the gene *CzDGAT1A* in *N. oceanica* under similar growth conditions resulted in accumulation of 58% more lipids than the empty vector after 10 days. Thus, manipulation of enzymes can favourably redirect the flux towards lipid biogenesis. Table 5 illustrates some of the metabolic engineering strategies with the underlying gene modulation concepts of algal strains under different growth conditions for increased TAG levels. Detailed summary report on gene modulation strategies by **Sun *et al.*** [19] have reported no significant increase in TAG accumulation with manipulation of genes

associated with FA synthesis pathway, but remarkable increase in TAG yield with overexpression of genes and transcription regulators of Kennedy pathway. The lack of understanding of the underlying mechanism involved, still calls for more in-depth research on these aspects. Identification of new genes and regulatory proteins, novel genome editing strategies, shuttle vector approach and use of TALENs for conducive expression of the endogenous transgene are expected to revolutionize the arena of classical metabolic engineering in near future [149].

4.3.2. *Metabolic model linked experiments*

Integrating data from GSMMs, high throughput experiments using reconstructed metabolic models could be designed to study the effect of different environmental parameters on TAGs accumulation [86]. Several metabolic models like AlgaGEM [150]; *i*CZ843 [75]; *i*LB1027 [88]; *i*CZ946 [151] were being constructed for experimenting and predicting the algal growth and lipid accumulation under different operational conditions. A recent study by **Li *et al.*** [152] used GSMM of *C. vulgaris* (*i*CZ946) to predict the minimum amount of nitrate necessary to increase lipid productivity without compromising growth under autotrophic conditions. The model predicted that 18% nitrate is sufficient enough to support growth under nitrogen restricted autotrophic conditions with 61% and 195% increase in FAMEs and lutein yield respectively after 549 h. The descriptive features of the metabolic models along with the inference obtained in different rational metabolic engineering studies by manipulating the nutrient levels are highlighted in Table 6. It could be concluded that stress conditions related to nutrients metabolically decreases the growth, redirecting the energy towards lipid accumulation. Quality and quantity of light influences the growth and kinetics of microalgae, thus understanding of the light driven metabolism is essential to assess the constraints associated with its reaction on carbon allocation mechanism [151]. The entire spectrum of light can be divided into specific bandwidth, each associated with a particular light-driven reaction depending on the phototrophic species [78]. **Baroukh *et al.*** [89] utilized DRUM to predict the metabolite accumulation in *T. lutea* under diurnal variation using metabolic representation of 4 microalgae (157 metabolites, 162 reactions). The model worked in conjugation with the experimental study predicting maximum growth and metabolite accumulation during day and consumption after sunset. Influence of photoperiod on *Chlamydomonas* sp. JSC4 was studied by **Kato *et al.*** [157] reporting higher lipid anabolism from CO_2 under light-dark intermittent conditions rather than continuous light cycles. Since, the influence of light on lipid yield is also dependent over the algal species, culture density and PBRs utilized, there is a need to redefine GSMMs accordingly to better assess its effect on intracellular metabolite levels.

4.4. *Molecular basis of the dynamic algal starch to lipid metabolic switch*

The metabolic pathways in microalgae for the production of energy rich molecules like biodiesel is based on lipid content, while the conversion of algal biomass and other cellular extracts under biorefinery concept into bioethanol, biogas and bio-hydrogen are mostly based on the biochemical content of starch/carbohydrates. Since, glyceraldehyde-3-phosphate (G3P) obtained via glycolysis acts as the common precursor, the metabolic production and conversion pathways of starch and lipids are correlated as shown in Fig. 1. Targeted production of the requisite bio-energetic molecule requires understanding of the culture conditions along with the intersecting and collateral biosynthesis pathways.

Similar to lipids, as summarized in the detailed review report by **Surendhiran and Sirajunnisa,** [158], the changes in algal growth conditions like the increase in pH, light intensity and temperature could accumulate more starch. Nutrient depletion is very often used for starch accumulation, however, the process is considered species-specific. For

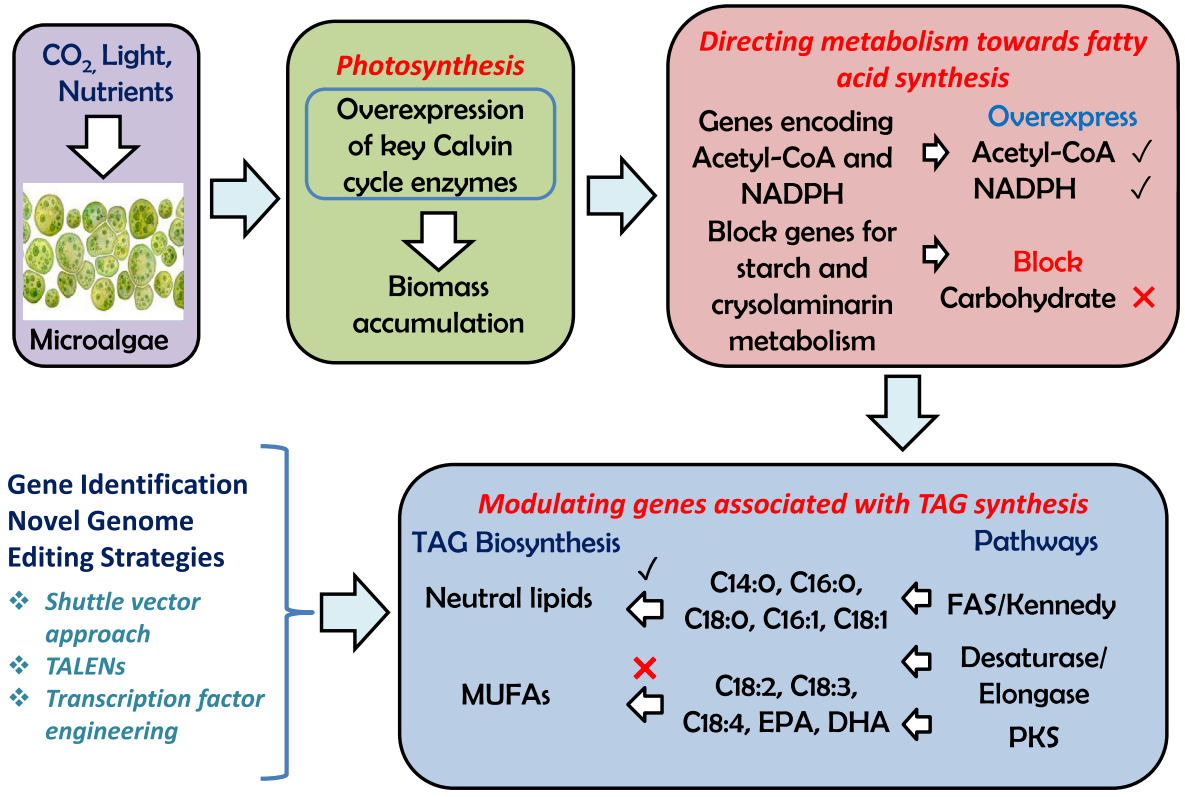

Fig. 3. Schematic representation of the approach and the techniques involved for metabolically engineering microalgae for biofuel. {Abbreviations: NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen; FAS: Fatty acid synthesis; PKS: Polyketide synthase; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid}.

