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Recent advances in biotechnology and bioengineering for efficient microalgal biofuel production

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ABSTRACT

Microalgal biofuels have emerged as a promising avenue for meeting the growing demands for clean and efficient energy. However, the integration of microalgae into the biofuel industry is still in the early stages, primarily due to low productivity and high production costs. To address these challenges, researchers are actively exploring innovative methods to enhance biomass, concurrently increasing lipid and carbohydrate content. This review paper discusses the unique attributes of microalgae that make them attractive candidates for biofuel production. Advancements in cultivation techniques, such as photobioreactor design, co-cultivation strategies (microalgae-microalgae, microalgae-bacteria, and microalgae-fungi), and the optimization of nutrient conditions (carbon, nitrogen, and phosphorus) as well as environmental factors (salinity, light, and temperature) were explored to enhance biomass and lipid productivity. Furthermore, genetic engineering tools (genetic elements, gene interference, genome editing, and genome reconstruction) and omics technologies (genomics, transcriptomics, and proteomics) were discussed to gain a deeper understanding of microalgal lipid synthesis metabolism. The application of these techniques in microalgae facilitates enhanced lipid productivity, improved stress tolerance, optimized carbon sequestration and utilization, and reduced harvesting and processing costs. The study also delves into the decision-making process related to software selection, with the overarching goal of improving performance, profitability, and sustainability while mitigating risks, operational costs, and environmental impacts. Additionally, this review highlights future perspectives on large-scale microalgal biofuel production and its industry.

1. Introduction

In recent years, microalgae have emerged as a promising bioenergy source due to their dual function in carbon sequestration and sustainable feedstock production [1,2]. Their remarkable CO_2 capture capacity, sequestering approximately 1.88 kg of CO_2 per kilogram of biomass, not only helps reduce greenhouse gas emissions but also supports global climate change mitigation efforts [3]. This is particularly crucial as modern society heavily relies on energy, and in recent decades, the surging energy demand has been accompanied by a concerning rise in

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CO_2 emissions, underscoring the urgent need for sustainable alternatives like microalgal biofuels. Over the past two decades, global primary energy consumption has witnessed a staggering 45 % increase, with projections pointing to a further 39 % rise in the next two decades. These figures underscore an unrelenting need for energy services, with anticipated annual growth rates of 1.7 % by 2030 and 1.3 % by 2040 [4,5]. These trends emphasize that efforts to enhance energy efficiency alone will not fully mitigate the ever-increasing demand for energy services. While these growth projections may seem tempered compared to the remarkable 2.3 % increase recorded in 2018, they continue to pose formidable challenges [5]. These challenges span a gamut, from the substantial upswing in energy-related emissions to the growing pressures on energy security, prompting a collective call to action.

In response to these complex energy challenges, renewable energy sources have gained significant traction, notably reflected in a concentrated effort to address the emission of greenhouse gases within the transportation sector through the biofuels production. Biofuels have emerged as a beacon of promise in the renewable energy landscape, primarily due to their remarkable propensity to be blended with gasoline at higher concentrations, up to 85 %, without necessitating engine modifications [6]. This unique feature has ignited a global scientific endeavor aimed at converting diverse raw materials into biofuels. Initially, first-generation biofuels obtained from edible plant biomass, including barley, beet, corn, potato, sugarcane, vegetable oils, and wheat were used as a replacement for fossil fuels and showed effectiveness in reducing CO_2 emissions in the atmosphere [7]. However, the use of these first-generation biofuel sources raised concerns about potential food shortages and conflicts between food and fuel production. To address this issue, second-generation biofuels were developed using non-edible feedstocks, such as agricultural waste, wood residuals, and bioenergy crops [8]. The carbon emissions associated with second-generation biofuels can be either neutral or negative. However, a drawback of these fuels is their dependence on the seasonal availability of raw materials. Microlgae have been categorized as third-generation biofuels, presenting a viable and advantageous alternative for biofuel production [9]. Microalgae have gained popularity over lignocellulosic biomass due to their rich biochemical composition, which includes lipids, proteins, carbohydrates, and other valuable compounds, all produced within an impressively short generation time [10,11]. Moreover, their cultivation can be seamlessly integrated into both indoor and outdoor settings, all without encroaching on valuable arable land. The adaptability of microalgae is remarkable, as they flourish across a spectrum of water sources, including waste, brackish, and saline waters [12]. Microalgae possess remarkable propensity to accumulate significant amount of neutral lipids, predominantly in the form of triacylglycerols (TAGs), making those prime candidates for the synthesis of biodiesel. In the presence of abiotic stresses, these microorganisms react by gathering excess lipids, providing a vital energy source essential for their survival and upkeep [11]. However, the commercial viability of third-generation feedstock remains limited due to challenges in cultivation, harvesting, and downstream processing. To address the limitations of previous biofuel generations, genetically engineered (GE) microalgae, referred to as fourth-generation biofuels, have been explored to enhance process efficiency and product yield [13]. Genetic engineering strategies targeting microalgal metabolism, physiology, stress response mechanisms, and carbon-energy balance, along with minimizing unnecessary ATP consumption, can significantly improve the production and yield of high-value metabolites [14]. Various microalgae species, including but not limited to *Chlamydomonas* sp., *Chlorella* sp., *Dunaliella* sp., *Monoraphidium* sp., and *Scenedesmus* sp., have demonstrated the capacity to produce excess lipids in response to a range of abiotic stressors [5,11]. These lipids are then converted into biodiesel through transesterification, a chemical process that replaces the glycerol in the lipid molecules with alcohol.

Microalgae, with their ability to convert sunlight into energy through photosynthesis, have garnered significant attention and considered prominent resource for biofuel production. However, to enhance the biomass yield or lipids, essential for extracting biofuels, innovative approaches such as microalgae co-cultivation and the imposition of nutrient and environmental stress have been emerged as key strategies. Microalgae co-cultivation involves cultivating different strains of microalgae together or with other group of microorganisms (bacteria and fungi), leveraging their complementary metabolic capabilities [15]. This synergistic approach enhances overall biomass productivity and lipid content. Furthermore, introducing nutrient and environmental stresses to microalgae cultures represents another effective strategy. Nutrient stress, achieved through the manipulation of nutrient availability like carbon, nitrogen, and phosphorus, prompts microalgae to accumulate lipids as an energy storage response [11]. Similarly, environmental stressors such as changes in light intensity, temperature, and salinity can stimulate lipid production as a survival mechanism [16]. These controlled stress conditions induce microalgae to prioritize lipid synthesis, making them a valuable resource for biofuel feedstock. Additionally, for the enhancement of biofuel production, a transition from traditional methods to a rational approach involving genetic and metabolic engineering of microalgae is crucial. Conventional techniques are slow and unpredictable, prompting the adoption of more targeted strategies. Genetic engineering modifies microalgal enzymes, transcription factors, fatty acid composition, and lipid secretion involved in lipid metabolism, enabling the display of specific traits [17,18]. This innovation facilitates processes like converting starch into energy producing molecules for biofuel production. Simultaneously, metabolic engineering optimizes productivity, expands carbon substrate utilization, and boosts stress tolerance in microalgae. Advancements in this field, aided by omics platforms and cutting-edge tools in algal genetic engineering (CRISPR/Cas9, ZFNs, RNAi, and TALENs) are revolutionizing biofuel production. Furthermore, in biorefinery operations, evaluating process equipment design involves complex analysis spanning quantitative, economic, and environmental dimensions. Conducting such assessments without specialized software is challenging due to intricate calculations and vast data. Dedicated software solutions are crucial, easing assessment burdens and enabling more accurate evaluations. In establishing biorefineries, strategic success hinges on adopting state-of-the-art software. The key lies in timely and methodical selection, ensuring a positive return on investment, risk mitigation, enhanced profitability, reduced operational costs, and steadfast environmental sustainability. Missteps in choosing simulation and modeling software can lead to long-term operational challenges. Addressing such issues can be formidable, emphasizing the importance of careful software selection.

Advancements in bioinformatics, gene targeting, genome reconstruction, genome sequencing, and omics technologies have collectively advanced the field significantly. These progressions play a vital role in expediting the effective metabolic engineering of various microalgal strains, allowing for the strategic engineering and alteration of metabolic pathways to enhance lipid production. Notably, metabolic engineering, particularly through gene editing, emerges as a highly efficient method for overcoming genetic limitations and boosting the production of target molecules. While the genetic toolkit for microalgae may be relatively constrained compared to other commercially important microorganisms, recent strides in synthetic biology and molecular toolkits have expanded the possibilities. Tools like Cre/loxP, CRISPR-Cas, RNAi, and modular cloning systems have significantly augmented the capabilities of bioengineering and cellular reprogramming in microalgae [19]. Despite existing limitations, the decreasing expense of genome sequencing coupled with advancements in computational techniques for analyzing extensive omics data hold the potential for a significant breakthrough in the field. Anticipated breakthroughs in genomic analysis and computational methodologies are poised to pave the way for the comprehensive programming of microalgae as cell factories. This forward-looking perspective has the potential to transform sustainable lipid production, making a valuable contribution to the rapidly growing

field of biofuel manufacturing.

Despite the promising potential of algae-based biofuels, commercialization faces significant challenges due to substantial financial investments required in both upstream and downstream microalgae production processes. To address these hurdles, a comprehensive strategy has been devised, involving the convergence of cutting-edge biotechnological and bioengineering innovations to overcome scalability, productivity, and cost-effectiveness obstacles in microalgal biofuel production. The urgency to explore sustainable alternatives to conventional fossil fuels underscores the significance of this review. By synthesizing recent advancements, the paper aims to provide a panoramic overview of the current state of microalgal biofuel production, emphasizing breakthroughs in genetic and metabolic engineering, cultivation techniques, and process optimization. The focus on sustainability aligns with global efforts to reduce environmental impact and transition towards cleaner energy sources, making this review instrumental for researchers, policymakers, and industry professionals seeking to propel biofuels into a more efficient and eco-friendly future.

2. Microalgae as green gold for renewable energy

Energy is a critical driver for economic development, societal progress, and the overall enhancement of human well-being. As global trends shaped by modernization and industrialization persist, the demand for energy continues to escalate on a worldwide scale. Projections indicate that global primary energy consumption is expected to reach 17,347 million tons of oil equivalents by 2050, with a projected plateau in the late 2040s [20]. A comprehensive report from the U.S. Energy Information Administration (EIA) reported significant growth in the global energy demand since 2012 to 2040 [21,22]. Global concerns have emerged regarding petroleum-based fuels due to the finite reserves of fossil fuels being at risk of depletion through overexploitation. Concurrently, current environmental conditions have become a pressing global issue, with the combustion of fossil fuels serving as a major contributor to the escalation of global CO_2 emissions. In response, global initiatives are underway to mitigate environmental degradation caused by the increasing levels of CO_2 emissions. On the basis of previous published report, significant enhancement in the CO_2 emissions have been reported from 32.2 to 35.6 billion metric tons in the years of 2012 to 2020 and this enhancement may reached to the level of 43.2 billion metric tons in 2040, which denotes 34 % enhancement over the period from 2012 to 2040 [23]. The challenges associated with an impending energy crisis, tied to the inexorable exhaustion of conventional fossil fuel reservoirs, coupled with the accrual of greenhouse gases in the atmosphere, have prompted a worldwide pursuit of innovative renewable energy sources.

Microalgae, an ancient group of photosynthetic microorganisms, have earned recognition for their remarkable traits, including swift expansion rates and their exclusively growing or surviving ability even in the adverse environmental conditions [24]. Their simplicity, often comprising single cells, has endowed them with the adaptability to thrive across diverse environmental contexts. The versatility in microalgae cultivation is noteworthy, as both freshwater and wastewater can serve as mediums for their growth [25]. In contrast, the pollutants found in wastewater stand as valuable nutrient sources, fostering the growth of microalgae. Notably, microalgae cultivation imposes minimal demands on arable land, avoiding competition with food production and freshwater resources [26]. In a world marked by a burgeoning population, the requisites for sustenance encompass both food and energy, underlining the urgency to seek alternative, sustainable energy sources. Confronted with the consequences of overexploitation and depletion of fossil fuels, there is a compelling drive to explore alternatives. Lignocellulose materials have been extensively scrutinized as potential biofuel feedstocks, yet the challenges posed by lignin recalcitrance and the generation of inhibitors are acknowledged as formidable barriers [27]. This recognition has sparked a fervent exploration of microalgae as an auspicious reservoir for the generation of a variety of biofuels.

A strain's ability to produce high lipid content, particularly under stress conditions, is essential for biodiesel production, while strains with high carbohydrate content are more suitable for bioethanol production, and those accumulating hydrogen can be harnessed for biohydrogen [28]. Identifying strains with the desired biochemical profile is therefore pivotal for economic feasibility, as it ensures compatibility with specific biofuel applications. To maximize productivity, these selected strains must also thrive under specific cultivation conditions, such as high salinity, temperature fluctuations, or nutrient limitations, which often mimic real-world operational environments [29]. Strains that demonstrate robust performance under such stress conditions not only enhance output but also reduce the need for stringent environmental controls, thereby lowering operational costs. Furthermore, scalability poses an additional challenge, as not all strains perform well in large-scale systems like open ponds or photobioreactors. Therefore, selecting strains with high resistance to contamination and stable productivity under non-sterile conditions is critical for ensuring consistent and reliable large-scale operations [30]. Additionally, strains capable of utilizing non-arable land, wastewater, or CO_2 from industrial emissions further enhance sustainability. This dual benefit of resource efficiency and environmental mitigation significantly reduces the overall ecological footprint of cultivation. By aligning biochemical potential, environmental adaptability, and scalability, the selection of appropriate microalgal strains becomes the cornerstone for advancing economically viable and sustainable biofuel production systems.

Traditional feedstocks like animal fats, jatropha oil, palm oil, soybean, and waste cooking oil have been conventionally used for biodiesel production [31]. Nevertheless, microalgae have emerged as a superior alternative due to their rapid growth and substantially higher lipid yield per hectare. One of the striking attributes of microalgae is their capacity to sequester CO_2, not solely from the environment but also from diverse wastewater outlets. For example, municipal wastewater from treatment plants serves as an exceptional nutrient source for cultivating microalgae, outperforming synthetic mediums for bioenergy generation. Microalgae efficiently utilize sunlight and environmental CO_2 to synthesize primary metabolites, including carbohydrates, lipids, and proteins (Table 1). They also produce a diverse array of commercially valuable products such as phycobilins, carotenoids, sterols, and vitamins [32]. Amidst this assortment of substances, neutral lipids and carbohydrates have surfaced as crucial biomolecules for biofuel synthesis. Microalgae sequester these lipids within distinct compartments, consolidating their potential as promising sources for third-generation biodiesel production [33]. Notably, microalgae cultivation does not encroach on food crop production, effectively addressing the ongoing debate of food versus fuel. Research has consistently demonstrated that microalgae boast an exceptional oil yield potential, estimated to be 25 times greater than that of conventional biodiesel oilseeds [34]. For instance, microalgae exhibiting elevated oil content, constituting 70 % of their biomass, and required only 0.1 m^2 of land per year to produce 1 kg of biodiesel [35]. Consequently, 1 ha of land cultivated with higher oil content microalgae can yield an impressive 121,104 kg of biodiesel annually [36]. Although the lipids retained by microalgae predominantly comprise of neutral fatty acids and characterized by relatively minimal degrees of saturation, which closely resembles with animal fats and vegetable oils. While lipid bodies are typically intracellular in microalgae, certain strains such as *Botryococcus braunii* secrete oils into their extracellular matrix [37,38]. These characteristics showed the uniquenss of microalgae for the selection as a resource for efficient biodiesel production.

Conversely, the extensive research endeavors into algal biorefineries have been met with formidable challenges, which have, to a great extent, obstructed their journey towards commercial-scale production and widespread application. These impediments span a wide range of issues, encompassing the composition of microalgae, the efficiency of photosynthesis, and the substantial energy consumption involved.