instance, nitrate depletion resulted in enhanced starch accumulation in *S. obliquus* CNW_N [55] while under the same conditions TAGs content increased following a temporal increase in starch accumulation in *C. zofinginesis* [159]. **Pick and Avidan,** [160] demonstrated that starch accumulated during initial nutrient starvation in *D. tertiolecta* degraded over long term starvation period into lipids. Over years, omics tools co-combined with enzymatic and genetic validations revealed partial picture of pathways and the carbon allocation between them facilitating strain engineering. Supressing adenosine 5′-diphoshospho glucose (ADP) phosphorylase (AGPase), the key enzyme catalysing conversion of glucose-1-phosphate to ADP glucose, which served as the building block for starch have been reported to have directed carbon flux towards increased TAG accumulation in *S. obliquus* [104]. **Vonlanthen *et al.*** [161] provided contradictory results projecting that the starch deficient mutant of *C. sorokiniana* failed to accumulate significantly higher lipids, showing that the overall process is strain-specific and much more complicated regulatory network governs the partitioning of carbon to these metabolites. **Tan *et al.*** [162] showed that the accumulation of starch/TAGs is highly perplexing and is often correlated to the evolutionary aspects of utilizing different carbon sources. Photoheterotrophs like *C. vulgaris* and *C. reinhardtii* assimilate organic carbon sources that can be easily metabolized to acetyl CoA [fatty acid (FA) synthesis precursor] and ATP providing reducing power for energy intensive TAG synthesis, while certain obligate photoautotrophs accumulate starch for rapid and efficient energy storage and utilization. The switch from starch accumulation during initial nitrogen replete to TAGs under subsequent starvation can be ascribed to the fact that the former metabolite is energetically easier to produce on per carbon basis and can be easily metabolized to TAGs.

Recent studies have also unfolded the enzymes involved during the switch from one metabolite to another. **Chen *et al.*** [163] proposed that triose phosphate isomerase (TPI) catalysing reversible conversion of glyceraldehyde-3-phosphate (G3P) to dihydroxy acetone phosphate (DHAP), favours G3P to DHAP and thereby into fructose-1,6 bisphosphate (FBP), while low TPI activity supresses the conversion favouring increasing G3P, which then enhances glycolysis increasing production of Acetyl-CoA, accelerating fatty acid synthesis. Based on the assertive assumption that starch degradation favours TAGs accumulation, reverse transcriptase studies have proven that under nutrient starvation, the metabolic levels of α, β and iso-amylases significantly increases providing the desired carbon precursors for lipid synthesis [164]. **Ho *et al.*** [165] projected that increase in salinity enhanced lipid synthesis activating amylases and starch phosphorylases. Increasing phosphorus concentration during nitrogen starvation decreases AGPase levels switching the metabolism from starch production to lipid synthesis [166,167]. Several transcription regulators/factors have recently been identified to control the switch from one metabolite to another. **Bajhaiya *et al.*** [168] reported that the overexpression of phosphorus starvation response 1 (PSR 1) factor increased starch accumulation and reduced neutral lipid content in *C. reinhardtii*. Another transcription factor, DNA binding one finger 11 (Dof11) acting as the metabolic switch for starch to lipid conversion was identified by **Salas-Montantes *et al.*** [169], which under sulphur and nitrogen starvation facilitated lipid accumulation with reduced starch content. Thus, manipulating the expression of genes or TFs could be used to engineer microalgae to accumulate the desired metabolite to generate the targeted biofuel. Since, the review is limited to the lipid enhancement strategies for microalgae, a thorough discussion over the biofuel pathways concerning bioethanol/biobutanol and biohydrogen are far beyond the scope of the paper. However, the readers are directed to refer the recent comprehensive reviews by **Naghshbandi *et al.*** [23] and **Banerjee *et al.*** [25] discussing the genetic engineering strategies concerning the above-mentioned biofuel. Nevertheless, understanding the critical limiting steps in the metabolite synthesis and degradation pathways along with the genes involved can definitely be researched further for simultaneous exploration of multiple biofuels in a biorefinery.

5. Engineering operational strategies for improving lipid productivity

5.1. Mode of reactor operation

5.1.1. Fed-batch mode of operation

Fed-batch mode of operation provides an intermittent addition of required nutrients and enhances the growth rate and end-product formation. Fed-batch cultivation strategy under mixotrophic mode

Table 5
Lipid content of metabolically engineered algal strains through gene modulation approach.

Genes involved	Modulation strategy	Algae and growth conditions	Observed effects	References
Genes for malic enzyme (ME gene)	Overexpression of ME gene to convert malate into pyruvate and release NADPH that act as cofactor for lipid synthesis	*C. protothecoides* grown in SE medium with 54.5 g/L $C_2H_3NaO_2$, 651.6 mg/L $NaNO_3$; 40 μmol/m^2/s white light at 25 °*C*	Lipid content in metabolically engineered microalgae increased by 2.8 fold, with no effect on biomass content	[142]
GmDof4* (DNA binding with one finger) from *Glycine max	Upregulation of 22 genes related to lipid metabolism and ACCase activity	*C. ellipsoidea* grown in ENDO medium under mixotrophic condition (40 μmol/m^2/s at 25 °*C*)	Improved lipid accumulation from 46 to 52% in transgenic strains with no effect on biomass content	[143]
***omega-3 desaturase* (*ω-3 FAD*)**	Overexpression of *ω-3 FAD* under the control of endogenous promoter facilitates desaturation of C18:3	*C. vulgaris* UMT M1 maintained in N deficient BBM with continuous T5 fluorescent light at 23-25°*C*	16–17% increment in oil content under N deficit conditions compared to wild strain; with enhanced linoleic acid content	[144]
The rhythm of chloroplast 40 (*ROC40* gene)	Insertional gene disruption (Control of circadian rhythm & MYB related regulation of TAG accumulation under N depletion)	*C. reinhardtii* cultured in N free mineral basal medium with KNO_3 replaced by KCl, 80 μmol/m^2/s with 18h light regime; 24 °*C*	Wild strains under nitrogen depleted conditions showed 11% increased lipids compared to mutant	[145]
Genes for carbon fixation, chloroplastic glycolysis and membrane transporters, components of the pyruvate dehydrogenase complex (PDHC)	Upregulation of genes involved in carbon assimilation to channelize Carbon into fatty acid (FA) synthesis	*C. sorokiniana* LS-2 grown at 26 °*C* with 100 μmol/m^2/s for12 h	Redirected the carbon flux towards lipid synthesis; 2-fold increase in intracellular lipids with 10% CO_2 in spite of the downregulation of genes associated with FA synthesis	[146]
Diacylglycerol acyltransferase 2 (Neo*DGAT2*)	Overexpression of NeoDGAT2 Cassettes (Promote TAG conversion from DAGs and acyl-CoA)	*N. oleoabundans* in BBM without $NaNO_3$ at 30 °*C*, 55–60 μmol/m^2/s (constant illumination)	1.8–3.2-fold increase in TAGs, with 49% enhancement of C16:0 fatty acids under N starvation	[147]
Glycerol-3-phosphate acyltransferase (*GPAT*) from *L. incisa*	Heterologous overexpression of *LiGPAT* ORF (key role in storage lipid formation)	*C. reinhardtii* N depleted TAP medium with 12.5 μg/ml paromomycin	1.5-fold increase in TAG content without compromising growth with enhanced oleic acid content	[148]

Table 6
Description of metabolic models of algal strains with observations obtained through model linked experiments.

Organism/ID	Metabolic model/Analysis	Model characteristics	Inferences	References
N. gaditana B31 iRJ1321	GSMM with FBA and FVA	1321 genes, 1918 reactions, 1862 metabolites; 4 compartments; 21% gene coverage; 6311 total annotated genes	With 5% nitrate of that required for growth, carbon flux was directed towards lipogenesis resulting in 0.213 mmol/g/h, however the growth rate declined by 5%	[71]
N. salina CCMP 537 iRC1080	GSM with dFBA	934 genes, 2345 reactions, 1985 metabolites; 10 compartments; 70% gene coverage; 17519 putative genes; 7205 genes with no functional annotations	Under limited nitrate below 0.01 mmol/L, TAGs upto 50% of dry weight of algal cells could be accumulated	[153]
C. prototheocoides	Core model with ^{13}CMFA	24 reactions; 19 metabolites; no compartmentalization	Grown in chemically defined medium, under high C/N ratios 53.8% w/w lipids accumulated compared to 25.2% w/w lipids obtained with low C/N ratio	[154]
***Tetraselmis* sp.M8**	GSMM with FBA and FVA	2249 genes; 1725 reactions; 1862 metabolites; 4 compartments	During growth in f/2 medium deprived of nitrogen in first 24 h of cultivation, genes of FA synthesis pathways was downregulated by 3–113 folds but accumulated lipid content increased	[155]
C. reinhardtii iCre 1355	GSMM with FBA	1355 genes (1460 transcripts); 2394; reactions; 1133 metabolites	Max. 0.36 g TAG/g CO_2 and 1.36 g TAG/mol of photon was observed, growth rate completely ceased after 8 h; TAG levels increased after 4 min of nitrogen starvation	[87]
C. vulgaris	Core metabolic model with FBA	347 enzymatic reactions; 195 transport processes; 258 metabolites; 4 compartments	Limited nitrate (5 mg/L in BG11) redirected the carbon flux towards lipid synthesis resulting in 43.6% w/w lipids	[156]

(glucose) with exponential feeding supply of nitrate having specific growth rate of 0.35 h^{-1} for *N. oleoabundans* grown in BBM showed 1.7-fold higher lipid yield than at specific growth rate of 0.42 h^{-1} [170]. The study suggested that as the nitrate consumption and growth rate is reduced, carbon flux is being redirected towards accumulation of TAGs. A recent study by **Sun *et al.*** [171] reported that the fed-batch strategy under mixotrophic mode (1 g/L glucose) enhanced 41.5% lipid productivity with 1084.3 mg/L/d biomass productivity for *Chromochloris zofingiensis* grown in Kuhl medium without nitrogen at continuous light intensity of 300 μmol/m^2/s for 7 days. The study also highlighted increased ATP and NADPH in central carbon metabolism to redirect the carbon flux towards lipid synthesis with higher C16–C18 FA content. **Fu et al.** [172] studied the influence of fed batch supply of phosphorous divided into 2, 4 and 8 parts (with a total concentration of 48 mg/L) under heterotrophic cultivation (10 g/L glucose) in BG11 having 50 mg/L nitrogen at 25 °Cover *C. regularis* for 8 days. The strategy implicated more complete utilization of intracellular phosphorous, upregulation of lipid biosynthesis genes leading to 4–16% increase in lipid content along with enhancement in desirable FA content. Further research is necessary to optimize the time flow of nutrients to overcome the limitations associated with self-shading due to the high cell density and biomass productivity in fed-batch mode [173].

5.1.2. Continuous mode of reactor operation

The continuous mode of cultivation combined with the phototrophic and mixotrophic growth can dilute the culture with fresh medium at intermittent periods, thus avoiding light attenuation and turbidity issues associated with high-density algal cultures. Compared to continuous cultivation, batch nitrogen starvation has been reported to accumulate 2-fold higher TAGs in *Acutodesmus obliquus* [174]. However, in batch mode often TAG accumulation occurs at an expense of decreased rate of cell division compared to continuous mode where simultaneous cell growth and TAG accumulation can persist with minimal nutrient feed strategies. **Zhu *et al.*** [175] showed that minimal urea addition (18 mg/L/d) to BG11 medium without nitrate, resulted in 88% and 74% more neutral lipid accumulation with 1.03 g/L biomass yield after 16 days in *Chlorella* sp., compared to the regular BG11 and nitrogen starvation media respectively. **Kandilian *et al.*** [176] reported that continuous feed of 3.65 mM nitrate with a dilution rate of 0.011 h^{-1} in flat panel PBR with bolds basal medium (BBM), exposed to 250 μmol photons/m^2/s resulted in an areal lipid content of 2.62 g/m^2 for *P. kessleri*. Though these systems are regarded as one of the cheapest and easiest methods at industrial level, issues related to the maintenance for obtaining consistent biomass and product yield with lesser wash-outs and aseptic conditions hinders their use, requiring more research in relation to the feed flow optimization techniques.

5.1.3. Semi-continuous mode of reactor operation

The strategy of semi-continuous mode is regarded as one of the most effective ways for operating photobioreactors to avert light limitation problems during the late exponential phase and low cell density in the decline phase. A dramatic increase in lipid productivity can be achieved under semi-continuous mode with appropriate nutrient management. 3.64-fold increase in lipid productivity (115 mg/L/d) of *C. sorokiniana* during semi-continuous cultivation with nitrogen limitation (200 mg/L $NaNO_3$ in modified F–Si medium) and pH (7) control was achieved by **Han *et al.*** [177] compared to batch mode. **He *et al.*** [178] have reported higher lipid productivity of 5.15 versus 4 g/m^2/d and, 5.35 versus 3.00 g/m^2/d for *Chlorella* sp. L1 and *Monoraphidium dybowskii* respectively under semi-continuous mode cultured in groundwater from desertification area with 1.41 ppm nitrate than during batch mode. A recent study by **Kong *et al.*** [179] reported that the semi-continuous addition of ferric (Fe^{3+}) ions (35 μm) resulted in 31.7% lipid content and facilitated easier harvesting in *Scenedesmus* sp, with a biomass yield of 1.06 g/L. Since, iron constitutes an essential cofactor for enzymes associated with lipid synthesis pathways, increased TAGs with 80% C16–C18 FA was observed. Even though, the available literature markedly suggests the semi-continuous mode as an essential strategy to be adopted at a real time scale, still, further experiments are required to confirm its actual potential.

5.1.4. Two-stage strategy for reactor operation

One of the major hurdles in commercialization of PBRs for algal biofuels is the lack of an optimized strategy for achieving higher lipid productivity without compromising the growth rate. Several studies with the application of engineering perspective have suggested the use of a two-stage process involving exposure of cells to two different culture conditions for enhancing the biomass content in the first stage and lipid yield in the second stage. This strategy is an advancement to the existing monoculture systems, where cells are exposed to two different growth conditions as summarized in Table 7.

In most cases (as evident from the research listed in the table), the strategy involves accumulating biomass in nutrient sufficient stage, then subjecting the cells to nutrient stress to redirect the carbon flux/metabolic switch of microalgae to accumulate lipids. Subjecting the cells to hyperosmotic stress in the second stage as in the study by **Xia *et al.***

[182] is also considered an essential strategy, where cells not only accumulate higher amount of lipids but also more oleic acid desirable for fuel application. **Wang *et al.*** [183] proposed a novel two-stage fed--batch strategy where high biomass content of 81.4 g/L was obtained due to the presence of external carbon source in first phase, followed by higher lipid accumulation due to increased ROS levels owing to hyper-osmotic stress and N depletion. Considering the fact that the phyto-spectrum influences the photosynthetic efficiency and the carbon allocation to lipids, combining two different light wavelengths is also being used to enhance lipid productivity. **Ra *et al.*** [41] utilized red/blue wavelength to accumulate biomass due to increased photosynthetic efficiency, and exposed the cells to green LEDs in the second stage to create stress redirecting the carbon flux towards TAGs accumulation. Increasing the biomass content by phototrophic cultivation and switching the inherent metabolism towards heterotropic/mixotropic mode reducing the requirement of light as demonstrated by **Ge *et al.*** [181] to be successful in accumulating higher lipids. **Nagappan *et al.*** [184] and **Aziz *et al.*** [24] have summarized the two stage cultivation methods involving integration of different growth modes and stress factors synergistically to enhance algal lipid productivity. The effects of different operational parameters on biomass and lipid productivity as well as the essentiality of the two-stage approach have been illustrated in Fig. 4. Even though the two-stage process seems to be promising, the complexity of control to be provided for maintaining two different culture conditions adds up to the cost, making it problematic to operate at large scale. Also, there is a need to optimize the conditions for each phase to counteract the conflicting feature for achieving the desired lipid productivity without hampering biomass content. Insights into some of these control strategies can be obtained from the recent review by **Aziz *et al.*** [24]**.**