Table 1
The varied composition of primary metabolites in microalgal species serves as the blueprint for biofuel production.

Microalgal species	Factors affecting productivity	Proteins (%)	Carbohydrates (%)	Lipids (%)	Biomass content (g/l/d)	References
Acutodesmus dimorphus	Salinity (NaCl) 200 mM	13.30	53.30	33.40	0.023	[39]
Botryococcus braunii	BG-11 medium, CHU medium	41.7	20	30.2	0.2	[40,41]
Chlorella ellipsoidea	ultrasonic waves at 50 Hz	5	16	84	0.79	[42,43]
Chlorella emersonii	BG11 culture medium	9.03	37.9	29.3	0.3–0.72	[44,45]
Chlorella FC2 IITG	Carbon sources (glucose (21 %–87 %), galactose (1 %–20 %), and mannose (2 %–46 %)	10.4	24.5	37.3	3.69	[44]
Chlorella pyrenoidosa	Nutrient-rich wastewater	57	26	2	0.25	[45,46]
Chlorella sp.	BG-11 medium	19.3	47.4	21.8	6.08	[40]
Chlorella sp. MF.1	BG-11 medium, photoperiods (8:16, 12:12, 18:6, and 24:0 h light: dark regimes)	39.8	23.7	21.1	0.815	[47,48]
Chlorella sorokiniana	Wastewater source, dairy wastewater	28.8	35.4	31.8	0.068	[49,50]
Chlorella vulgaris	BG11 culture medium	18.3	50	21.9	1.37	[45]
C. vulgaris	BBM culture medium	56.1	14	12.5	3.86	[51]
C. vulgaris	Wastewater from the oil industry	58	17	22	0.03	[52]
C. vulgaris	BG-11 medium supplemented with glucose	35.1	16.8	9.81	1.00	[53]
Chlorella zofingiensis	Flat plate photobioreactors	11.2	11.5	56.7	0.058	[54]
Chlorogloeopsis fritschii	Carbon sources (glucose (21 %–87 %), galactose (1 %–20 %), and mannose (2 %–46 %)	41.8	37.8	8.2	0.72	[44]
Dunaliella bioculata	Carbon sources (glucose (21 %–87 %), galactose (1 %–20 %), and mannose (2 %–46 %)	49	4	8	0.71	[44]
Desmodesmus brasiliensis	BG-11 medium	42.2	32.7	14.5	0.042	[40]
Dunaliella parva	Response surface methodology	6.46	6.31	29.33	0.95	[55]
Dunaliella salina	Closed tubular photobioreactor	57	32	6	0.080	[56]
Desmodesmus sp.	Temperature, light and photoperiod	38.4	36.7	19.4	0.04	[57]
Dunaliella tertiolecta	Glycerol and Triethylamine	29	14	11	0.07	[58]
Parachlorella Kesslari	Microalgae–bacteria co-culture	15	25	30	2.0	[59,60]
Scenedesmus obliquus	Wastewater source	19.5	35	30.8	0.080	[49]
Scenedesmus platensis	Tubular photobioreactor system at 12 % CO_2 concentration	53.7	9	8.1	1.03	[61]
Scenedesmus quadricauda	Different LED wavelengths and photoperiods	56	25	13	2.369	[62]
Scenedesmus sp.	Wastewater of the Dairy Industry	49	23.3	12.1	1.84	[63]
Scenedesmus sp. BHU1	Sodium bicarbonate ($NaHCO_3$) 12 mM	8	56	34	0.059	[64]
Scenedesmus sp. BHU1	Salinity (NaCl) 0.4 M	12	42.16	38.10	1.3	[11]
Scenedesmus sp. CCNM 1077	Salinity (NaCl) 400 mM	24.53	35.91	33.13	1.2	[65]
Synechococcus sp.	Light intensity and Na_2CO_3 concentration	63	15	11	0.012	[66]
Tetraselmis maculata	Harvesting methods	52	15	3	3.2	[67]

Furthermore, the absence of large-scale commercial facilities, coupled with various technological bottlenecks, including species cultivation, efforts to reduce harvesting cost, and strategies to minimize CO_2 diffusion losses, adds to the complexity of the task [68,69]. The amalgamation of these predominantly technological challenges has led to a surge in production costs, the establishment of unfavorable energy and CO_2 balances, and a decrease in the efficiency of downstream processes in microalgae biorefineries and biofuels production. While substantial research has been devoted to microalgae for energy applications, the high costs associated with constructing and operating microalgae cultivation systems, coupled with the ongoing lack of consensus regarding their environmental impact, continue to impede the market introduction of microalgae-based biofuels. However, recent years have witnessed a growing prominence of microalgae biorefineries as a compelling alternative of traditional petroleum refineries. The fundamental concept of a microalgae biorefinery aligns closely with the operations of petroleum refineries, involving the transformation of biomass into commercially viable compounds including oil. Although between the biorefinary and petroleum refinery significant differnces have been reported in terms of feedstock and the machinery utilized. This paradigm shift towards microalgae biorefineries underscores the escalating demand for sustainable products and the mounting concerns over environmental pollution, which has repositioned biorefineries as a pivotal solution. In response to these dynamics, a multitude of methodologies have been proposed to harness the vast potential of microalgae biomass. These methodologies encompass liquefaction and the extraction of valuable compounds, thereby opening doors to an expanded market appeal for microalgae products. Crucially, these innovative methods unveil the potential for the concurrent production of biofuels, pharmaceuticals, and other coveted chemicals [70,71]. However, this journey of microalgae from challenge to innovation holds the promise of a sustainable and diversified bio-based future.

3. Genetic engineering progress to boost microalgae lipid production

In the dynamic field of microalgal biotechnology, there exists a traditional approach for enhancing microalgae strains that relies on evolutionary engineering techniques. These techniques encompass methods such as random mutagenesis, achieved through exposure to agents like UV radiation, chemical mutagens, charged particles, neutral reactive species, or free radicals. Additionally, genome shuffling, involving processes like sexual recombination or protoplast fusion, is employed to promote genetic diversification. These strategies are often favored for their relative simplicity compared to genetic engineering, as they require only limited knowledge of the species genetics and life cycle. While these methods have demonstrated success in improving microalgal strains, it is essential to recognize their inherent challenges. They are notably time-consuming, labor-intensive, and entail numerous iterations. Extensive screening under specific conditions is necessitated because the desired mutations tend to be rare, and any enhancements in phenotypes are typically incremental.

Over the years, the realms of genetic and metabolic engineering have played a pivotal role in enhancing lipid productivity in a diverse range of organisms, including bacteria, yeasts, fungi, and plants. Recent advances in genetic tools and the increased availability of omics data for numerous microalgal species have ushered in a new era in microalgal biotechnology [72]. These advancements have been further catalyzed by

the development of robust genetic transformation techniques for over 50 microalgal species. These techniques empower researchers to efficiently express foreign genes and manipulate crucial metabolic pathways, revealing intricate relationships between genes, proteins, and metabolites (Fig. 1). Contemporary genetic engineering endeavors aimed at enhancing microalgae can be broadly categorized into five key areas. Firstly, there's the drive to augment lipid yield by modifying lipid biosynthesis pathways. Secondly, researchers aim to divert the metabolic flow towards lipid biosynthesis by obstructing competing pathways. Thirdly, there's a focus on amplifying biomass production by enhancing the efficiency of photosynthesis and carbon fixation. Additionally, genetic engineers are delving into the engineering of transcription factors as a means of control. Lastly, the manipulation of transporters and lipid secretion is gaining prominence.

3.1. Genetic elements

Constructing expression cassettes is a critical stage in genetic and metabolic engineering, especially when managing multiplex cassettes with multiple target genes. The increasing global interest in molecular design engineering, coupled with extensive exploration of microalgal genomes, has yielded a rich array of genetic elements for designing synthetic gene cassettes, allowing precise protein targeting to various cellular locations. In this context, promoters, integral components of expression vectors, play a crucial role by interacting with gene regulatory factors, thereby governing genetic expression. Constitutive promoters play a vital role in maintaining microalgae transformants at a basal level, while inducible promoters offer the ability to regulate conditional transgene expression, preventing metabolic burdens. However, multicopy transgene integration can pose challenges, leading to position effects and transgene silencing. To address this, a proposed solution involves the use of a hybrid *HSP70A-RbcS2* promoter [73]. Numerous thoroughly studied constitutive promoters specific to microalgae, including *Actin*, *CvpsaD*, *EPPSII*, *HSP90*, *Lhcf*, *PsaD*, and *TUB*, contribute to the diversity of regulatory elements. Nitrogen-responsive promoters, such as *NR* is identified in *Chlorella* and *Nannochloropsis*, offer adaptability to varying environmental conditions [74,75]. Additionally, salt-responsive and green-light-inducible promoters (*CrGPDH3* and *cpcG2*) in *C. reinhardtii* offer cost-effective regulatory options [76,77]. Further demonstrating adaptability, other inducible promoters such as Ammonium-induced (*NIT1*), CO_2-induced (*CA1*), and iron-deficiency-inducible (*ATX1*) play diverse regulatory roles in microalgae [78–80]. This diverse array of promoters is essential for designing multi-gene constructs, preventing potential genetic rearrangements that could

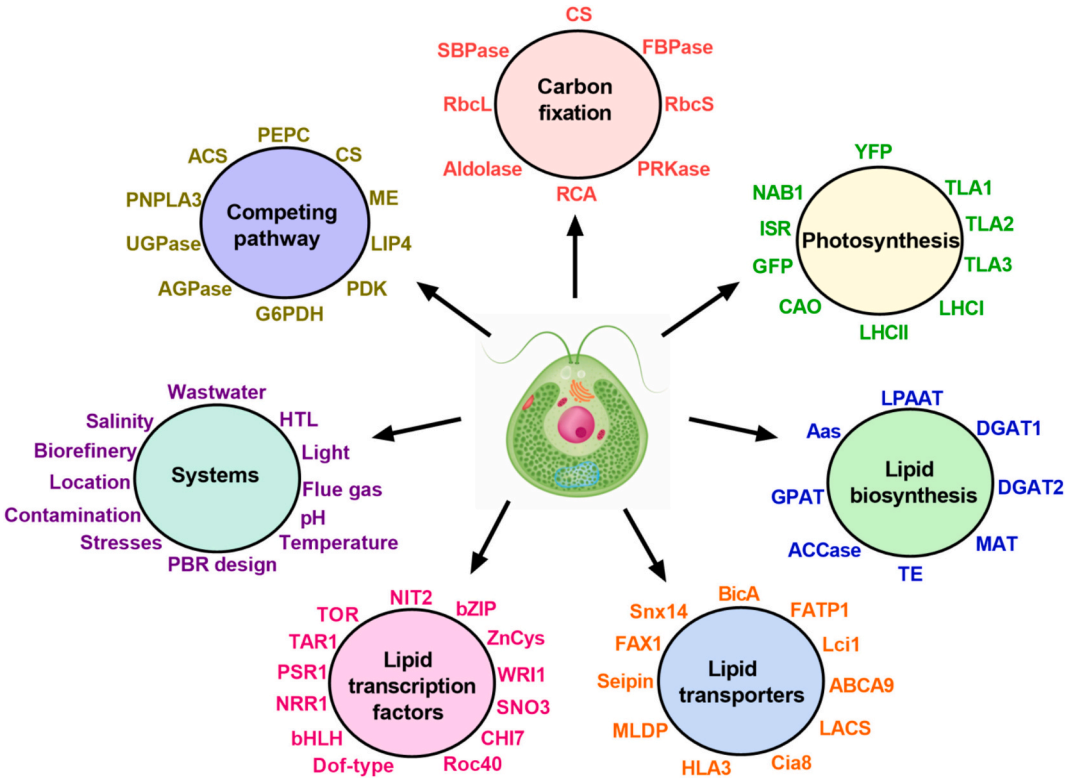

Fig. 1. Exploration of genetic engineering strategies aimed at enhancing lipid and biomass production in microalgae by optimizing carbon fixation, photosynthesis, lipid biosynthesis, lipid transport mechanisms, transcriptional regulation, and balancing competing metabolic pathways. Different abbreviations represents: CS (citrate synthase), FBPase (fructose-1,6-bisphosphatase), RbcS (ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit), PRKase (phosphoribulokinase), RCA (Rubisco activase), RbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), SBPase (sedoheptulose-bisphosphatase), TLA (truncated light-harvesting antenna), LHC (light harvesting complex), CAO (chlorophyll *a* oxygenase), GFP (green fluorescent proteins), YFP (yellow fluorescent protein), ISR (intracellular spectral recompositioning), NAB1 (nuclear acid binding protein1), LPAAT (lysophosphatidic acid acyltransferase), DGAT (diacylglycerol acyltransferase), MAT (malonyl-CoA ACP transacylase), TE (acyl-ACP thioesterase), ACCase (acetyl-CoA carboxylase), GPAT (glycerol phosphate acyltransferase), Aas (acyl-ACP synthetases), BicA (bicarbonate A), FATP1 (fatty acid transporter protein 1), Lci1 (low-carbon-induced protein 1), ABCA9 (ATP-binding cassette sub-family A member 9), LACS (long-chain acyl-CoA synthetase), Cia8 (carbon inorganic accumulation protein 8), HLA3 (high light-activated 3), MLDP (major lipid droplet protein), FAX1 (fatty acid exporter 1), Snx14 (sorting nexin 14), NIT2 (nitrogen response transcription 2), bZIP (basic leucine zipper), ZnCys (zinc cysteine), WRI1 (WRINKLED1), SNO3 (S-nitrosylation 3), Roc40 (rhythm of chloroplast 40), bHLH (basic helix-loop-helix), NRR1 (nitrogen response regulator 1), PSR1 (phosphorus stress response 1), TAR1 (triacylglycerol accumulation regulator 1), TOR (target of rapamycin), HTL (hydrothermal liquefication), PBR (photobioreactor), PEPC (phosphoenolpyruvate), ME (malic enzyme), LIP4 (lipase 4), PDK (pyruvate carboxylase kinase), G6PDH (glucose-6-phosphate dehydrogenase), AGPase (ADP-glucose pyrophosphorylase), UGPase (UDP-glucose pyrophosphorylase), PNPLA3 (patatin-like phospholipase domain-containing protein 3), and ACS (acetyl-CoA synthetase). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

arise from repeated use of the same promoter. The exclusivity of identified endogenous cis elements for chlorophytes in commercially important microalgae, such as *Chlamydomonas*, *Dunaliella*, and *Nannochloropsis*, underscores their practical significance.

Additionally, the golden gate modular cloning (MoClo) technique has simplified the design and assembly of cloning vectors for various industrially significant microorganisms. Customized for *C. reinhardtii*, the MoClo toolkit consists of 119 publicly available genetic components. These include promoters, terminators, UTRs, tags, antibiotic resistance genes, reporters, and introns, strategically arranged for optimal modularity [81]. This toolkit is positioned to streamline the formulating codon-optimized gene expression cassettes, aiming for increased gene expression in *Chlamydomonas* by leveraging standardized synthetic biology tools [82]. Similarly, a gene stacking toolkit with a large capacity has been developed specifically for *Nannochloropsis*, enhancing the predictability and efficiency of gateway-compatible assembly for various configurations of multigene expression vectors in this microalga [83]. Moreover, computational approaches are now employed to enhance the efficacy of codon-optimized gene delivery systems. It is noteworthy that freely available software for optimizing sequences in the nucleus and chloroplast is accessible for *C. reinhardtii*. This exclusivity necessitates tailored genetic construct approaches for these microalgae in industrial applications, emphasizing the importance of their unique genetic makeup in optimizing processes for biofuel production and other industrial uses.

3.2. Gene interference

Gene-interfering tools, such as RNA interference (RNAi), act by suppressing gene transcription without altering the gene nucleotide arrangement. RNAi utilizes miRNA or siRNA, forming base pairs with the transcribed RNA of the target gene, and activating the RNA-induced silencing complex (RISC), involving the enzymes Dicer and the protein Argonaute within the host cell. This activation induces RNA control through either cleaving or inhibiting the target mRNA translation. In the realm of microalgae, RNAi has been applied as a functional genomics by reverse genetics, targeting mRNA molecules to examine genes and their roles in various microalga strains. Beyond transcriptional repression and mRNA degradation, RNAi in microalgae has been observed to induce additional changes. For example, studies on *P. tricornutum* revealed that the introduction of RNAi led to the repression of translation [84]. In *C. reinhardtii*, RNAi was deliberately activated by lowering ammonium levels, leading to the expression of inverted repeats under the control of the *NIT1* promoter [85]. Gene silencing via RNA interference has identified and characterized several genes in microalgae, and its application in investigating the impact of nitrogen limitation on lipid biosynthesis has been reported. In *P. tricornutum*, silencing the gene that encodes nitrate reductase through RNAi resulted in increased lipid content [86]. Despite the effectiveness of RNAi in gene suppression and modifying gene functions, it has disadvantages, including the potential for inducing off-target effects, suppressing desired traits, influencing the RNAi construct, and partial suppression of transformants and target genes. Because of limitations such as poor effectiveness and non-specific targeting in RNAi for microalgae, the innovative CRISPRi approach has emerged as a fully controllable alternative technique. Additionally, an innovative RNA-dependent system has been established to supervise gene expression in microalgae, employing riboswitches composed of a cis-repressed RNA (crRNA) and a trans-activating RNA (tracRNA). These elements work together to control the translation process of the target mRNA by managing the small ribosomal subunit's access through interactions between RNA molecules.

3.3. Genome editing

In classical genetic engineering, the typical method involves introducing the target gene along with the necessary regulatory elements and markers, integrated into the plasmid vector backbone. However, the challenges associated with the non-integrated transgene expression, such as chronological fluctuation and the potential for unfavorable mutagenesis, have driven the development of novel techniques for the efficient gene integration into the host genome. This has led to the adoption of targeted nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like endonucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems, marking a revolutionary shift in genome editing (Fig. 2). These enzymes may be precisely designed to pinpoint specific DNA sequences, providing a powerful tool for creating knockout, deletion or insertion, mutants.

In the pursuit of advanced genome editing methods, scientists have led innovation in configurable sequence-specific nucleases (SSNs), with ZFNs emerging as notable entities. Originating from the *Fok*I endonuclease in *Flavobacterium okeanokoites*, ZFNs are protein-based enzymes featuring N-terminal DNA-binding motif and a non-specific C-terminal DNA-binding motif. This endonuclease forms the core of ZFNs, which are designed by integrating zinc finger proteins with the C-terminal of *Fok*I. These resulting nucleases can Identify and attach to the 3-bp site on the target sequence. Upon the dimerization of the two FokI domains, DNA cleavage occurs, inducing the formation of double-strand breaks (DSBs). Subsequently, the DNA repair mechanism is triggered, mending the breaks through non-homologous end joining or homologous recombination. The adaptability of ZFNs is evident in their application for site-specific genome modification across more than 11 organisms, spanning mammals, cell culture lines, fishes, insects, and plants. For instance, in a specific application, ZFNs were inserted into a mutated *Chlamydomonas* cell, where the insertion of the *cop3* gene into an aphVIII marker had made the marker nonfunctional. The insertion of ZFNs led to the cleavage of the gene, effectively reestablishing the function of aphVIII [87]. However, the effective utilization of ZFNs necessitates the development of arrays with high affinity and precision for each target sequence, demanding individual programming for each target. Additionally, the functionality of ZFNs hinges on the dimerization of the *Fok*I domains. Drawbacks of ZFNs encompass the costly construction process, the potential for off-target effects, and the intricacy of the designing process.

CRISPR is a precise and cost-effective genetic engineering tool that allows scientists to modify specific genes in microalgae, enhancing their growth, stress tolerance, and lipid accumulation. This is particularly important because one of the biggest challenges in microalgal biofuel production is the relatively low natural lipid content of many strains. By leveraging CRISPR, researchers can directly target and modify genes involved in lipid biosynthesis, enabling the development of strains with higher biofuel-compatible lipid yields [88]. Moreover, these genetic modifications not only enhance lipid production but also improve overall microalgal growth rates and resilience to harsh environmental conditions. As a result, CRISPR-based advancements contribute to reducing production costs and increasing scalability, making microalgal biofuels a more viable and sustainable energy source.

TALEN is used to introduce highly specific genetic modifications with minimal unintended effects, making it a reliable tool for engineering desirable traits in microalgae. By precisely targeting genes involved in lipid biosynthesis, TALEN enables the development of strains that produce higher amounts of biofuel-compatible lipids [88]. Furthermore, this targeted approach can enhance microalgae's resilience to environmental stresses, leading to improved biomass yield and overall production efficiency.

TALENs consist of three domains: DNA binding, transcriptional activation and nuclear localization signal (NLS) and are constructed by combining a DNA binding domain with a *Fok*I cleavage domain, similar to ZFNs. While both provide sequence specificity, TALENs are considered simpler than ZFNs since each domain identifies a single nucleotide rather than DNA triplets. This complexity makes the construction of TALENs challenging but less difficult and time-consuming compared to

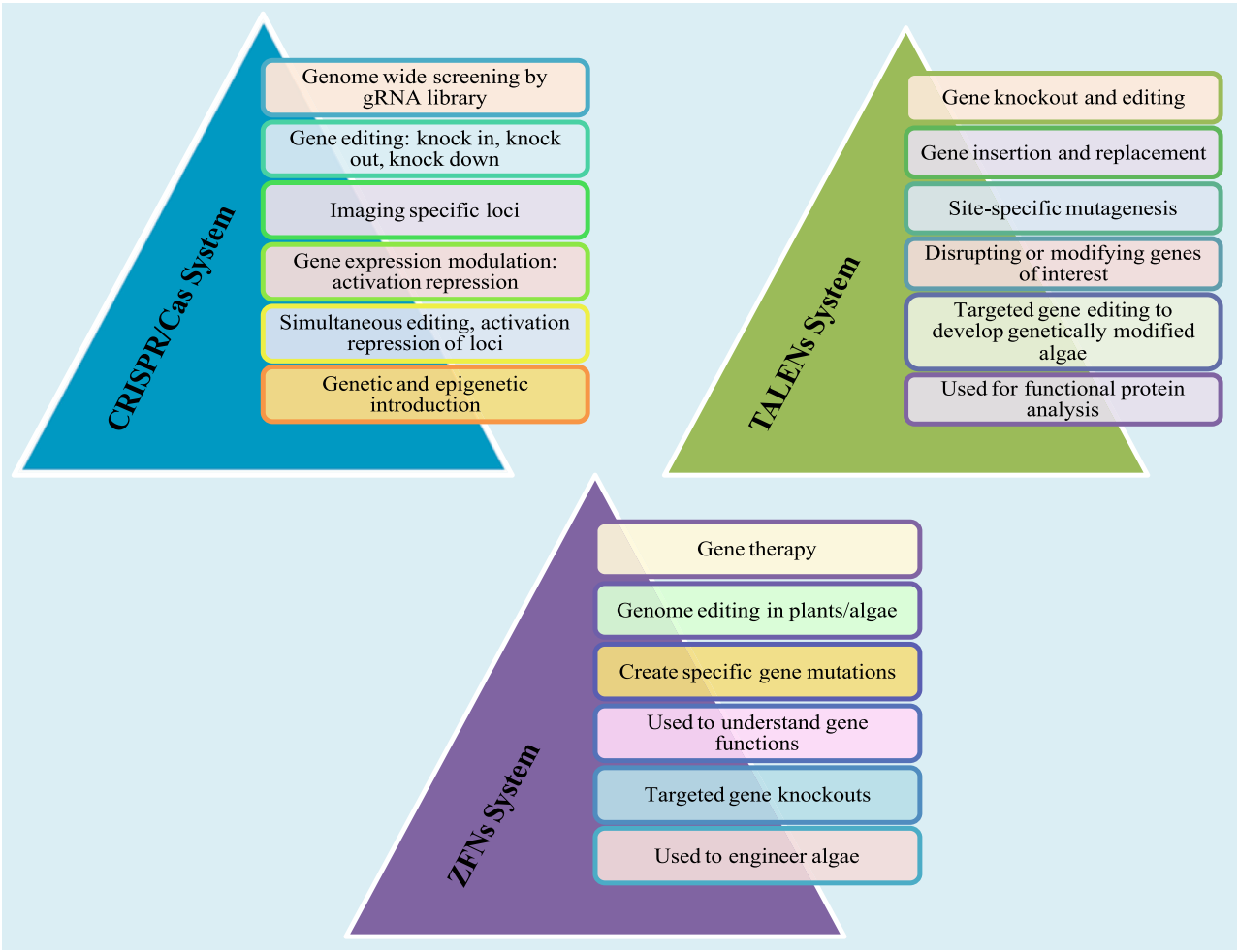

Fig. 2. Illustration to showcase the multiple uses of transcription activator-like endonucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR)/Cas and zinc-finger nucleases (ZFNs) systems to validate its role as a powerful tool in applied and basic research.

engineering ZFNs. Moreover, TALENs offer a genome-wide detection of off-target sites. Achieving potent binding of *Fok*I and mutation efficacy necessitates careful consideration of the length of the target site and the space between two TALEN arms. In a study involving *C. reinhardtii*, engineered TALEs (dTALEs) were assessed for their ability to activate genes [89]. Inorganic carbon accumulation in one instance suggested its role in transporting bicarbonate in CO_2 limited environments. Subsequent studies with the promoter led to the upregulation of genes like *ARS1* and *ARS2*, resulting in increased levels of mRNA and protein. During the investigation of the carbohydrate storage pathway in *P. tricornutum*, researchers noted a 45 % rise in productivity following the targeted disruption of a gene using TALENs [90]. Both TALENs and ZFNs are extensively employed in gene editing, with numerous success stories documented in mammals, plants, insects, and microalgae like *C. reinhardtii*. The Table 2 provides a detailed comparison of CRISPR and TALEN based on their efficiency, benefits, and drawbacks.

3.4. Transplastomic technology

The compact size and simple structure of the chloroplast genome make it an excellent candidate for genome editing through homologous recombination, allowing for precise gene insertion. However, while microalgal chloroplasts are proficient in protein folding and disulfide bond formation, they lack the ability to glycosylate proteins, which limits the expression of glycosylated proteins. This limitation underscores the challenge of achieving stable and homoplasmic transformants in microalgae like *C. reinhardtii*, given the presence of multiple identical copies of the plastome in each chloroplast. Despite the array of methods available for chloroplast transformation, developing an efficient delivery system for plastids remains challenging, particularly due to the intricacies involved in preparing protoplasts. On the topic of lipid biosynthesis, the chloroplast serves as the primary site for these processes, allowing genetic and biochemical modifications to be contained within this organelle and minimizing their impact on the rest of the cell. However, the potential formation of chimeric plastomes resulting from unintended DNA recombination between native and introduced copies necessitates precautionary strategies such as selectively eliminating native DNA before transformation. Despite the lower rates of recombinant protein accumulation in microalgal chloroplasts compared to their plant counterparts, and the confinement of plastid-produced enzymes within the organelle, restricting the range of introducible proteins, the concept of stacking transgenes in synthetic operons presents intriguing possibilities for metabolic engineering of photosynthetic organisms using transplastomic technology.

The method of engineering the chloroplast genome of *Chlamydomonas reinhardtii* in yeast, then transitioning to bacteria for production before reintroducing it into Chlamydomonas cells, is intriguing due to its ability to facilitate complex genetic modifications systematically and simultaneously within the cell. This technique's functionality was demonstrated by successfully integrating core photosystem subunits from *S. obliquus* into various sites within the *Chlamydomonas* plastid genome [113]. This innovative approach to manipulating the entire plastome holds promise for creating customized plastids containing essential genes that can transform microalgae into efficient lipid

Table 2
Comprehensive comparison of CRISPR and TALEN focusing on their efficiency, advantages, and limitations.

Aspect	CRISPR	TALEN	References
Mechanism	Uses a guide RNA (gRNA) to direct the Cas9 nuclease to a specific DNA sequence, enabling targeted genetic modifications.	Uses custom-designed proteins containing DNA-binding TAL effectors and a FokI nuclease to induce DNA breaks at specific sites.	[91,92]
Efficiency	Highly efficient due to the simplicity of gRNA design and rapid implementation. Suitable for large-scale and high-throughput applications.	Efficient but less so than CRISPR due to the time-consuming process of engineering specific TAL effector proteins for each target.	[93,94,99]
Specificity	Moderate specificity, but off-target effects can occur due to short (20-bp) gRNA sequences. Improved Cas9 variants help reduce errors.	Higher specificity due to longer recognition sequences (14–20 bp), resulting in fewer off-target effects.	[95,96]
Ease of design	Simple and quick—requires only a gRNA sequence to target DNA. Can be easily modified for different targets.	Complex and time-consuming—requires engineering a new TALEN protein pair for each target.	[97,98]
Multiplexing ability	Excellent—multiple genes can be edited simultaneously by introducing multiple gRNAs.	Limited—each target requires a separate TALEN pair, making multiplexing impractical.	100]
Target site flexibility	Limited—requires a nearby Protospacer Adjacent Motif (PAM) sequence for Cas9 binding, restricting possible target sites.	More flexible—does not require a PAM sequence, allowing targeting of a broader range of genomic sites.	[96,101]
Cost	Cost-effective—designing and producing gRNAs is inexpensive compared to engineering TALEN proteins.	Expensive—requires labor-intensive protein synthesis and engineering, increasing costs.	[102,103]
Application range	Widely used in gene knockout studies, disease modeling, gene therapy, synthetic biology, and agricultural modifications.	Preferred in therapeutic applications where precision is crucial, such as correcting genetic disorders with minimal off-target effects.	[104,105]
Performance in complex genomic regions	Less effective in GC-rich or repetitive sequences and epigenetically modified DNA.	Performs well in GC-rich, repetitive, or epigenetically modified DNA regions, making it useful for challenging genomic targets.	[100,106]
Off-target effects	Higher—due to short gRNA sequences and the potential for mismatches, leading to unintended mutations.	Lower—longer recognition sequences reduce the likelihood of off-target modifications.	[96,107]
PAM dependency	Requires a PAM sequence (e.g., NGG for SpCas9), limiting targeting options.	No PAM dependency—can target virtually any DNA sequence.	[108]
Scalability	Highly scalable—suitable for high-throughput experiments and genome-wide studies.	Less scalable—time-consuming protein engineering limits large-scale applications.	[109,110]
Repair mechanism	Primarily relies on non-homologous end joining (NHEJ) or homology-directed repair (HDR) for genome modifications.	Similar to CRISPR, but its higher specificity improves precision in gene corrections.	[111,112]

producers. However, it's important to note that organellar genome engineering doesn't extend to manipulating the expression of nuclear-encoded genes like transcription factors. By targeting the microalgal chloroplast, avenues for enhancing photosynthesis efficiency are opened up, such as introducing additional light-harvesting pigments, improving photoprotective mechanisms, incorporating recombinant photosystems, or optimizing carbon fixation through manipulation of carbon concentration mechanisms (CCM) and Calvin cycle enzymes [114]. Essential modifications like designating plastid-specific untranslated regions (UTRs), intercistronic spacers, and refining codon usage are crucial for achieving these enhancements.