5.2. Hybrid reactors and process control

A promising alternative to alleviate unsatisfactory reactor performance involves the utilization of two/more configurations popularly termed as a hybrid reactor with an appropriate height to diameter ratio, high surface area to volume ratio with increased working efficiency, and better volumetric biomass and lipid productivity. **Martin *et al.*** [185] have recently reported a hybrid system composed of PBR in the first stage to increase the inoculum amount (0.64 g/L) and second stage of raceway ponds, leading to a total of 54.4% w/w lipids and TAG productivity of 1.2 mg/L/d after 19 days for *H. coffeaeformis*. A recent study by **Vu *et al.*** [186] projected 75% nutrient removal, >97% chemical oxygen demand removal from anaerobically digested effluent accumulating 700 mg/L biomass for *C. vulgaris* in a hybrid reactor system consisting of membrane PBRs. Though the available literature in relation to the use of hybrid reactors for lipids are limited, with most studies reporting higher biomass yield with efficient nutrient removal, these strategies can be integrated with the mode of reactor operation and nutrient stress in the second phase to coerce the cells for enhancing TAGs.

Apart from selecting an energy efficient reactor, different infrared and fluorescence based controllers are being employed for onsite monitoring to facilitate better control over the biomass and lipid productivity [187]. **Andrade *et al.*** [188] have developed a sliding module pH controller using total inorganic carbon as reference for evaluating the transient disturbances in pH. Fluorescence-based approach for online flow control was used to maintain a constant dilution rate in PBR, operated in continuous mode for culturing *Scenedesmus* AMDD to maximize productivity (0.11 g/L/d) resulting in 70% improvement in performance compared to turbidostat mode [189]. Nevertheless, application of linear and non-linear predictive models with feed-back and feed-forward controllers described by **McGinn *et al.*** [189]**; Ifrim *et al.*** [190] for regulating the dilution rate during continuous operation are essential for managing the hydraulic and solid retention time to optimize the algal productivity. These online monitoring systems can be utilized to appropriately control the nutrient regime and operational mode for increased efficiency of reactors concerned with high lipid productivity.

5.3. Simulation and modelling studies on algal behaviour

The advancement in the biochemical technologies triggering biomass and lipid yields and interdisciplinary integration with the mathematical sciences has led to the development of several kinetic and predictive mathematical models. These simulative models are based on either Monod's equation for predicting the growth rate of microalgae or Droop's equation to evaluate the algal growth based on the internal substrate consumption. Several models as summarized in the review by **Darvehei *et al.*** [191] considered mostly the growth limiting nutrients like nitrogen, phosphorus as well as the photosynthetic efficiency based on light intensity or temperature. Most of these studies are based on the steady state assumptions and are limited to single influential factor. However, the algal metabolism is complicated and is governed by the dynamic interaction between several influencing variables under different cultivation conditions. Based on the above-perspectives, recent models described by **Bekirogullari *et al.*** [192,193] have explored multi-parameter interactions with time-variations and could realistically predict the variation in algal growth, biomass and lipid productivity. Contrary to the segregated and structured models, which consider each algal cells as individual units and are mostly used for flux analysis studies for intracellular starch and lipid globules, unsegregated and unstructured kinetic models considered as simple and much less expensive have mostly been restricted to project lipid productivity in large-scale algal systems. Most of these models based on the central carbon pathways in microalgae assume that excess carbon is used for lipid synthesis, rather than other metabolites, and restricted for mostly autotrophic [194] or heterotrophic cultivation [195]. Recent study by **Figueroa-Torres *et al.*** [196] used nitrogen limitation and variation in organic and inorganic carbon substrate (mixotrophic) conditions proposing 261% and 66% increase in starch and lipid respectively due to acetate boost. This study is an advancement to dynamic mixotrophic kinetic model by **Adesanya *et al.*** [69] considering starch and lipid as single component. **Manhaeghe *et al.*** [197] have further used a respirometry-titrimetry technique to model and quantify the growth under different cultivation regimens proposing that microalgae prefers to grow phototrophically but shifts to heterotrophic condition (when grown mixotrophically) to avoid ill-impacts of stress due to photorespiration. This information could be taken as the baseline to further understand the carbon allocation under stress conditions to trigger lipid accumulation without decline in growth rate.

Since, large-scale algal cultivation specially in high rate algal ponds (HRAPs) utilizes wastewater as the nutrient source along with the symbiotic interaction between bacteria-microalgae, the river water quality model 1 [198], Sah model [199] and the recent BIO_ALGAE model [200] are mostly utilized for predicting algal growth. While RWQM1 model considered only nitrogen and phosphorus as the source of nutrients for algae-bacteria growth and the Sah model took into account the effect of multiple factors like temperature, solar radiation. Many of the above-mentioned models do not consider the interactive effects of nutrients especially the inorganic carbon, dissolved oxygen over the algal-bacterial growth. The new model BIO_ALGAE developed by **Solimeno *et al.*,** [200] is an improvement to the previous models and is one of the exhaustive and comprehensive model framed considering all nutrient based and operational parameters. The model simulated the dynamic population of bacteria and microalgae, projecting light intensity and inorganic carbon concentration as the most influencing factors for optimizing the design and cultivation in HRAPs.

Another, worth mentioning advancement is the utilization of these basic interactive models as the framework with dynamic spatiotemporal abiotic factor variation to forecast the biomass and lipid productivity in future. Site-specific models developed explicitly for PBRs *i.e.* open ponds

Table 7
Two stage cultivation strategies to improve algal lipid yield.

Microalgae	1st stage operational strategy	2nd stage operational strategy	Biomass/Lipid productivity (BP/LP)	Outcomes obtained at the end	References
S. obtusus	Photoautotrophic outdoor cultivation with 3.4 mM urea and 0.34 mM phosphorous for 12 days	20 g/L NaCl during second step (8 days)	BP: 212 mg/L/d LP: 60.7 mg/L/d	1.4 times higher lipids with 1.7 fold higher oleic acid content	[145]
C. protothecoides	Fed-batch strategy with nitrogen starvation (after 108 h of inoculation) and supplementation with 10–20 g/L glucose	Hyperosmotic stress (1300 mOsm/kg after 132 h of inoculation) in the second stage	BP: 452.22 mg/L/h LP: 177.3 mg/L/h	39.2% lipids after 180 h, which was almost twice more than one stage approach without stress	[146]
Chlorella sp.,	5 days of nitrogen replete BG11 with 180 μmol/m^2/s illumination and 1% (v/v) of CO_2 supplied at 25 °C	4 days of nitrogen starvation	BP: 591.1 mg/L/d LP: 216.9 mg/L/d	1.2-fold increase in lipid content (36.7% w/w) compared to single stage culture	[147]
C. pyrenoidosa	Nutrient replete cultivation for 9 days [23 °C; 8500 lx; 16:8 h photoperiod, aerated with 15% CO2 at 0.1 vvm]	Nitrogen starvation with addition of phosphorus (10–70 mg/L) in continuous mode over 12 days	BP: 261.90 mg/L/d LP: 191.32 mg/L/d	1.3 and 2.2 times higher biomass concentration and lipid productivity respectively	[180]
N. oceanica	Blue LED lights (465 nm); 100 μmol/m^2/s for 12 h in sterilized sea water with f/2 medium; 20 °C; 2 days	Green LED lights (520 nm)	Biomass content: 0.92 g/L Lipid content: 56% w/w	57% higher lipid accumulation compared to single phase exposure to green LEDs	[41]
C. vulgaris	Phototrophic condition; raw centrate water with 120 μmol/m^2/s continuous light	Fed batch with feeding of 2 g/L glycerol and 2 ml sterilized raw centrate water after 5 and 10 days (Mixotrophic)	Biomass content: 1.89 g/L LP: 24.7 mg/L/d	Higher lipid productivity with desirable FA content under autotrophic-mixotrophic mode	[181]