3.5. Omics tools

Advancements in omics technologies have allowed for the seamless integration of transcriptomics, proteomics, metabolomics, and interactomics data using mathematical models and computational tools within the realm of systems biology. This integration has yielded a more nuanced understanding of the biochemical composition of microalgal strains across various physiological conditions. By meticulously mapping genes, proteins, and metabolites associated with metabolic pathways that govern cellular functions in response to environmental stimuli, a novel approach to efficiently manipulate microalgal genomes has emerged. This method empowers the tailored production of desired bioproducts, such as lipids for biofuels, while streamlining resource allocation. The adoption of liquid chromatography, gas chromatography, mass spectrometry, nuclear magnetic resonance, and other omics tools has significantly bolstered our capacity to compare lipid types and quantities across diverse microalgal species, both qualitatively and quantitatively (Fig. 3). These tools also facilitate the identification of shifts in global lipidomes under varying cultivation conditions, providing invaluable insights for process optimization and yield enhancement in microalgal biotechnology. Moreover, several integrated online platforms like ChlamyCyc, Greenhouse, Diatom EST database, Alga-PrAs, and Cyan-Omics offer unrestricted access to omics data crucial for advancing microalgal research. Noteworthy resources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) furnish extensive data on genome sequencing, annotations, enzymes, reactions, and biosynthetic pathways pertinent to microalgae, serving as a pivotal tool for pinpointing genes and pathways relevant to microalgal lipid engineering [115]. Additionally, tools like the Algal Functional Annotation Tool and Phytozome provide comprehensive analysis suites and meticulously curated genome data, facilitating the functional interpretation of microalgal genes and unraveling biological themes related to lipid production and other metabolic processes [116].

3.6. Genome reconstruction

Reconstructing the metabolic network is a crucial tool for genome engineering. In genome-scale reconstruction, all pertinent metabolic information, including genes, enzymes, and reactions, is collected and analyzed using a mathematical model that considers the entire network [117]. This process typically involves utilizing genomic databases, biochemical texts, metabolic pathway databases, and archived journal articles. Bioinformatic software is also used to predict enzyme localization within the cell, while flux balance analysis (FBA) predicts fluxes through linear programming, considering reaction stoichiometry, biomass composition, and constraints related to metabolite transport

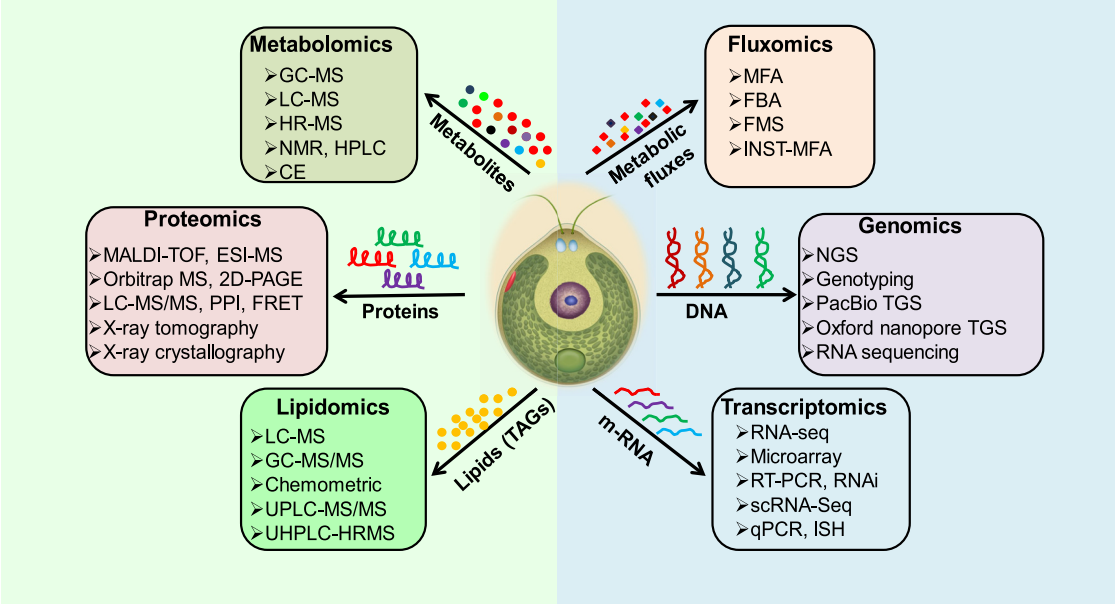

Fig. 3. Various omics approaches and instrumentations for microalgal metabolic and genetic engineering. Different abbreviations represents: MFA (metabolic flux analysis), FBA (flux balance analysis), FMS (fluxomics with mass spectrometry), INST-MFA (isotopically nonstationary metabolic flux analysis), NGS (next-generation sequencing), PACBio TGS (pacific biosciences third-generation sequencing), Oxford nanopore TGS (oxford nanopore third-generation sequencing), RNA-seq (RNA-sequencing), RT-PCR (reverse transcription polymerase chain reaction), RNAi (RNA interference), scRNA-seq (single-cell RNA sequencing), qPCR (quantitative real-time PCR), ISH (RNA in situ hybridization), LC-MS (liquid chromatography–mass spectrometry), GC–MS/MS (gas chromatography-tandem mass spectrometry), UPLC-MS/MS (Ultra-high performance liquid chromatography-mass spectrometry), UHPLC-HRMS (Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry), MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight), ESI-MS (electrospray ionization mass spectrometry), Orbitrap MS (orbitrap mass spectrometry), 2D-PAGE (two-dimensional gel electrophoresis), LC-MS/MS (liquid chromatography with tandem mass spectrometry), PPI (protein-protein interaction), FRET (forster resonance energy transfer), GC–MS (Gas chromatography–mass spectrometry), LC-MS (liquid chromatography-mass spectrometry), HR-MS (high-resolution mass spectrometry), NMR (Nuclear magnetic resonance), HPLC (High-performance liquid chromatography), CE (capillary electrophoresis).

rates and thermodynamics [118]. Various genome-scale models have been developed for microalgae, such as AlgaGEM and iRC1080 for *C. reinhardtii*, DRUM for *Tisochrysis lutea* and *Chlorella sorokiniana*, iLB1027_lipid for *Phaeodactylum tricornutum*, iCZ843 for *Chlorella vulgaris* UTEX 395, iN934 for *Nannochloropsis salina*, iRJL321 for *Nannochloropsis gaditana*, and iSyp 821 for *Synechococcus* sp. PC 7002 [119,120]. AlgaGEM, for example, has been effectively used to enhance hydrogen productivity and model various light sources in *C. reinhardtii*, demonstrating its practicality and accuracy [121]. These models enable system-level metabolic engineering, pathway design, and in silico analyses for improving microalgae strains in a comprehensive approach, contributing to a deeper understanding of microalgae's genetics and biophysical properties. With the increasing efficiency and precision of genome editing, the potential of integrating artificial intelligence and biotechnology, although in early stages, is substantial for enhancing biological systems beyond natural evolution. This integration could lead to the development of a mathematical framework encompassing all biological potentials.

4. Advancement of cultivation system

In the recent past, significant progress has been reported in the microalgal cultivation systems, propelled by rising demand for sustainable sources of food, biofuels, and various high-value products. These developments aim to enhance the efficiency, scalability, and economic viability of microalgal production. One key area of advancement lies in the design of cultivation systems that optimize the growth conditions for microalgae. Traditional open pond systems have been widely used for microalgal cultivation, but they face challenges such as contamination and limited control over environmental variables. To address these issues, closed photobioreactor systems have gained popularity. These systems provide a controlled environment, allowing precise regulation of temperature, light intensity, and nutrient availability. Additionally, closed systems minimize the risk of contamination, leading to higher biomass yields and improved product quality. Furthermore, the advancement of microalgal co-cultivation and staged cultivation systems represents a significant stride towards optimizing productivity, resource utilization, and the versatility of microalgae in various applications. Co-cultivation involves cultivating multiple microalgal species together or with bacteria and fungi, creating symbiotic relationships that enhance overall productivity and provide additional benefits. In co-cultivation systems, microalgae can complement each other by utilizing different nutrients or byproducts, leading to a more efficient use of resources. For example, one species may produce certain nutrients that are essential for the growth of another, creating a mutually beneficial environment. This approach helps to address challenges such as nutrient depletion and competition for resources, ultimately increasing biomass yields. Furthermore, co-cultivation systems can be designed to produce specific compounds or bioactive substances by taking advantage of the metabolic interactions between different microalgal species. This holds relevance in the pharmaceutical, nutraceutical, and bioenergy sectors, where the synthesis of valuable compounds is a subject of significant interest. Additionally, staged cultivation systems involve manipulating the growth conditions of microalgae at distinct phases of their life cycle to maximize productivity. This approach recognizes the diverse physiological requirements of microalgae during various growth phases. For instance, a two-stage cultivation system may involve an initial stage with optimal conditions for rapid cell division and a subsequent stage with altered conditions to induce the accumulation of desired compounds or trigger specific metabolic pathways. These advancements underscore the potential of microalgal cultivation systems to contribute to sustainable practices and address global challenges. As the understanding of microalgal biology deepens and technology continues to evolve, co-

cultivation and staged cultivation strategies are poised to significantly contribute to unleashing the complete capabilities of microalgae for a diverse array of applications.

4.1. Hybrid photobioreactors

The efficiency of the cultivation system is critical for the practical production of lipid for biofuel from microalgae. Microalgae can be mass cultivated in two main types of systems: open ponds and closed photobioreactors (PBRs) [122,123]. However, open pond systems face limitations in regulating crucial working conditions such as light intensity, temperature, nutrient levels, and CO_2 availability. Furthermore, they are susceptible to contamination risks, which can adversely affect the productivity and purity of the microalgae [124]. In contrast, closed cultivation systems, such as PBRs, offer several advantages over open systems. They exhibit high biomass productivity due to superior photosynthetic efficiency and enable better control over maintaining a monoculture. This control optimizes growth conditions and minimizes contamination risks [125]. PBRs also have the benefit of minimal water evaporation compared to open ponds, and they can attain higher cell concentrations, leading to increased biomass production potential. Nevertheless, closed cultivation systems do face challenges, including operational expenses associated with mixing, cooling, and energy demands [123]. To address these challenges and reduce the operational expenses of photobioreactors, ongoing research is developing novel approaches to improve their efficiency. The upcoming sections of this review will delve into improving process design and operation to optimize reactor performance and facilitate scale-up. We will explore essential design parameters crucial for photobioreactor scalability and introduce innovative photobioreactor designs aimed at enhancing overall productivity.

Hybrid PBRs represent a cutting-edge approach to microalgal cultivation, combining the advantages of different cultivation systems to optimize growth conditions and enhance overall efficiency. These innovative reactors aim to overcome the limitations associated with traditional PBRs and open pond systems, offering a versatile and scalable solution for microalgal biomass production. Hybrid PBRs, a recent innovation in photobioreactor technology, aim to merge the strengths of different traditional PBRs while addressing their inherent limitations [126]. One key feature of hybrid PBRs is their ability to integrate both natural sunlight and artificial lighting sources, such as light-emitting diodes (LEDs). This combination allows for continuous cultivation irrespective of environmental conditions, ensuring a stable light supply for photosynthesis. Natural sunlight provides a cost-effective and energy-efficient source, while artificial lighting enables precise control over light spectra and intensity, promoting optimal growth rates and biomass yield. A notable feature of hybrid PBRs is the incorporation of low-cost open ponds into a closed-loop system, creating a novel hybrid system with superior control over growth conditions [127]. Closed PBRs are adept at preventing contamination and maximizing biomass production, while open ponds excel in inducing nutritional stress and encouraging the synthesis of desired metabolites. By combining these systems, hybrid PBRs can significantly boost both overall productivity and metabolite yields. Furthermore, the hybrid approach extends to the incorporation of multiple cultivation modes, such as attached growth systems or immobilized microalgae on substrates within the reactor. These strategies enhance nutrient uptake efficiency and offer supplementary surface area to facilitate algal adhesion, contributing to increased biomass productivity. This potential is realized through careful adjustments, including the optimization of factors such as the ratio of surface area to volume and the ratio of height to diameter in the design [128]. These design enhancements not only improve productivity but also minimize both costs and energy consumption.

In the realm of innovative hybrid PBRs, various research papers and publications have proposed intriguing concepts. For instance, [129] conducted a study focusing on the biomass productivity of *Chlorella homosphaera* within a novel hybrid PBR design. This particular hybrid PBR ingeniously combined elements such as an open tank; a flat plate PBR, with series arranged two vertical bubbles. To optimize light distribution, transparent materials were employed throughout the system, and a carefully positioned light assembly ensured uniform illumination over the PBR tank. The integration of these diverse components was facilitated by a network of connecting pipes, enabling efficient circulation of algae and the exchange of gases. Notably, this hybrid PBR design outperformed conventional open tanks in terms of biomass productivity. The improvements were attributed to enhanced flow dynamics and more efficient gas transfer within the hybrid PBR system. The [130] introduced a hybrid PBR that integrated an airlift reactor and an external tubular loop within a thermostatic water pond. This unique setup offered advantages such as degassing capabilities and the incorporation of probes for controlling other critical culture variables. Similarly, [131] developed a comparable PBR featuring a tubular light-harvesting system. This design not only enhanced culture control but also improved productivity and energy efficiency. However, earlier designs often faced challenges such as the requirement for large land areas and associated costs for land and tube bundles. To overcome these limitations, [132] introduced an innovative horizontal floating PBR design. This more efficient and cost-effective system could be easily scaled up to produce large quantities of microalgae. It employed two sealed plastic films, one at the top and one at the bottom of the PBR, connected to vertical airlift units, ensuring effective cultivation. Furthermore, researchers recognized the advantages of attached cultivation systems for superior biomass harvesting efficiency compared to suspended systems. A capillary-driven PBR system utilized capillary action to deliver nutrients and water into a polyester microfiber media [133]. As a result, the produced biomass had a higher proportion of carbohydrates to lipids, making it suitable for biofuel production. Similar experiment performed by [134] pioneered a hybrid PBR system tailored to increase working volume and optimize CO_2 bioconversion by *Scenedesmus obliquus*. This innovative system integrated a bubble column reactor with an illumination unit, resulting in a dynamic setup that achieved a remarkable maximum specific growth rate of 0.96 d^{-1} during the bioconversion of CO_2 by *S. obliquus*. These diverse approaches in hybrid PBR design demonstrate ongoing efforts to optimize microalgae cultivation for various applications, ranging from biomass production to biofuel generation. The versatility of hybrid PBRs makes them adaptable to various microalgal species and cultivation goals. Whether the objective is biomass production, biofuel generation, or the extraction of valuable compounds, these systems offer a flexible and efficient platform for microalgal growth. As research and technology continue to advance, hybrid PBRs are poised to play a crucial role in sustainable microalgal cultivation, aiding in the advancement of financially feasible and ecologically sustainable solutions for a range of industries.

4.2. Microalgae-microalgae co-cultivation

In the recent past, co-cultivation of microalgae strains has been frequently used for the enhancement of biomass and lipid content. This favorable outcome results from selecting microorganisms with complementary metabolic capabilities that can coexist in a shared environment without inhibiting each other's growth. To enhance lipid production, it is advantageous to pair a lipid-producing microorganism with another species capable of providing essential nutrients or metabolizing by-products. Interestingly, different cultivation modes can cause varying effects on lipid productivity when co-culturing microorganisms like *Chlorella* sp. with *Monoraphidium* sp. [135]. The lipid yield of these microalgae significantly increased from 93.4 to 223.42 g m^{-3} d^{-1} when the two microorganisms were co-cultivated under heterotrophic conditions. However, the lipid yield decreased to 21.23 g m^{-3} d^{-1} when the co-cultivation was carried out under mixotrophic conditions. This suggests that optimizing cultivation conditions is crucial, and in this case, heterotrophic conditions were more conducive to higher lipid

productivity than mixotrophic conditions. The success of co-cultivation systems lies in harnessing the metabolic interactions between the organisms. For instance, one organism may produce growth-promoting factors, vitamins, or enzymes that can benefit the growth and lipid accumulation of another organism. Moreover, it is possible to fine-tune carbon and nutrient allocation between the co-cultured organisms to maximize both biomass and lipid production. This can be achieved by adjusting the composition of the growth medium, ensuring the availability of carbon sources, and optimizing nutrient ratios to favor lipid accumulation in the desired organism. Consistently, previous studies have supported the notion of a synergistic effect between microalgae strains in promoting lipid production under co-cultivation conditions. Overall, these findings underscore the importance of well-designed co-culture systems to enhance microalgal biomass and lipids.

Co-culturing microalgae offers a significant advantage over monocultures because it facilitates the development of denser cultures with accelerated growth rates. This increased interaction between microorganisms in a binary culture leads to the production of more extracellular polymeric substances (EPS) [136]. EPS production serves as a microbial adaptation strategy to cope with unfavorable conditions. However, it is essential to exercise caution when conducting co-culture experiments because excessive EPS can obstruct the flow of nutrients and dissolved CO_2 to microorganisms, thereby hindering their growth. Therefore, the careful selection of microalgal-microalgal consortia and the cultivation system becomes crucial, especially when considering potential limitations for applications at an extensive scale. Although other factors like temperature, pH, light intensity, and agitation for both organisms, is some crucial factors for their optimum growth. In addition to enhancing biomass and lipid production, co-culture systems can serve other purposes, such as the bioremediation of various waste materials and the promotion of bioflocculation, which enhances biomass harvesting efficiency [137]. Notably, [138] found that co-cultivating *Chlorella* sp. with *Ettlia* sp. resulted in enhanced biomass and lipid yields. Furthermore, employing a co-cultivation technique with *Leptolyngbya tenuis* and *C. ellipsoidea* showed 2–3-fold enhancement in the biomass production and 2-fold enhancement in total lipid in compared to monocultures [139].

Although microalgae binary cultivation holds promise for improving biomass and lipid yields, further research is needed to elucidate the underlying symbiotic mechanisms. Such studies can provide valuable insights into enhancing productivity. To achieve this, effective monitoring techniques are essential to assess the growth and lipid production of each organism within the co-culture. These techniques may encompass biomass measurements, lipid quantification, and molecular analyses such as gene expression. Using monitoring data, it becomes possible to make real-time adjustments to cultivation parameters, optimizing the co-culture process. Moreover, evaluating the scalability of the co-culture strategy involves considerations such as reactor design, process control, and downstream processing requirements. Nevertheless, it is critical to carefully select the appropriate consortium to ensure positive outcomes. Recognizing that the specifics of a co-culture strategy can vary depending on factors such as the microorganisms used, the target compounds, and the overall process requirements, it is important to adopt a comprehensive approach, including literature review and experimental trials. This is essential to develop an effective co-culture strategy tailored to specific objectives.

Microalgae-microalgae co-cultivation, while offering advantages like enhanced biomass productivity and resource utilization, presents several limitations. One major challenge is competition for nutrients and light, where dominant species may outcompete others, leading to imbalanced growth [140]. This competition is further exacerbated by species compatibility issues, as differences in growth rates, metabolic interactions, and environmental preferences make it difficult to maintain stable co-cultures. In addition to competition, allelopathic interactions, where one species releases inhibitory compounds can negatively impact the growth of co-cultivated strains, further destabilizing the system [141]. Moreover, the complexity in optimizing culture conditions adds another layer of difficulty, as different microalgae species may require distinct pH, temperature, and salinity levels, complicating large-scale cultivation [142]. This complexity extends to harvesting challenges, where variations in cell size and density make separation inefficient. Compounding these issues is the limited knowledge of microbial interactions in co-cultures, which restricts precise control over community dynamics. Addressing these limitations requires integrated biotechnological strategies and a deeper ecological understanding to ensure the successful implementation of microalgae-microalgae co-cultivation systems.

4.3. Microalgae-bacteria co-cultivation

The mutualistic relationship between microalgae and bacteria in a co-culture system is crucial for enhancing biomass and lipid productivity. Bacteria play a pivotal role by providing essential nutrients that promote microalgae growth. In return, microalgae release oxygen and organic carbon, benefiting bacterial metabolism. This nutrient cycling mechanism reduces reliance on external nutrient sources, making co-culture systems more sustainable. For instance, co-cultivating *Ankistrodesmus* sp. with *Rhizobium* sp. resulting a significant 30 % enhancment in biomass and lipid yield [143]. After optimizing various parameters, lipid productivity reached an impressive 112 mg L^{-1} d^{-1}. Bacteria contributions go beyond nutrient provision; they also secrete enzymes and bioactive compounds that influence microalgae growth and lipid metabolism. Co-cultivating *C. reinhardtii* with *Bradyrhizobium japonicum* resulted in a substantial increase in microalgal growth [144]. This co-culture system exhibited a remarkable 26 % rise in biomass yield and a 14-fold increase in hydrogen production compared to monoculture. This enhanced microbial interaction underscores the potential of co-cultivation in biotechnology and biofuel production.

Furthermore, bacteria play a dynamic role in modifying the microenvironment within co-culture systems, which can profoundly impact the growth and lipid production of microalgae. This environmental modulation is conducive to the accumulation of biofuel precursors. In the case of the co-culture between *C. reinhardtii* and *B. japonicum*, the creation of an anaerobic atmosphere resulted in increased Fe-hydrogenase activity and, consequently, higher production of H_2 [144]. This highlights how changes in oxygen levels can directly influence biofuel-related processes. Zhao et al. [145] demonstrated the capability of a combined consortium of microalgae and bacteria in landfill leachate, achieving noteworthy biomass output and lipid productivity. This suggests that co-culture systems can thrive in diverse environmental conditions, such as those found in landfill leachate. Innovative approaches, such as the symbiotic relationship between *B. braunii* and *Rhizobium* sp., have led to substantial increases in microalgal biomass yield and specific lipid production [146]. These advancements underscore the versatility and adaptability of microalgae-bacteria co-culture strategies. Furthermore, the utilization of microalgae and bacteria co-culture in water remediation efforts, as mentioned in the context of cleaning up polluted water, has demonstrated significant improvements in lipid production under different growth conditions [147]. This indicates the potential for addressing environmental challenges while concurrently enhancing biofuel precursor production. In conclusion, microalgae-bacteria co-culture shows great potential for enhancing biomass and lipid production for biofuels. Nonetheless, it is essential to emphasize that further research and development are required to optimize these co-culture systems, unravel the underlying mechanisms, and eventually scale up the process for industrial applications.

Microalgae-bacteria co-cultivation systems face several challenges despite their potential for enhanced biomass production and nutrient cycling. One major issue is establishing a stable, efficient symbiotic relationship, as bacteria provide essential nutrients to microalgae; however, competition for nutrients and space can hinder growth [148]. This challenge is compounded by differences in metabolic activities and

environmental requirements, such as light, temperature, and pH, which further complicate the optimization of conditions for both organisms [149]. Additionally, the accumulation of toxic byproducts from bacteria may inhibit microalgal growth or even cause cell death, disrupting the symbiotic relationship [150]. These factors also complicate the scaling up of the system for industrial applications, as efficient harvesting, maintaining consistent biomass production, and ensuring cost-effective nutrient management become more difficult [151]. Thus, addressing these interconnected challenges is essential for the successful commercial implementation of microalgae-bacteria co-cultivation systems.

4.4. Microalgae-fungi co-cultivation

The co-culture strategy involving microalgae and fungi is considered as one of the promising approach to enhance the microalgal biomass and lipids. Microalgae and fungi possess distinct metabolic pathways and capabilities that can be mutually beneficial when co-cultivated. For instance, fungi have the capacity to secrete enzymes that effectively break down complex organic compounds, making them more accessible as nutrients for microalgae [152]. Simultaneously, microalgae release oxygen and organic carbon compounds during their growth, providing essential support for fungal development. Furthermore, the interaction between fungi and microalgae can induce lipid accumulation in the microalgae through various mechanisms, such as signaling molecules or metabolic interactions [153]. This lipid accumulation is highly desirable for biofuel production. However, achieving optimal results in a microalgae-fungi co-culture system necessitates careful consideration of cultivation environment. Factors like pH, temperature, intensity of light and nutrient availability should be fine-tuned to create an environment that fosters the growth and productivity of both microalgae and fungi.

Dash and Banerjee [154] initiated their study by exploring symbiotic associations between the microalga *Chlorella minutissima* and the oleaginous fungus *Aspergillus awamori*, a novel approach in the field. Notably, they made a significant departure from conventional methods by substituting glucose with pure glycerol as the organic carbon source, a choice that yielded reduced production costs. This innovative strategy yielded remarkable results. When comparing microalgal monocultures to co-cultures with *C. minutissima*, there was a notable rise in both biomass yield and lipid accumulation, showing respective increments of 2.6 and 3.4 folds. However, even more impressive were the findings within the co-culture system of *C. minutissima*, where a nearly 3.9-fold higher biomass increment and a 5.1-fold increase in lipid yield were observed. The major fatty acids identified in both co-culture systems were C16:0 and C18:1, underscoring the potential of this approach to enhance lipid production [154]. Building upon success, [155] conducted their own study on a co-culture system, this time featuring *Spirulina platensis* and *Rhodotorula glutinis*. In this case, the co-culture system outperformed monocultures, demonstrating significantly increased total lipid and biomass production. Similarly, [156] investigated mixed consortia composed of *Chlorella* sp. and oleaginous yeasts, specifically *Torulaspora globosa*. The decision to employ sugarcane juice as a carbon source in a mixotrophic mode led to staggering 96 % increase in lipid production and a significantly higher biomass yield. These studies collectively highlight the potential of co-culture approaches to achieve substantial biomass yield and lipid production. For example, the co-cultivation of *Ambrosiozyma cicatricose* yeast with the microalga *Isochrysis galbana* resulted in a noteworthy biomass yield of approximately 20.71 g m^{-3} [157].

Furthermore, [135] reported about the consortia of *R. glutinis* and *C. vulgaris* which during co-cultivation significantly boosted overall lipid yield, enhancing biomass yield by 17.3 % and lipid productivity by 70.9 % compared to monocultures. In another study, [158] reported about the mixture of yeast and *C. vulgaris*, which showed substantial yields of 4.63 g L^{-1} biomass and 2.88 g L^{-1} lipid production, after utilization with molasses from sugarcane. Expanding on this body of research, [159] revealed after study that consortia of *T. obliquus* and *R. glutinis* can increased the biomass by 40–50 % and lipid yield by 60–70 % in compared to single batch cultures. Similarly, [160] reported that a co-culture system of *Chlorella* sp. and *Rhodotorula toruloides* achieved a biomass yield of 26.9 g L^{-1} and a lipid content of 70.94 %, marking a 1.8-fold and 2.13-fold increase, respectively, over monoculture systems. These findings highlight the effectiveness of microbial co-cultures in boosting biomass and lipid productivity, a crucial factor in improving the feasibility of algal biofuels. Beyond enhancing biomass and lipid accumulation, co-culture strategies also play a key role in improving downstream processing efficiency. Al-Hothaly et al. [161] examined the self-flocculating activity of *Aspergillus fumigatus* at a pilot scale (500 L) and observed an impressive harvesting efficiency of up to 98 % for *Botryococcus braunii*. This fungal-assisted flocculation addresses one of the major bottlenecks in algal biofuel commercialization—the challenge of efficiently harvesting microalgal biomass from dilutes cultures. When combined with the improved biomass and lipid productivity observed in microbial consortia, such advances in harvesting techniques further strengthen the potential of co-culture systems as a sustainable and scalable approach for biofuel production.

Microalgae-fungi co-cultivation presents several challenges that must be addressed for successful implementation, especially in large-scale biofuel production. One major issue is ensuring a stable, mutually beneficial interaction between the organisms. Fungi, while providing organic carbon sources or serving as biocontrol agents, have different growth requirements such as temperature, pH, and nutrients as compared to microalgae [162]. Balancing these conditions can hinder growth and productivity. Additionally, fungi can outcompete microalgae for nutrients or space, potentially inhibiting microalgal growth, especially under conditions favoring fungal proliferation [163]. Furthermore, fungi may produce secondary metabolites or toxins that adversely affect microalgae [164]. Another challenge is optimizing nutrient cycling in co-cultures, as fungi may complicate nutrient availability and impact microalgal metabolism [165]. Scaling up microalgae-fungi co-cultivation for industrial applications also faces difficulties, such as efficient biomass separation and harvesting, long-term culture stability, and cost-effective nutrient management [15].

4.5. Two-stage cultivation

A two-stage cultivation system for microalgae aimed at biofuel production involves a carefully orchestrated process to optimize biomass and lipid yields (Fig. 4). This method typically consists of two distinct phases: the phase of growth and the phase of lipid accumulation [11]. In the initial stage, microalgae are cultivated under conditions conducive to rapid growth. Open pond systems or closed PBRs are commonly employed, providing the microalgae with the necessary nutrients, light, and CO_2 for robust biomass production. This phase is characterized by exponential cell division, resulting in a significant increase in microalgae biomass. Once the microalgae reach the desired biomass concentration, they are harvested and transitioned to the second stage. Harvesting methods vary and may involve centrifugation, filtration, or flocculation to separate the microalgae from the culture medium. In the second stage, the focus shifts from rapid growth to lipid accumulation within the microalgae cells. The cultivation conditions are manipulated to induce stress, typically through nutrient deprivation or salinity stress [16]. This stress triggers the microalgae to redirect energy towards synthesizing and accumulating lipids, particularly TAGs, within their cells. Once the microalgae have undergone sufficient lipid accumulation, they are harvested again. After this step, the process involves separating the lipid from the microalgae biomass. Diverse methods, such as solvent extraction or mechanical pressing, can be utilized for lipid extraction from microalgae cells. The obtained lipids, abundant in fatty acids, function as the foundational material for biofuel synthesis. Through processes like transesterification, the lipids can be converted into biodiesel. This final stage transforms the accumulated lipids from the microalgae into a usable and sustainable biofuel. The two-stage

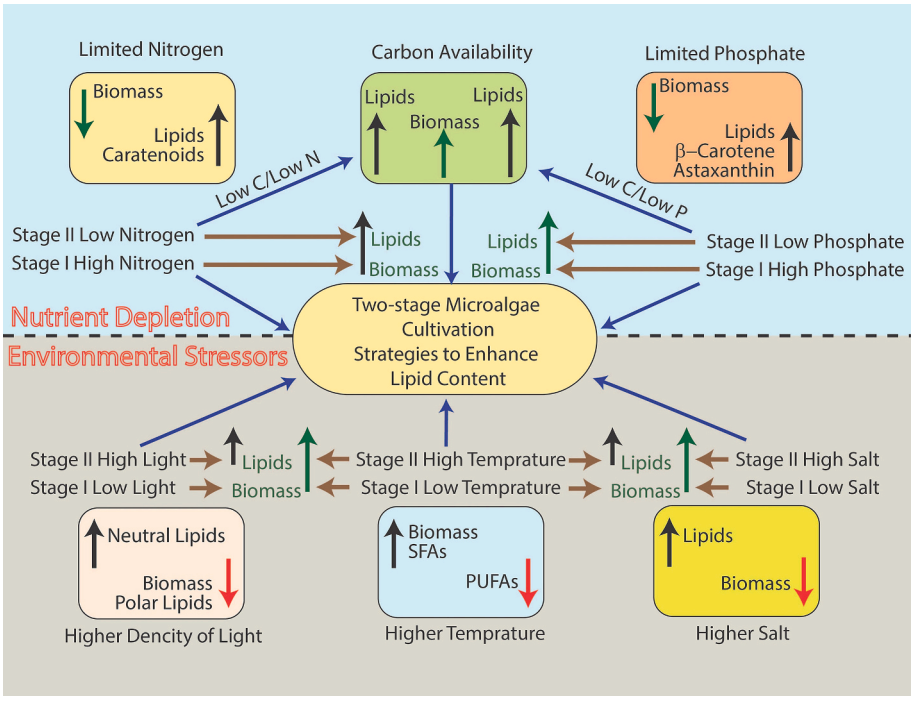

Fig. 4. Model showing the impact of nutritional and environmental stressors on the synthesis of biomass and lipids in microalgae under two-stage cultivation. This figure is modified from Sun et al. [168].

cultivation system is designed to maximize both biomass and lipid production, ensuring an efficient and economically viable process for microalgae-based biofuel production. This process often incorporates salinity stress during the lipid accumulation phase to enhance lipid content in the microalgae.