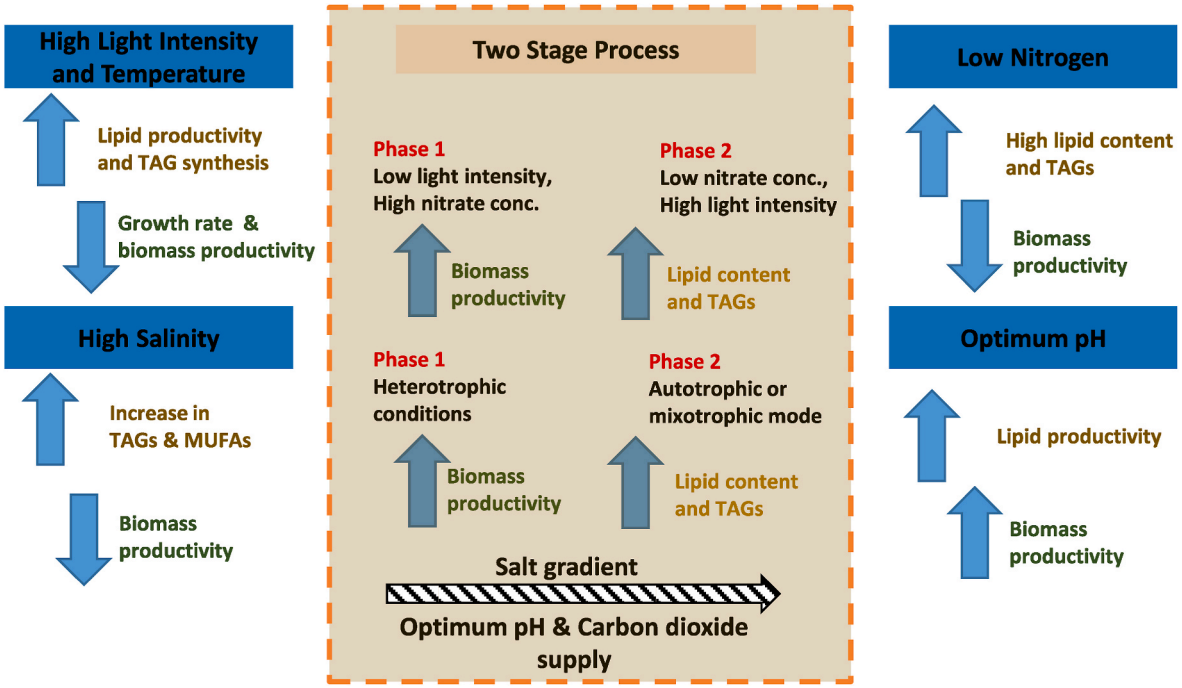

Fig. 4. Schematic representation of the advantage of two stage hybrid cultivation process over other growth conditions on algal biomass and lipid productivity [Upward and downward pointed arrow represents increase and decrease respectively].

[201], flat PBRs, tubular reactors [202,203] help to better decide the appropriate reactors to be implemented at a particular location. To the best of author's knowledge, no such studies has been done for implementing hybrid reactors or HRAPs considering the influence of other protozoans and zooplanktons. Also, very limited research has been done with respect to modelling the growth of genetically modified (GM) microalgae except the one by **Flynn *et al.*** [204] who reported that biofuel productivity could be enhanced 5-folds via GM algae incorporation with enhanced photosynthetic efficiency and limited nutrient quota. However, the authors through simulations reported an escape into environment with concentration much beyond a capacity to be controlled by zooplanktons causing eutrophication. Nevertheless, more such detailed studies including all the above-mentioned excluded factors with both wild and mutated strains would aid in better optimization and design of reactors for scale up of algal cultivation aiming for biofuel production.

5.4. Techno-economic and life-cycle analysis

Algal industry although regarded to have a broad impact over the global market due to rise in demands of alternative fuel and irresistible needs for mitigating climate change effects, often experiences unwanted competition due to high exit costs and commercialization related profitability struggles [102].

Techno-economic studies by **Xin *et al.*** [205] and **Hoffman *et al.*** [206] have been done to evaluate the algal biofuel production process mainly using phototrophic wild microalgal strains. The conclusive summary from most of these papers proposes to utilize technological innovation and management skills to reduce the production cost of algal biodiesel. Cost economics of algal biodiesel is often governed by several influencing factors like processes under considerations, technological improvements and merit scale. Results of the existing techno-economic analysis (TEA) often vary due to the different assumptions and unit production costs. A review of the algal biofuel based TEA done before 2013 by **Nagarajan *et al.*** [207] projected biodiesel costs of 0.42–0.97 $/L with an average biomass productivity of 30–60 g/m^2/d and a lipid content of 50%, while the TEA considering the process starting from cultivation to biodiesel production as summarized from the studies until the year 2015–16 by **Banu *et al.*** [208] showed a unit production cost ranging from 150 to 6000 $/tonne.

As per most of the techno-economics reports, the commercialization of algal technology is often hindered by the cultivation process *i.e.* the lack of strains with higher biomass and lipid productivity. **Yang *et al.*** [209] reported that the higher biomass and lipid productivity have a positive impact on biofuel cost price. Most of the biochemical lipid enhancement strategies implementing stress conditions with a decline in biomass content often lead to overall decline in lipid productivity, thus are expected to increase the overall process costs. Two phase cultivation strategy enhancing biomass content during the initial controlled/nutrient replete phase and lipid productivity under physiochemical stress/nutrient deprivation stage seems to be a promising strategy. Even-though TEA studies related to stress induced biphasic lipid enhancement are limited, undoubtedly utilizing the optimal combination of nutrients and physiochemical stress factors will appropriately trigger the lipid concentrates in microalgae, without inhibiting the biomass concentration, thereby reducing the operational costs**. Judd *et al.*** [210] suggested that integrating algal cultivation with waste resources as nutrient/substrates along with CO_2 from flue gas could bring the costs by 36–95%. Inclusion of an additional harvest step in two-stage stress induction strategy to re-suspend microalgae is also considered a costly step requiring high capital costs. Nevertheless, with high biomass content in first step and inclusion of low cost alkaline flocculation having efficiency similar to centrifugation can cut down the overall expenditure of the bioprocess.

Similarly, studies related to the use of engineered algal strains with enhanced metabolite accumulation in field scale production of industrial chemicals/biofuels are recently emerging. TEA analysis of bioenergy production scenario using a genetically engineered *Synechocystis* sp., PCC 6803 showed that even with increased production capabilities, only bioenergy production scenarios are not techno-economically feasible [211]. However, with biorefinery approach and better utilization of coproducts, the economic feasibility of the process improves with better returns on investments. The authors have also concluded that even if the algal strains are genetically modified to increase the biofuel yield, the overall economic feasibility of algal fuel only option would still not be feasible compared to the cultivation of wild algal strains for value added products. Expression of bacterial ethylene-forming enzyme (EFE) gene in cyanobacterium to produce ethylene and other renewable fuels through carbon fixation and techno-economic analysis of the process showed that the project costs are mostly determined through yield of the targeted product which in turn is dependant over the photosynthetic efficiency and carbon reallocation [212]. The choice of algae, the nature and extent of modification will definitely influence the requisite infrastructure and the yield of product will influence the revenues earned.

To the best of authors knowledge, until now none of the studies have evaluated the environmental impacts associated with the GM algal strains for biofuel or value added products. Nonetheless, lifecycle impact assessment studies for outdoor scale cultivation of microalgae for biofuels by **Dutta *et al.*** [213] have shown that most of the energy and environmental impacts are associated with the downstream process of harvesting and extraction and the cultivation stage. **Collet *et al.*** [214] and **Azari *et al.*** [215] reported that technological breakthroughs to increase biomass productivity with the use renewable energy mix during the upstream and downstream process can further reduce the climate change impacts. **Togarchetti *et al.*** [216] reported that increasing the biomass productivity by 2.5 fold could bring down the energy requirements by 58%. An important affirmation made by the study is that even-though the energy source is same, the geographical location specific energy demand also influences the life-cycle impacts. The use of algal strains capable of growing in waste resources or with limited nutrient can also reduce the GHG emissions. PBR design and configuration also influences the environmental impacts. **Azari *et al.*** [215] suggested that the carbon footprints of cultivating microalgae in heterotrophic mode in open raceway ponds for biofuel is 72% less than that of the competitive fossil fuel. The cultivation in raceway ponds is 4 times less capital intensive and have lower environmental footprints [24]. But, these systems might also increase the unintended spread of the engineered strains through natural agents thereby causing eutrophication and compete with native biodiversity. Thus, there is a need to explore other low cost reactor configurations like industrial scale glass house reactors or polythene tunnels to access the environmental and ecological risks involved during contained cultivation.

6. Challenges and prospects associated with lipid enhancement strategies

Strategies discussed in the previous sections, based on biochemical, engineering and molecular principles nonetheless seems to be promising in improving lipid accumulation in microalgae. However, these methods are dawned with several technical and ethical externalities.

6.1. Constraints and advances linked with biochemical and engineering strategies

The general process for lipid enhancement involves the use of locally acclimatized, fast growing strains accumulating lipids as the part of evolutionary survival mechanism. However, most of the algal strains with such capacity remains undiscovered due to the expensive and time-consuming screening process. Recently, **Huang *et al.*** [217] described a cheap methodology of growing microalgae in an enrichment medium (N depleted) followed by screening lipids qualitatively and quantitatively by BIODPY assay and Bligh and Dyer method along with subsequent isolation by micromanipulator. This strategy will certainly be applied to screen algal strain with high lipid content. Most of the stress inducer based biochemical strategies often cause an unwanted decline in biomass content while improving the lipid accumulation [7]. This is mainly attributed to the decreased photosynthetic metabolic efficiency due to the limitations associated with the light adsorption by photosynthetic apparatus and CO_2 metabolism [174]. Further, the reduced biomass content makes the process of harvesting tedious and decreases the bulk oil yield obtained after extraction. Thus, there is a need to explore alternate strategies to reduce the negative impacts of different abiotic stress conditions. One of the recently prophesized approach involves exogenous addition of phytohormones. Combination of indole-3-acetic acid (IAA), diethyl-aminoethyl hexaonate (DA-6) under minimal dose into *C. sorokiniana* culture with 12.5% N, increased the biomass and lipid productivity by 49% and 84% respectively [218]. **Yu *et al.*** [219] projected that supplementing the growth media with phytohormones can alleviate the oxidative damage due to nitrogen starvation, thus, maintaining the biomass concentration leading to 3-fold higher lipid productivity in *Chlorella* sp., and *Scenedesmus* sp. Phytohormones increase the photosynthetic RuBisCO activity leading to high CO_2 biofixation and growth, along with appropriate maintenance of redox potential, metabolite levels of antioxidants and antioxidases favouring TAG accumulation [22]. Other approaches like bicarbonate

addition to balance the carbon flux between the biomass and TAG accumulation as listed by **Ra *et al.*** [41] also seem promising. The study by **Sajjadi *et al.*** [8] have discussed the individual stress factors must be decisively combined under optimal operational conditions to achieve the maximal lipid productivity with favourable economics.

Engineering approaches utilizes the principle of coercing the cells to nutrient sufficient or deficit conditions in reactors under different cultivation regimes to obtain desirable yield. Most of these are hindered by economic limitations due to lack of proper optimization [187]. Use of wastewater though have been postulated to improve the process economics for algal cultivation, but the review by **Ferreira *et al.*** [220] projected that, in most cases, utilization of waste streams reduces lipid accumulation with no much effect over the FA composition, while supply of CO_2 can counteract the effect of decline in lipid content. **Ma *et al.*** [221] reported supplementing 10 g/L pre-treated glycerol from scum derived oil into synthetic wastewater, increased the nutrient removal efficiency, biomass productivity and lipid content of *Chlorella* sp., by almost 2, 5 and 4 folds respectively, compared to control. Glycerol therefore could act as a low cost waste resource for promoting TAGs accumulation with saturated FA desirable as biofuel. A new engineering approach involving the supplementation of the growth medium with optimal doses of iron/magnesium oxide [222] and silicon based [223] nanoparticles projected an increase in the lipid content by over 40% due to rapid substrate utilization under stress and improved acetyl CoA activity.

Also, the nutrient limitation technologies for algal growth though perform well under laboratory condition, but often fail when extrapolated to field scale [11]. There is a need to extend the modelling studies to identify the specific limiting pathways and operational conditions, optimize them accordingly in order to make the process feasible in terms of energy and economics. The implementation of the advanced bioprocess strategies with a more in-depth understanding of the optimization and dynamics will help in extrapolating the lab scale studies to commercial level. As highlighted by **Lammers *et al.*** [187], improvisation of engineering strategies must focus on environmental friendly and energy efficient reactor designs built on the preliminary analysis of computational flow dynamic studies, with online/inline monitoring sensors, provision for nutrient recycle along with optimal feed and stress management. Another, key advance in this regard as mentioned by **Lammers *et al.*** [187] and also researched by **Behera *et al.*** [201] is the utilization of biophysical predictive models formulated on site-specific climatological data and experimentally predetermined algae specific growth parameters to predict annual biomass and lipid productivity. Such predictive studies with TEA [3] can help identify potential strains and suitable locations for scale up.

6.2. Constraints and advances related to genetic engineering strategies

Random mutagenesis though postulated as an efficient method for strain selection and propagation is often time consuming and have provided moderate success [174]. The knowledge of underlying metabolic pathways with related transcripts and key regulatory genes, have revolutionized the genetic approaches for strain improvement. However, the lack of systematic approach, along with insufficient gene annotations have limited its successful applicability only in few strains as evident from previous sections. **Unkefer *et al.*** [224] reported that the biofuel based genetic engineering studies have been restricted to the key strains like *C. reinhardtii*, *P. tricornutum*, *B. brauni*., *Chlorella* sp., and *Nannochloropsis* sp. The chloroplast transformation process is only well established for *C. reinhardtii* along with few studies on *D. tertiolecta* and *P. tricornutum* [225]. Since, chloroplast and mitochondria are the key functional organelles in microalgae, there is a significant need to develop the transformation methods for more species [105]. The techniques of targeting single gene believed to be rate limiting, through suppression or overexpression have increased TAG levels as highlighted earlier, but the success is limited due to lack of insight into multiple interconnected carbon based metabolic pathways.