Microalgal lipids are crucial for biofuel production, but achieving optimal lipid accumulation while maintaining cell growth presents a challenge. Staged cultivation systems provide a solution by balancing favorable growth conditions with stress-induced lipid accumulation. This two-stage approach involves nutrient-rich conditions in the first phase for robust biomass production and stress conditions in the second phase to boost microalgal lipid production [11,65]. Among stress conditions, nitrogen starvation stands out as a reliable inducer of lipid synthesis within staged cultivation. For instance, deprivation of nitrogen during the two-phase cultivation process of *Chlorella* sp. resulted in a significant 50.43 % increase in neutral lipid content [166]. Similarly, an elevated carbon-to-nitrogen ratio acted as a nitrogen deficiency stressor during the second stage of the two-phase culture system *Chlorella protothecoides*, resulted in the enhancement of 58 % of lipid content [167]. Stressors such as phosphorus or silica starvation can potentially boost lipid accumulation in microalgae, but their efficacy depends on the specific species of microalgae.

The economic feasibility of nutrient-starvation-based staged cultivation systems for microalgal lipid production can be hindered by the costs associated with transitioning microalgal biomass from nutrient-rich to nutrient-limited conditions during the lipid accumulation phase. However, staged cultivation systems rooted in environmental stress conditions provide a more practical approach, particularly for large-scale applications. For example, a two-stage cultivation approach utilizing light-induced stress conditions achieved an impressive lipid content of 56 % in *Nannochloropsis oculata* during the second stage of culture [169]. Similarly, the application of saline stress, achieved by directly introducing NaCl, significantly increased the lipid content in *Scenedesmus obtusus*, reaching 47.7 % under specific circumstances [170]. Large-scale application of saline stress approaches, particularly at 140 l, proved feasible and efficient, boosting *S. obtusus* lipid content to 42.1 %. Nevertheless, it is essential to note that while heightened salinity stress conditions can enhance lipid production, microalgae have limited tolerance to high salinity, potentially inhibiting growth even during the lipid accumulation phase. In the second stage of *Scenedesmus* sp. BHU1 cultivation, the highest levels of carbohydrates (42.16 %) and lipids (38.10 %) were achieved in an 8-day-old culture [11]. To mitigate biomass inhibition due to high salinity stress and further boost lipid accumulation, strategies such as a salinity gradient approach have been proposed, resulting in a peak lipid content of 59.4 % in marine *Chlamydomonas* [171]. Moreover, complex staged cultivation systems that combine various stress conditions, such as glucose-fed batch culture during the cell growth phase and hyperosmotic stress coupled with nitrogen starvation during the lipid accumulation phase, have yielded substantial improvements in lipid content, yield, and productivity. The application of a staged cultivation system that induces nitrogen starvation stress has proven reliable in enhancing microalgal lipid production. In industrial-scale settings, environmental stress approaches are more practical. Consequently, integrating environmental stress methods while microalgae are under nitrogen starvation stress can substantially improve lipid production during large-scale staged cultivation. It is paramount to efficiently optimize and oversee the complete stress-integrated cultivation process to ensure the successful commercialization of microalgal lipid production.

5. Advancement of nutrient and environmental conditions

In the domain of microalgal biofuel production, the quest for sustainability critically revolves around optimizing nutrient and environmental conditions. This intricate procedure involves the precise adjustment of various factors: nutrient supply, light quality and intensity, temperature control, and pH regulation. This meticulous orchestration plays a vital role in enhancing both microalgal growth and lipid production. Researchers and biofuel manufacturers collaborate in a concerted effort to enhance the efficiency of microalgal biofuel production. The ultimate goal is to offer an environmentally responsible and economically viable alternative to traditional fuels. This collective endeavor is in perfect alignment with the broader mission of paving the way for a sustainable biofuel revolution. By doing so, it not only reduces

the environmental impact of energy generation but also ensures a reliable and sustainable energy source for the future.

5.1. Carbon availability

Carbon is an essential nutrient for microalgae cultivation, existing in both organic and inorganic forms. Microalgae utilize fixed carbon for respiration, energy generation, and cell formation. Autotrophs primarily rely on inorganic carbon sources like carbon dioxide (CO_2), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}), while heterotrophs depend on organic carbon such as glucose, glycerol, and sodium acetate for their nutritional needs. The availability of carbon significantly influences the growth, lipid content, and composition of microalgae, playing a pivotal role in shaping these aspects scientifically. This biological process is exemplified in *Chlorella protothecoides*, which leverages external organic carbon sources to enhance the production of neutral lipids while simultaneously reducing chlorophyll levels [172]. Furthermore, an increase in environmental CO_2 levels significantly influences the enhancement of photosynthetic lipid content. Notably, species such as *Scenedesmus obliquus* and *Chlorella pyrenoidosa* demonstrate increased lipid content under 50 % CO_2 conditions compared to 0.03 % CO_2 [173]. Beyond the influence of CO_2, various microalgae species exhibit substantial improvements in lipid content across different cultivation methods. For instance, *C. sorokiniana* manifests a remarkable 2.4-fold surge in maximum lipid content when cultivated heterotrophically with 20 g/l glucose [174]. Under mixotrophic conditions with 8 g/l glucose, this increase extends to an impressive 3.9-fold. Furthermore, under autotrophic conditions with 12 mM sodium bicarbonate, *Scenedesmus* sp. BHU1 accumulated 56 % carbohydrate and 34 % lipid content [64]. Exploring unconventional organic carbon sources, such as molasses for *C. vulgaris* and sodium gluconate for *Haematococcus pluvialis*, yields a significant boost in lipid production [175,176]. Furthermore, targeted approaches to address nutrient deficiencies, such as using sodium acetate in *C. reinhardtii*, result in a substantial 93 % increase in lipid production [177]. Li et al. [175] showed that adapting *Chlorella* sp. to CO_2 levels between 1 % and 30 % leads to a remarkable increase in lipid content.

The genome sequencing of *C. pyrenoidosa* demonstrated that the change from relying on heterotrophic to adopting photoautotrophic nutrition resulted in a noteworthy increase in the activation of crucial genes [178]. These genes were essential in functions such as carbon fixation, photosynthesis, fatty acid production, and starch breakdown. Consequently, this metabolic shift led to the accumulation of lipids, constituting approximately 35 % of the dry cell weight, and proteins, accounting for about 40 % of the dry cell weight. Lakshmikandan et al. [179] extended this exploration into microalgae by studying *C. vulgaris*. Their investigation focused on the impact of varying CO_2 levels, with an emphasis on higher CO_2 concentrations (up to 8 %) combined with light exposure. The results were striking, showcasing a significant surge in 94 % biomass and a substantial boost in 54.8 % lipid productivity compared to the control group. Additionally, their findings suggested that the application of gentle pressure and exposure to mild heat shock can further improve the extraction of lipids by 21 %, underscoring the positive role of carbon sources in fostering microalgae growth and lipid content. Furthermore, the role of the malic enzyme, primarily associated with pyruvate metabolism and carbon fixation, was uncovered. Overexpressing the malic enzyme in transgenic *Phaeodactylum tricomutum* led to 2.5-fold enhancement in the lipid content [180]. In another study, [181] explored the potential for carbon capture by microalgae through carbonic anhydrase (CA), an enzyme that facilitates the conversion of CO_2 into bicarbonates. Their work demonstrated the exciting potential of CA in *C. sorokiniana* and *C. vulgaris*, resulting 2.2-fold enhancement in the lipid content in compared to the wild strain.

5.2. Nitrogen depletion

Nitrogen depletion disrupts the synthesis of essential cellular components, resulting in a notable rise in lipid yield. Although various nitrogen sources like ammonium, nitrate, yeast, peptones, and urea etc. have been reported in the growth medium that directly influence cell metabolism. Microalgae demonstrate lipid accumulation when experiencing nitrogen deficiency. However, in the recent studies, it has been emphasize enhancements in energy equivalents during early nitrogen deprivation. Nitrogen stress induces a range of biochemical and metabolic alterations, alongside other cellular processes, fostering lipid accumulation in microalgae. These findings underscore the importance of nitrogen management in optimizing microalgae lipid production. Extensive research efforts have been directed towards augmenting lipid production in various microalgae types through the induction of nitrogen stress. These collective investigations consistently demonstrate substantial increases in lipid accumulation. For instance, *Neochloris oleoabundans* exhibited a 2-fold rise in lipid content when grown in nitrogen-deficient environments [182]. In a similar vein, *Auxenochlorella protothecoides* displayed heightened lipid productivity when cultivated with reduced nitrate levels [183]. Moreover, a separate study illustrated a notable upswing in the overall lipid content of *C. vulgaris*, from 20 % to 53 % under limiting or starvation of nitrogen [184]. Moreover, research endeavor by [184], they effectively optimized lipid synthesis in *Selenastrum* sp. This optimization was achieved by subjecting the microalgae to nitrogen deprivation, resulting in an increase from 16.12 % to 48.6 % of the dry cell weight in terms of lipid content. Likewise, *Eustigmatos vischeri* exhibited an enhancement in lipid content from 49.5 % to 52.3 %, in the nitrogen deprived medium [186]. The consistency of this pattern was further observed during co-cultivation of *Chlorolobion* sp. and *Chlorella* sp. under nitrogen-deprived conditions, underscoring the enhancement of lipid production [187].

Additionally, [188] reported *Chlorella* exhibit significantly higher lipid content in response to nitrogen limitation. Moreover, the highest lipid levels are achieved under nitrogen-deprived conditions in *Chaetoceros muelleri* and *Dunaliella salina* [189]. This occurrence can be ascribed to nitrogen scarcity, which affects microalgae by diminishing cell division and redirecting the lipid biosynthesis [188]. Consequently, this may result in a decrease in biomass production, ultimately affecting lipid accumulation. To enhance lipid production, a recommended approach involves combining nitrogen deficiency stress with carbon enrichment. The [190] exemplify this strategy by showing that combining nitrogen deprivation with additional carbon sources, such as acetate, increases the lipid content in *C. minutissima* and *C. pyrenoidosa*. Similarly, [191] reported that a combination of high glucose and low nitrogen enhances the lipid content of *C. sorokiniana* under heterotrophic cultivation conditions. In a study by [192], *C. reinhardtii* was subjected to 144 h of nitrogen starvation, resulting in 3.8-fold increase in total fatty acids. Moreover, the research revealed that nitrogen deprivation in microalgae stimulates the accumulation of intracellular lipids. Specifically, when transgenic microalgae, *P. tricornutum*, were exposed to 96 h of nitrogen deprivation, they exhibited a substantial 31 % increase in neutral lipid content [180]. In a separate study conducted by [193], the biochemical response of *Scenedesmus acuminatus* to varying nitrogen levels (low at 3.6 mM $NaNO_3$ and high at 18 mM $NaNO_3$) was investigated. Remarkably, under low nitrogen conditions, a substantial lipid accumulation of 53.7 % of dry weight was observed. These pivotal studies collectively shed light on the intricate relationship between nitrogen depletion and lipid production in microalgae, offering valuable insights for a wide range of applications.

5.3. Phosphorous depletion

Phosphorus is a vital nutrient for microalgae, playing a central role in their growth and essential cellular processes, including photosynthesis, nucleic acid synthesis, phospholipid formation, and ATP generation. In

the life cycle of microalgae, phosphorus plays a dual role: it is used to construct crucial organic constituents within cells, such as phospholipids, and any surplus is directed towards the generation of inorganic polyphosphate granules [194]. These microorganisms store phosphorus in the form of polyphosphate or orthophosphate to build critical cellular components. Furthermore, phosphorus serves as a key regulator in metabolic pathways. When phosphate is limited, it triggers increased biosynthesis of diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS), a pivotal intermediate in glycolipid synthesis [195]. A recurring trend becomes evident across various studies: microalgae experience a significant increase in lipid production when exposed to phosphorus stress. This effect is particularly pronounced when phosphate levels are reduced, leading to a notable rise in the overall lipid content across different microalgae species. For instance, in the case of *Phormidium* sp., the lipid content exhibits a substantial increase, climbing from 17 % to a significant 27 % of the dry cell weight as a result of reduced phosphate concentrations [196]. An earlier investigation conducted by [185] presents compelling evidence of this phenomenon. They demonstrated a substantial 31 % boost in lipid production in *Selenastrum* sp. under phosphate depletion. Furthermore, recent research has shed additional light on this relationship, revealing that when phosphorus was deprived in the growth medium, there was a remarkable increase of approximately 53 % in the accumulation of cellular lipids within *Scenedesmus* sp. [197].

Additionally, phosphorus deficiency results in elevated production of eicosapentaenoic acid in the microalgae *Nannochloropsis oceanic* [198]. Similar type of observation reported in *Nannochloropsis oculata* under phosphorous limiting condition [199]. Although lipid production is increased individually by phosphorus and nitrogen deficiencies, they frequently result in a decrease in overall biomass, impacting the production of algal oil and biodiesel. Recent research has explored the synergistic effects of combining nitrogen starvation with phosphorus supplementation, which has proven effective in simultaneously increasing biomass and lipid productivity. For example, *C. vulgaris* cultures exposed to nitrogen starvation and supplemented with phosphorus experienced a significant enhancement in biomass by 10.2 % and lipid content by 39.3 % [200]. The concurrent increase in both biomass and lipid production under nitrogen depletion and phosphorus supplementation can be attributed to the vital role of phosphorus in photosynthesis and the synthesis of energy molecules. This combination shifts microalgal metabolism from protein synthesis towards the production of lipids, nucleic acids, and ATP. These consistent findings collectively emphasize the pivotal role of phosphorus availability in shaping lipid biosynthesis in microalgae, offering a promising avenue for enhancing lipid production in these microorganisms.

5.4. Salinity stress

Salinity stress significantly impacts the growth and lipid production of microalgae, influencing their physiological and biochemical processes. High salinity levels in the growth medium can lead to osmotic stress on microalgae cells. This stress disrupts water balance, affecting cellular turgor pressure and metabolic activities. In response to salinity stress, microalgae often undergo alterations in their morphology, cellular structure, and biochemistry. Growth rates are generally hindered as the cells invest more energy in osmoregulation mechanisms rather than biomass accumulation. Furthermore, salinity stress can influence the photosynthetic efficiency of microalgae. Chlorophyll content and photosynthetic pigments may be affected, leading to a decline in photosynthesis and overall carbon fixation. This, in turn, affects the availability of carbon precursors for lipid biosynthesis. To resolve this issue, some researchers applied a two-stage cultivation system for microalgae growth and lipid production. For instance, in a two-stage cultivation, lipid content increased by 24.77 % and 38.10 % in *Scenedesmus* sp. under 400 mM NaCl [11,65]. Under salinity stress, microalgae often modulate their lipid composition and content as a survival strategy. Some species accumulate higher amounts of lipids in response to stress, possibly as a protective mechanism or to store excess energy. In response to salt-induced stress, microalgae undergo metabolic adaptation, transitioning from synthesizing starch to storing lipids as an energy reserve. Notably, halotolerant microalgae such as *Scenedesmus* sp. IITRIND2 exhibit physiological and metabolic responses to salinity, including lipid accumulation and altered metabolic flux [120]. Research on freshwater microalgae, such as *C. sorokiniana* and *Desmodesmus* sp., has shown that the addition of $CaCl_2$ effectively enhances lipid accumulation, resulting in remarkable gains [201]. In marine microalgae like *Tetraselmis suecica*, varying salinity levels significantly impact total lipid accumulation, with a notable boost observed at higher salinity levels [202].