Pilot scale experiments on genetic engineering tools to improve microalgae are often limited by the lack of information and assessment tools to the researchers, industrialists, policy makers and regulators to evaluate the associated risks. Limited studies have been done on full scale cultivation of genetically modified microalgae except the one by **Hamilton *et al.*** [226], where transgenic *P. tricornutum* with enhanced eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was cultivated in 550 L closed photobioreactor and 1250 L raceway ponds. Maximum EPA of 12.8% was achieved in raceway ponds and 7.1% DHA was obtained in PBR under artificial illumination. A recent study by **Sarayloo *et al.*** [227] cultured mutagenic *C. vulgaris* with 35% and 67% higher biomass and lipid content than the wild strain in a 25 L bench-scale flat panel photobioreactor projecting 4-fold higher MUFAs. Apart from the improvements, the associated constraints of ecological spread linked with the large-scale cultivation of genetically modified microalgae was reported in the study by **Matsuwaki *et al.*** [228]. The authors during outdoor cultivation of genetically modified *Pseudochoricystis ellipsoidea* with enhanced lipid content under nitrogen stress in a 7.5 L raceway ponds over 35 days have shown the wind-borne spread of microalgae over 150 m in the surrounding areas influencing the growth of paddy. Similar to the above study, **Szyjka et al.** [229] carried out the first Environmental Protection Agency (EPA) approved outdoor field trial of GM algae *A. dimorphus* in outdoor raceway ponds for a period of 50 days. The GM microalgal strains with gene encoding for fatty acid accumulation and green fluorescent protein demonstrated its colonization in nearby trap ponds, which was proportional to the distance from the point of inoculation. The study also showed that the GM algae did not outcompete the native strains or had any adverse impacts over the local biodiversity. Taking the framework of such initial risk assessment protocols, there is a need for more such studies with appropriate government approval to evaluate the real time-risks and challenges associated with the potential utilization of such GM strains facilitating their responsible application by the industrialists and policy makers.

The new generation lipid improvement strategies often utilize the biochemical strategies in conjugation with the omics approach, to take the advantage of operational principles and advantages of both the approaches shown in Table 8. Nevertheless, these integrative methods have helped to better understand and predict the growth and oil yield under abiotic stress.

7. Practical implications and recommendations

Isolating native algal strains, enriching in low cost nutrient medium with application of nutrient or operational stress is the most common approach followed by researchers to trigger lipid accumulation. Manipulation of the cultivation regimen and scale up in HRAPs have provided the potential for successful commercialization. Environmental stress however, inhibits the cell growth, declines the algal biomass and lipid productivity, thereby increasing the economics related to bulk oil production. The use of two-stage cultivation strategies recently could suitably provide a solution to the anomalies associated with conventional techniques. There is a need to understand the critical points of shifts in biosynthesis pathways and underlying metabolism to obtain the targeted metabolites. Also, the feasibility of these hybrid systems must be evaluated in a comprehensive manner to provide the baseline information regarding the process economic, environmental and energy impacts at suitable geographical site. The economics and environmental impacts of the two-stage cultivation process can be improvised by exploring multiple bioenergy option or coproduct allocation. For, instance, the defatted microalgae can be subjected to fermentation to utilize the remaining starch for bioethanol/biohydrogen production or can be subjected to hydrothermal processing to obtain biocrude oil, biochar/hydrochar.

Approaches of gene editing can be successfully applied to improve the targeted traits of naturally occurring microalgae in terms of the

biomass and oil content, thus making the overall harvestability and extractability feasible. Undoubtedly, the vast availability of whole genome information of over 20 different microalgae integrated with technological development related to omics tools has facilitated the design of novel strains with requisite traits. Recently, designed mutant strains very often shown a trade-off between lipid accumulation and growth rate. Most of the transgenic studies on microalgae done so far utilize constitutive promotors, which expresses the gene of interest during algal metabolism. However, the extra-metabolic burden often impairs the overall genetic make-up, thereby toxically reducing their growth rate. To increase the efficacy of gene editing for achieving desired yields, it is essential to increase the utilization of codon optimization techniques along with tightly regulated promotors having a large range of control genes. There is a need to use biochemical and omics technology for bioprospecting locally acclimatized strains with ability to propagate in waste resources for industrial application. Although several projects are directed in the area of genomics, transcriptomics and metabolomics information with many industrially relevant algal strains, it is still vital to fully annotate the algal genes and decipher the metabolic pathways to further harness their potential for obtaining multiple products in a biorefinery. This approach would make the biofuel economics competitive with the petroleum market. In this regard, high throughput chemical genetic strategies can be redirected to perturb the biological pathways, in order to better understand the phenotypic changes during manipulation of the TAG biosynthesis. More insights into the use of small precursor compounds/phytohormones or even engineered nanoparticles postulated to block/activate the rate limiting reaction, increase cell permeability and regulate oxidative stress thereby increasing lipids without compromising growth are essential. Comprehensive screening of oleaginous strains has been achieved by integrating traditional technologies like U-mutagenesis, with novel approaches of microfluidic based high throughput screening studies and whole algal genome resequencing. Though this approach is promising, but creates mutations at several loci, making identification of responsible gene becomes difficult. Alternatively, random site directed insertional mutagenesis having a traceable sequence along with an antibiotic resistance gene combined with fluorescence activated cell sorting could help in detection of the requisite responsible target gene easier. Also, rather than targeting a single gene believed to be associated with FA synthesis, multiple genes and pathways could be altered to obtain desirable yields.

Since, each lipid enhancement strategies have their pros and cons; the authors propose to utilize the bioprocess and molecular engineering methods in a strategically planned manner, based on the note derived from the tasks outlined by the National Alliance for Advanced Biofuels and Bio-products (NAABB) committee. The use of a consolidated planned, step by step process (Fig. 5) with predictive modelling, strain improvement using molecular and synthetic biology approach and further, an appropriate mode of nutrition regime in energy efficient reactors with sensors and controllers can significantly enhance lipid productivity without compromising the growth rate. The information gained from each step could be used in a feed forward/backward loop to improvise the previous step. For example, the data obtained through lab-scale studies on potential algal strains linked to the growth rate, biochemical content could be coupled with stoichiometric kinetic models to direct more target specific genetic manipulation of the underlying algal metabolic pathways concerning higher TAGs accumulation. The modified strain along with the annotated information about genes and enzymes involved could be rationally utilized to simulate experiments to provide insights on the flux distribution under stress conditions. These strains can be further acclimatized in lab to obtain the specific growth rate which can be fed into the predictive biophysical and techno-economic models to access their feasibility at outdoor location. The process could then be scaled up to obtain bulk algal lipid yield. Thus, the advantage of one process could be utilized in well phased manner for obtaining better results. Nevertheless, more research into the

Table 8
Insights on TAG yield obtained from the combination of biochemical stress and omics approaches.