High salinity stress significantly affects the lipid and carbohydrate concentrations in microalgae, potentially impeding the growth of various species such as *Scenedesmus* sp. *Dunaliella* sp., and *Chlorococcum* sp. [11,203,204]. When microalgae face immediate salt stress, they adapt by accumulating lipids or starch, depending on the specific species. A study conducted by [204] serves as an example, where an increase in salt concentration from 0 % to 2 % using NaCl resulted in a substantial rise in lipid content from 10.3 % to 29.8 % in *Chlorococcum* sp. Furthermore, salt stress alters both the lipid accumulation pattern and the fatty acid composition in microalgae. Often, heightened salt stress decreases polyunsaturated fatty acids while elevating saturated and monounsaturated fatty acids. Malonyl CoA-acyl carrier protein transacylase (MAT) is associated with lipid accumulation in cells, particularly when *C. reinhardtii* is subjected to NaCl-induced stress [205]. For instance, exposing microalgae to NaCl stress (250 mM for 7 days) results in significantly higher levels of fatty acid methyl esters (70.2 %) and an upregulation of genes related to fatty acid biosynthesis. Another study conducted by [206], involving integrated cytomic and lipidomic analysis, demonstrates substantial increases in various types of lipids under NaCl stress. Additionally, the choice of the cultivation medium is a critical factor. In one study, inducing salt stress by adding salt to autoclaved municipal wastewater increased total lipid accumulation in *Parachlorella kessleri* by 31 % compared to the BBM medium [207]. This highlights the importance of considering the cultivation environment when identifying target genes for metabolic engineering. Researchers also investigate how salinity, either alone or combined with other environmental stressors, affects the achievement of high lipid productivity. These findings advance our understanding of how salinity influences lipid production in microalgae and its potential applications, particularly in sustainable biofuel production.

5.5. Light intensity

The reliance of microalgae on light as their primary energy source in photoautotrophic cultivation underscores the pivotal role of light availability. Various methods for managing light significantly influence both the biomass and lipid accumulation of these microorganisms. The process of photosynthesis in microalgae is a remarkable engine that generates substantial energy in the form of ATP and NADPH. This energy is then harnessed to convert atmospheric CO_2 into glyceraldehyde-3-phosphate (G3P), a critical precursor for synthesizing starch and TAG. However, when microalgae face environmental stressors, such as changes in light intensity, a competition ensues between starch and TAG synthesis. Neutral lipids, serving as crucial energy reserves for microalgae, play a pivotal role in enabling these organisms to continuously harness more light for their sustenance. High light intensities have consistently been associated with significant lipid accumulation, as demonstrated in various studies. For instance, elevating the intensity of light exposure in the cultivation of *Desmodesmus* sp. and *S. obliquus* led to a simultaneous increase in the synthesis of biomass and fatty acids [208]. Similarly, when *C. vulgaris* was grown in greenhouse facilities using red and white LED lamps with high light intensity, the result was a heightened growth rate and augmented total lipid content [209].

Another investigation delved into the influence of photoperiods on the lipid accumulation of *Verrucodesmus verrucosus*, revealing that a 12-h light and 12-h dark photoperiod under high light intensity (2000 Lx) yielded over 50 % lipid accumulation [210].

In the realm of microalgae research, their remarkable ability to harness chemical energy from light through a diverse array of pigments, including chlorophylls, carotenoids, and phycobilins, remains a focal point. These pigments serve as adept photon absorbers, facilitating efficient energy capture. While green microalgae, or Chlorophyceae, are known for harboring chlorophylls a and b, it is noteworthy that the majority of cyanobacteria predominantly feature chlorophyll *a*, phycoerythrin, and phycocyanin underscoring the versatility of these pigments in adapting to varying light wavelengths and intensities. Within this intricate interplay of pigments and light, the pivotal influence of light's intensity and wavelength on microalgal biomass production comes to the forefront. For instance, exposing *Scenedesmus* sp. to the dual spectrum of red and blue light, results in a remarkable 50 % surge in cell propagation when contrasted with the effects of exposure to white light [211]. Meanwhile, *C. vulgaris* reveals its propensity for heightened cell growth when exposed to blue light, outstripping its performance under white, red, and green light [212]. This illumination-dependent growth dynamic unveils light intensity as a pivotal determinant, not only influencing cell proliferation but also governing the accumulation of essential carbohydrates and lipids, as well as the vital CO_2 fixation process. Diving deeper into the fine-tuning of light conditions, research discoveries provide insights into the optimal thresholds. Gradually increasing the light intensity from 140 to 540 μmol m^{-2} s^{-1} reveals a crucial turning point at 420 μmol m^{-2} s^{-1}, where the microalgae demonstrate their peak growth rate and CO_2 fixation efficiency [213]. Beyond this threshold, a subsequent decline is observed, shedding light on the delicate balance in optimizing growth conditions. In an era where sustainable practices and resource efficiency are paramount, the implications of these findings extend to practical applications. Another compelling revelation emerges from research, highlighting the advantages of continuous artificial lighting for microalgal cells. These cells exhibit heightened nutrient uptake compared to those subjected to a traditional alternating 12-h light and 12-h dark regimen under natural sunlight [214]. These intertwined facets underscore the intricate relationship between microalgae, their photonic environment, and the potential for optimizing their growth and biochemical processes, offering valuable avenues for further exploration and application in sustainable biotechnology and biomass production.

Variations in both light intensity and wavelengths profoundly affect lipid biosynthesis in microalgae, resulting in notable changes to their lipid profiles. For instance, *Tichocarpus crinitus* displayed an increase in membrane lipid production under reduced light intensity, while higher light intensities prompted a rise in the concentration of TAGs [215]. In response to elevated light intensity, microalgae appeared to enhance TAG production, potentially as a protective response. In a different study involving *Chlorella* sp. and *Monoraphidium* sp., optimal production of neutral lipids was achieved at a light intensity of 400 μmol photons m^{-2} s^{-1} [216]. This specific light intensity allocation favored carbon allocation for lipid biosynthesis while reducing carbohydrate content. Moreover, the wavelength of light emerged as a critical factor in lipid accumulation in microalgae. An investigation revealed that the biomass growth and lipid synthesis of *Chlorella* sp. responded differently as the culture was exposed to various wavelengths, including red, blue, yellow, and green fluorescent paint solutions [217]. Exposing growing cells to light within the blue range (450–475 nm) and red range (630–675 nm) led to increased absorption of light by chlorophyll pigments, consequently enhancing overall performance of both photosystems I and II [218]. These findings collectively underscore the intricate interplay of light conditions and wavelengths in influencing microalgal lipid production and composition.

In their pioneering research, scientists delved into the growth dynamics of *S. obliquus* under unceasing light conditions, uncovering an intriguing pattern [213]. They observed a striking surge in the growth rate at the onset of the experiment, intricately linked to variations in light intensity. As the luminance increased from 60 to 420 μmol m^{-2} s^{-1}, there was a remarkable 3-fold increase in biomass productivity. However, this progressive ascent had its limits. Beyond a threshold of 540 μmol m^{-2} s^{-1}, an abrupt decline manifested in this activity, characterizing a phenomenon widely recognized as photo-inhibition. In a seminal study by [215], an intriguing revelation surfaced regarding the intimate relationship between light intensity and the lipid composition of microalgae. The research illuminated that the delicate balance between storage and structural lipids within microalgae is highly responsive to fluctuations in light intensity. Notably, the study demonstrated that optimal light conditions fostered the accumulation of TAG, marking a pivotal discovery in the realm of microalgal research. More recent research endeavors have delved into the synergistic effects of light intensities in conjunction with various abiotic stresses, including temperature, salinity, and chemical-related factors. In a study by [219], an illuminating investigation explored the intricate interplay between temperature stress and *Scenedesmus* sp. employing a sophisticated multi-omics approach, their study uncovered a fascinating pattern. The highest biomass accumulation was witnessed post-cell division with the zenith of growth occurring around 25 °C. Their functional genome analysis unveiled the orchestration of genes associated with the lipid synthesis.

5.6. Temperature

Temperature exerts a critical influence on microalgae cultivation, and its pivotal role cannot be overstated. In regions characterized by temperate climates, the pronounced temperature fluctuations pose a particular challenge for outdoor cultivation facilities, with temperature extremes ranging from a low of 10 °C to a high of 45 °C. Optimal temperature conditions are key to unlocking the full potential of microalgae. Within this range, microalgae exhibit heightened photosynthetic and metabolic activities, primarily attributed to the increased enzymatic functions tied to the Calvin cycle. To quantify the relationship between temperature and microalgal growth, the Arrhenius equation offers precise insight, indicating that for every 10 °C increase, microalgae respond by doubling their growth rate and biomass accumulation until the optimal threshold is achieved.

However, the story does not end within the optimal temperature range. Beyond this point, the downside emerges as enzymatic activities start to dwindle due to protein denaturation, culminating in the inhibition of algal growth. This knowledge is integral to understanding the dynamics of microalgae in various cultivation scenarios. Indeed, several studies underscore the indispensable role of temperature in shaping the biomass and lipid accumulation of microalgae. For instance, consider *Chaetoceros* sp., a standout species that thrives in Thailand's tropical climate [220]. Its remarkable ability to flourish across a broad temperature spectrum, including in the scorching heat of up to 40 °C, makes it a compelling subject of study. In a parallel line of research, *Nannochloropsis* sp. exhibits peak performance in growth, biomass production, and lipid accumulation when temperatures hover in the range of 25 to 30 °C [221]. In previous research, temperature-induced alterations in the lipid profiles of various microalgae species have unveiled intriguing findings. Notably, when the temperature was decreased from 30 °C to 12 °C in the case of *D. salina*, a remarkable 20 % surge in unsaturated lipid concentration was observed [222]. This highlights the sensitivity of *D. salina* lipid metabolism to temperature variations, making it a promising avenue for further investigation. Furthermore, a separate study focusing on *N. oculata* revealed that a slight temperature adjustment from 20 °C to 25 °C resulted in a notable 14.92 % boost in the overall lipid content [223]. Such a relatively modest change in temperature yielding significant lipid gains underscores the potential for fine-tuning cultivation conditions to optimize lipid production in microalgae. In a study different algal groups like *Porphyridium*

purpureum, *Fistulifera* sp., and *C. vulgaris*, showed enhancement in lipids upto 20 °C in compared to their standard of 10 °C [224]. This collective observation suggests a common trend of enhanced lipid production at the 20 °C temperature range, underlining the influence of temperature as a universal factor in microalgae lipid biosynthesis. In a more recent study focusing on the cold-water marine diatom *Porosira glacialis*, a range of sub-control temperatures was examined. Surprisingly, the highest lipid content was recorded at an unusually low temperature of 2 °C, with a remarkable increase of 33.4 % [225]. This intriguing result challenges conventional temperature preferences for microalgae cultivation and prompts further exploration of extreme conditions for lipid optimization. Similarly, *Cylindrotheca closterium* exhibited an optimization in total lipid content when subjected to a lower temperature of 11 °C instead of the customary 20 °C control temperature [226]. The findings emphasize that temperature modulation can have profound effects on various microalgae strains, potentially offering new insights into tailoring lipid production for biotechnological applications. These cumulative discoveries underscore the significance of temperature management in lipid production by microalgae and open doors for sustainable lipid-based applications.

6. Advancement in metabolic engineering

The biochemical and molecular analyses of microalgae have illuminated the intricacies of TAGs and lipid metabolism. Metabolic engineering of microalgae holds immense potential for enhancing lipid production through precise manipulation of metabolic pathways (Fig. 5). However, the intricate character of lipid biosynthesis pathways and the restricted availability of molecular transformation tools have hindered the exploration of microalgal lipid engineering, posing challenges to its commercial application in sustainable biofuel production. These obstacles include low productivity unsuitable for commercial-scale demands, diminished oleaginous biomass, and variations in fatty acid composition that influence fuel properties. To address these challenges, it is imperative to identify and modify crucial metabolic nodes in target microalgal strains, emphasizing the optimization of precise metabolic engineering strategies. Advances in bioinformatics tools and the existence of fully sequenced genome databases have improved our understanding of genetic enhancements in microalgae. However, it is noteworthy that the genetic toolkit can vary among microalgal species. Various genetic engineering approaches can be employed to enhance microalgal lipid accumulation, necessitating a thorough comprehension of lipid metabolic pathways, the identification of crucial lipogenic genes, the establishment of a reliable genetic transformation toolkit, and the meticulous selection of suitable target species for algal metabolic engineering.

Accessible algal genomes, including those of *C. reinhardtii* and various other species, provide invaluable support for ongoing research efforts. Specifically, *C. reinhardtii* stands out as a premier model for lipid research, benefitting from extensive study. In-silico approaches have been employed to construct the complete acyl-glycerol pathway of *C. reinhardtii* that provide a broad overview of lipid biosynthesis. These advancements in genomic knowledge and metabolic insights facilitate more precise and efficient metabolic engineering of microalgae, enabling the amplification of lipid production and bringing us closer to sustainable biofuel solutions. Genomics has become a crucial tool,

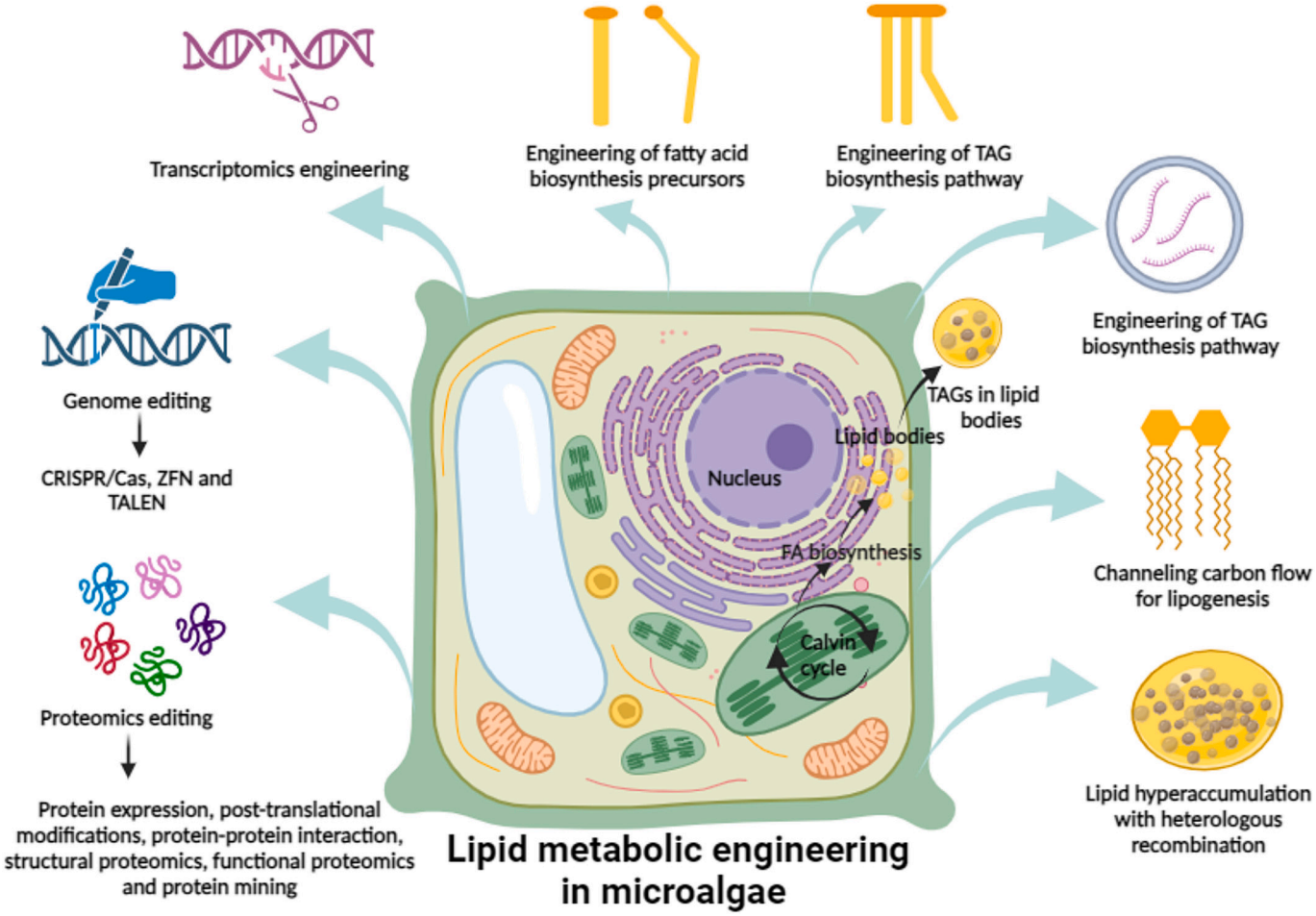

Fig. 5. Modifying lipid metabolic pathways in microalgae via molecular transformation techniques to boost lipid synthesis.

revealing insights into the exploration of adaptation and responses of microalgae under the different stress conditions. This investigation has not only validated the involvement of both shared and distinct metabolic pathways in critical physiological processes and generation of value-added products. Fayyaz et al. [90] has also paved the way for practical applications. Studies have revealed that the manipulation of various genes associated with lipid synthesis can significantly boost lipid production in microalgae. A notable example comes from [227], who successfully performed gene knock-out in the oleaginous model *N. oceanica* IMET1 using CRISPR technology. This breakthrough showcases the potential for precise genome editing in microalgae, opening new doors for genetic improvements. Moreover, the utility of CRISPR/Cas9 technology has extended to other microalgal species, including the green algae *C. reinhardtii,* and *Scenedesmus* sp. [90,228]. This demonstrates the growing interest in harnessing genetic tools to unlock the full potential of microalgae in various applications.