Microalgae	Growth conditions	Stress factors	Omics tools	Inferences	References
***C. reinhardtii* CC125**	MM medium without acetate, 100 μmol/m^2/s at 25 °C	Heat stress with cells subjected to 42 °C for 60 min	Lipidomics; Trancriptomics	Increase in TAG levels from 0.1 to 0.35 μg/million cells, with third fatty acid from phosphatidylethanolamine or a diacylglyceryl-O-4′-(N,N,N,-trimethyl)-homoserine betaine lipid species; increased gene expression of phospholipase A2 homolog and DAG acyltransferase DGTT1	[230]
***Scenedesmus* sp.**	Soil extract medium at 24 °C, with continuous illumination of 120 μmol/m^2/s	Addition of 2 mg/L $NaH_2PO_4.2H_2O$ every 2 days over a period of 9 days	Transcriptomics	Maximum lipid production of 350 mg/L and 41% lipid content; Upregulation of genes encoding for DGAT and pyruvate kinase, repression of carbohydrate metabolism to redirect the flux towards TAG synthesis	[231]
***D. tertiolecta* LB999**	f/2 medium without vitamin solution under high/low light intensity and nitrogen sufficient and deficient condition	Light intensity from 50 to 300 μmol/m^2/s with 0 mM and 2.68 mM nitrate under nitrogen sufficient and deficient cond respectively	Transcriptomics (Peptidome analysis)	Under high light intensity (300 μmol/m^2/s) and nitrogen deficit condition (0 mM) lipid content declined by 2.3% but carbohydrate accumulation increased by 28.3%; 33 transcripts expression was altered with growth conditions	[232]
***Tetraselmis* sp. KCTC12432BP**	Sterile three folded f/2-Si medium, with 55 W daylight fluorescent lights at 100 μmol/m^2/s illuminated continuously with 10–30 °C temperature, with 2% v/v CO_2 at a flow rate of 0.4 vvm	Temperature stress with culture exposed to 10, 20 and 30 °C variation in temperature	Transcriptomics; Genomics	Compared to mid temperature range, low and high temperatures decrease the FAME content by 2.9% and 4.1% respectively. 26,245 protein-coding transcripts of which 83.7% had putative functions, 681 genes were expressed under temperature variation influencing biochemical content	[233]
***N. oceanica* IMET1**	Modified f/2 medium; 50 μmol/m^2/s light intensity, continuous illumination at 25 °C, bubbled with 1.5% CO_2	Nitrogen starvation (f/2 medium without nitrogen source)	Transcriptomics; Lipidomics	Under N depletion TAG synthesis increased by almost 400-fold within 96 h; Upregulation of 7 DGAT genes and other Kennedy pathway genes, shunting of carbon from protein and carbohydrate metabolic pathways into glycerolipid synthesis	[234]

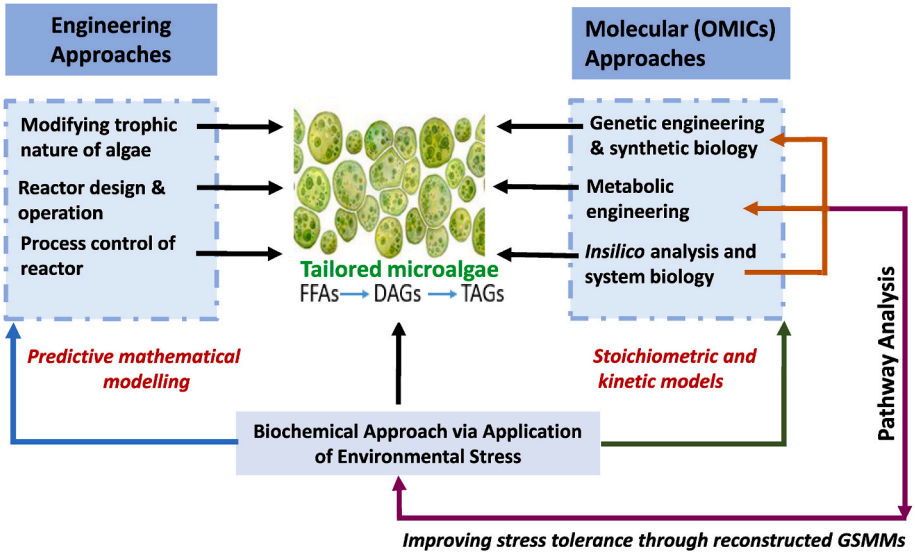

Fig. 5. Systematically planned utilization of bioprocess engineering and molecular approaches for tailoring microalgae with high TAGs {black arrows: direct application of each approach; starting from the biochemical approach (green arrow) the data obtained could be used to develop metabolic models (purple arrow) guiding molecular engineering of microalgae with desired TAG levels, the conclusive data from cellular pathways analysis (purple arrow) and reconstructed GSMMs could be used to acclimatize microalgae to stress under lab conditions and integrated with predictive model (dark blue arrow) and engineering strategies could be scaled up at desired location for bulk lipid yield**}.** (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hypothesized coupled methodology will make the real-time application of microalgae possible in future.

8. Conclusion and future directions

Catering the emerging energy demands of people requires a huge amount of biodiesel to substitute the currently used and declining fossil fuels reserves. Economic aspects related to implementation of algal biodiesel requires an increment in lipid content of microalgae. Microalgal biotechnology has improved over decades, thus the application of biochemical, engineering and molecular biology approaches for lipid enhancement achieved enhanced TAG accumulation with desired FA profile. These approaches though moderately successful are often linked with reduction in algal growth and biomass content, thus making them unsuitable for real time applications. Nevertheless, the ongoing research and recent advancements in engineering and molecular strategies have offered tremendous opportunities for tailoring microalgae with high TAG productivity, however, the lack of in-depth knowledge behind the principle involved often limits the targeted research towards commercialization. Thus, in an attempt to promote better forecasts on different bioprocess and molecular strategies to enhance algal lipids, the present study summarized and evaluated the available contemporary reports and findings, further pooled them together to analyse the associated challenges. As identified by comprehensive analysis, the following aspects have to be researched in future to improvise the currently available technologies for algal lipid enhancement:

1. Biochemical approach of coercing the microalgal cells to abiotic stress conditions guided with insights from rational metabolic flux analysis to identify the optimum condition of abiotic stress or nutrient limitation to be applied for accumulating lipids without sacrificing algal biomass content
2. Understanding the genes, their associated products at cellular levels and inter-conversion of metabolites to lipids along with intracellular carbon partition for TAG accumulation
3. Efficient gene candidate identification with desired traits mostly linked with the current technical and ethical constraints of GM microalgae
4. Development of sophisticated analytical tools to decipher the genomic datasets to reconstruct more comprehensive GSMMs for rational design and prediction of lipid yields under simulated growth conditions
5. Use of recyclable or multiple markers for stacking traits and multigene modulation to identify the interplay of various enzymes acting together in governing lipid yields must be researched
6. Synergistic combinations of strategies with predictive biophysical, stoichiometric and kinetic models followed by iterative rounds of experimentation at lab scale to achieve more productive results during scale up
7. Development of energy efficient and environmental friendly innovative culture test beds/PBRs to act as conduit for growing and studying the effect of GM microalgae
8. Collaborative efforts of biologists, biotechnologists and chemical engineers to accelerate the development of engineered strains for commercial application
9. Comprehensive techno-economics and lifecycle feasibility study with normalized assumptions exclusively for two-stage culture techniques, hybrid PBRs and GM algae
10. Exploring government policies and site-specific feasibility based on the energy source and demands attracting investors and venture capitalists before commercial scale-up

The coupling of biochemical, engineering and biotechnological perspectives can alleviate the associated risks making the commercialization of algal biofuels a reality in future. More laboratory and field-scale validation studies with economic feasibility analysis are required for assessing the pros and cons of these integrative methodologies. The systematic and collaborative utilization of lipid enhancement strategies are expected to revolutionize the future of sustainable algal biofuel industry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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