6.1. Engineering of fatty acid biosynthesis precursors

The initiation of lipid biosynthesis hinges on the availability of essential metabolic precursors, particularly acetyl-CoA and malonyl-CoA (Fig. 6). These compounds are fundamental building blocks for fatty acids, which are synthesized through a sequence of enzymatic reactions. A pivotal and rate-limiting step within this process involves the carboxylation of acetyl-CoA, a reaction catalyzed by the enzyme acetyl-CoA carboxylase (ACCase). This yields malonyl-CoA, which holds a crucial role as the primary carbon donor for elongating acyl chains. In essence, the conversion of acetyl-CoA to malonyl-CoA represents a critical juncture in the journey of fatty acid biosynthesis. Following this conversion, malonyl-CoA undergoes a significant transformation into malonyl-acyl carrier protein (ACP) facilitated by the enzyme malonyl CoA-acyl carrier protein transacylase (MCAT). This conversion is particularly noteworthy because malonyl-ACP becomes a pivotal substrate for the enzyme fatty acid synthase (FAS), which is responsible for extending acyl chains during the synthesis of fatty acids.

Researchers seeking to enhance the production of fatty acids have identified two key enzymatic targets: ACCase and MCAT. When ACCase is overexpressed, it has been observed to increase the fatty acid content by approximately 11.3 % in engineered strains of the microalga *Schizochytrium* [230]. Furthermore, when ACCase was overexpressed in the *Scenedesmus quadricauda*, which results in the 1.6-fold enhancement in the fatty acid content [231]. Intriguingly, in different scenarios, glucose has been discovered to have a role in stimulating ACCase gene in *Chlorella zofingiensis*, which leads to fatty acids accumulation, particularly oleic acid [232]. Additionally, there are intriguing suggestions that phytohormones hold potential for enhancing both biomass and lipid productivity in microalgae, notably in *C. sorokiniana*, through the elevation of intracellular levels of ACCase [233]. Taking the research, a step further, researchers working with *C. reinhardtii* have cloned and overexpressed an endogenous ACCase gene, which results in 1.16-fold enhancement in the fatty acid [234]. Further in a study, overexpression of MCAT in oleaginous microalga *N. oceanica* results in a substantial 31 % enhancement in lipid content [235]. Furthermore, the introduction of a heterologous form of MCAT from *Schizochytrium* into engineered yeast strains led to an impressive 62 % increase in the accumulation of fatty acids [236]. In *H. pluvialis*, elevated temperatures triggered an 8.7-fold enhancement in MCAT gene expression, resulting in a remarkable 24 % boost in overall fatty acid accumulation [237]. Importantly, these overexpression strategies did not hinder the growth rates of the transformed microalgae. Collectively, these instances underscore the central role of ACCase and MCAT in metabolic engineering, with the potential to significantly elevated lipid production in various microalgae species, each holding unique promise for biofuel industry.

6.2. Engineering the TAG biosynthetic pathway

TAGs are integral in the realm of energy storage due to their highly dense acyl-molecular composition, and they hold the promise of being a valuable resource for potential biofuel production. The intricate process of TAGs biosynthesis is chiefly orchestrated within the endoplasmic reticulum and involves a well-coordinated series of steps, all aimed at producing TAGs efficiently. This process is orchestrated with the indispensable involvement of three crucial enzymes: glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase

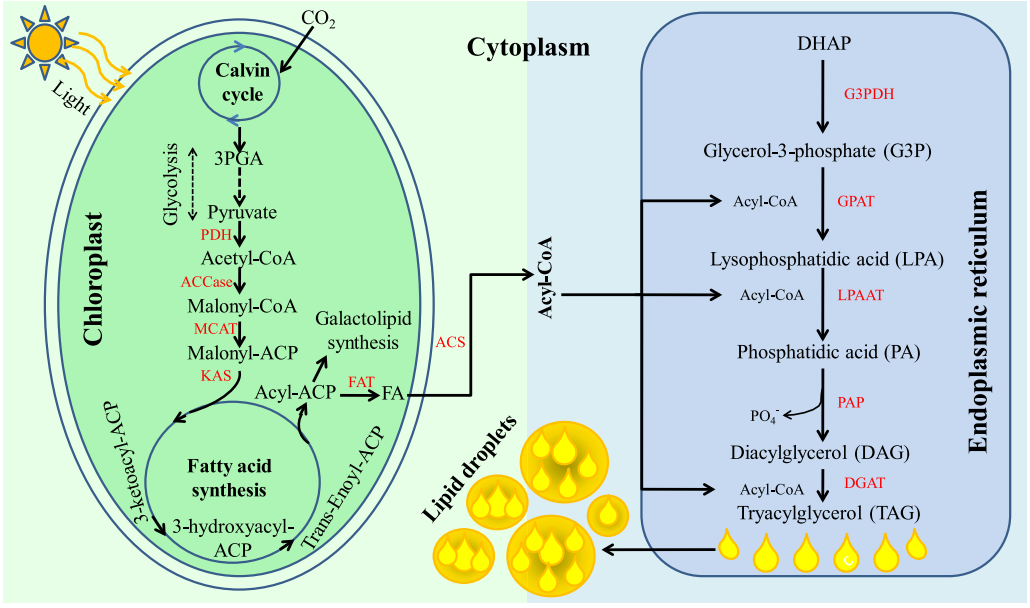

Fig. 6. Schematic representation of the metabolic pathways associated with lipid synthesis in microalgae. 3-phosphoglycerate (3PGA), pyruvate dehydrogenase complex (PDH), acetyl-CoA carboxylase (ACCase), malonyl-CoA: acyl carrier protein transacylase (MCAT), 3-ketoacyl-ACP synthase (KAS), fatty acyl-ACP thioesterase (FAT), fatty acid (FA), Acyl CoA synthetase (ACS), dihydroxyacetone phosphate (DHAP), gycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP), diacylglycerol acyltransferase (DGAT). This figure modified from Zhu et al. [229].

(LPAAT/AGPAT), and diacylglycerol acyltransferase (DGAT). The journey towards TAGs formation commences with the synthesis of fatty acids, a step initially executed by ACCase, MCAT, and FAS. Following this, the esterification of the fatty acids with glycerol-3-phosphate occurs at the *sn*-1 position catalyzed by GPAT. As a result, lysophosphatidic acid (LPA) is formed, which subsequently undergoes a transformative process into phosphatidic acid (PA) under the guidance of LPAAT. The journey of PA takes a crucial turn as it undergoes dephosphorylation by phosphatidic acid phosphatase (PAP), leading to the generation of diacylglycerol (DAG). This DAG is a vital precursor for the production of TAG. The conversion of DAG into TAG, a pivotal step in the process, is achieved through the transfer of an acyl group from acyl-CoA, facilitated by the DGAT enzyme. What further accentuates the significance of this pathway is the recognition of DGAT as the primary rate-limiting enzyme in TAG biosynthesis. This realization has spurred considerable interest among researchers who are eager to engineer the TAG metabolic pathway to bolster TAG content. By honing in on and optimizing the function of DGAT, it becomes feasible to increase TAG production. TAGs are not only instrumental in energy storage but also hold immense promise in the biofuel production sector. Consequently, this form of engineering has the potential to significantly impact various industries that rely on TAGs and biofuels, thus making it a topic of substantial scientific interest and exploration.

The successful genetic engineering of TAG pathways has shown remarkable potential in enhancing lipid production and customizing fatty acid profiles in microorganisms. Overexpressing GPAT in engineered *P. tricornutum*, results in 2 fold enhancement in lipid content, highlighting the role of this enzyme in lipid accumulation [238]. Similarly, in study, overexpression of AGPAT led to a 1.8-fold enhancement in the in *Tetradesmus obliquus* TAG content [239]. These studies showed the feasibility of targeting specific enzymes to boost lipid production and modify the composition of lipids, essential for various applications, including biofuel production. Recent research has unveiled an intriguing facet of TAG biosynthesis in microalgae, revealing the existence of a chloroplast-localized TAG biosynthetic pathway alongside the conventional ER-localized TAG pathway [240]. These enzymes were found to accelerate the accumulation of lipid droplets in the *C. reinhardtii* chloroplasts and cytosol. Additionally, the excessive expression of chloroplast-targeted AGPAT1 led to an overproduction of TAG, particularly with a rise in C16:0 at the *sn*-2 position accompanied by the buildup of plastoglobuli and cytosolic lipid droplets in *P. tricornutum* [241]. These findings emphasize the importance of chloroplast-localized acyltransferases in TAG biogenesis, offering exciting possibilities for the overproduction of TAGs with engineered fatty acid profiles. This development is particularly appealing for generating algal strains tailored to meet the requirements of various industries. Turning our attention to the overexpression of type 2 DGAT, the results are equally promising. Overexpressing type 2 DGAT in engineered *N. oceanica* led to a substantial 69 % increment in lipid content, highlighting the potential of this enzyme in enhancing lipid production [242]. Similarly, the overexpression of type 2 DGAT had a significant impact, led to a 35 % rise in neutral lipid content and an augmentation in the quantity of oil bodies in engineered *P. tricornutum* [243]. These outcomes indicate that type 2 DGAT is a valuable target for improving lipid production in microalgae. Zhang et al. [244] identified PtDGAT1 as a highly expressed transcript under N-deprivation among its isoforms in *P. tricornutum*. The localization of PtDGAT1 in the chloroplast, combined with its overexpression, resulted in a 2.3-fold increase in lipid content in transgenic cells compared to the wild type, with minimal impact on the growth rate. In *N. oceanica*, both DGAT1A and DGAT1B types are found, but it is noteworthy that when DGAT1A is overexpressed, it demonstrates acyltransferase function and substantially boosts TAG production by about 2.4 times. This enhancement was accompanied by a specific enrichment of C16:0 and C18:1 at the *sn*-1/*sn*-3 positions and C18:1 at the *sn*-2 position [245]. Intriguingly, overexpression of DGAT2A, 2C, and 2D revealed heterogenous substrate specificity of the mentioned enzymes during TAG biosynthesis. These comprehensive findings provide valuable insights into potential molecular candidates for enhancing TAG production with engineered fatty acid profiles, ultimately expanding the versatility of the end product [246].

To further advance these genetic engineering strategies, a deeper understanding of the key biochemical circuits and their regulatory nodes is essential. The application of multi-omic analysis can provide comprehensive insights into the target metabolic pathway, enabling scientists to identify key metabolic targets for TAG enhancement on a genome-wide scale [247]. The intricacies involved in redirecting carbon precursors among subcellular organelles, coupled with the presence of TAG pathways not only in the endoplasmic reticulum but also in plastids, emphasize the necessity for a more comprehensive comprehension of TAG pathways and the influence exerted by subcellular organelles in regulating them. This approach has the potential to significantly enhance lipid production and tailor the fatty acid composition of lipids, with implications for various applications, including biofuels and industrial use.

6.3. Redirecting carbon flow for lipogenesis

The intricate interplay between carbohydrates and lipids in their competition for shared metabolic carbon precursors and reducing power underpins a widely adopted strategy for augmenting the production of desired biochemical products. Central to this strategy is the strategic modulation of key enzymes within competing metabolic pathways, resulting in the redirection of carbon flux towards the intended end products. A noteworthy illustration of this approach is the complex relationship between starch and lipid biosynthesis pathways, which share a common precursor known as G3P [248]. By effectively curtailing the competitive starch photosynthetic pathway and rerouting carbon flux towards lipid biosynthesis, this methodology has demonstrated remarkable efficacy in elevating lipid production. To exemplify the impact of this strategy, we can turn our attention to a fascinating case study involving a starchless mutant of *Chlamydomona* sp. When exposed to circumstances characterized by high light intensity and nitrogen deprivation, this mutant displayed an astonishing 10-fold increase in the intracellular accumulation of TAGs compared to its wild-type [249]. In a separate study conducted by [250] insights were gained by investigating a starchless mutant of *S. obliquus*. Under their scrutiny, this mutant exhibited a substantial 41 % boost in total fatty acid productivity than wild type. Most impressively, when subjected to nitrogen-deficient conditions, this mutant achieved a TAG yield that reached an astounding 49.4 % of the dry cell weight. Additionally, another captivating dimension of this strategy comes to the fore through the development of a *C. reinhardtii* strain with strategically down-regulated phosphoenolpyruvate carboxylase (PEPC). This modification led to a significant transformation, as the modified strain demonstrated a remarkable 74.4 % increase in lipid content as compared to wild-type cells [251]. These illuminating case studies underscore the remarkable effectiveness of strategies designed to mitigate the competition between carbohydrate and lipid biosynthesis pathways. Through the fine-tuning of these interconnected pathways, we unlock the potential to significantly amplify the production of lipids.

In the pursuit of enhanced lipid accumulation, an effective metabolic engineering strategy involves the strategic inhibition of competing lipid catabolism pathways. As an illustration, [252] created genetically modified species of *Thalassiosira pseudonana*, with their specific objective being the multifunctional enzyme that includes lipase, phospholipase, and lysophosphatidic acyltransferase. This intervention yielded remarkable results, with lipid yields reaching 3.3-fold higher levels during the exponential growth phase than those observed in wild-type cells. Impressively, under silicon deficiency stress conditions, the transgenic strains exhibited an even more astounding outcome, with a 4.1-fold increase in lipid yield. Taking an alternative approach, certain studies involved the selective silencing of crucial genes in the

carbohydrate biosynthesis pathway, effectively redirecting carbon precursors in mutants to amplify lipid production. For instance, [253] introduced the concept of targeted knockdown through RNA interference (RNAi), focusing specifically on phosphoenolpyruvate carboxykinase. This genetic manipulation had a pronounced effect on carbon flux, favoring lipogenesis in *P. tricornutum* mutants. Notably, these mutants displayed lower photosynthetic efficiency but exhibited increased lipid production. Further demonstrating the versatility of this strategy, the inhibition of the gene encoding UDP-glucose pyrophosphorylase, a crucial enzyme in *chrysolaminarin* biosynthesis, led to reduction in their content. Simultaneously, it correspondingly increased lipid content within *P. tricornutum* mutants, all without affecting the specific growth rate [254]. Similarly, the inhibition of pyruvate dehydrogenase kinase using antisense techniques led to a notable outcome: elevated lipid content, although accompanied by a marginally reduced growth rate in mutants of *P. tricornutum* [255]. In a different context, the RNAi-mediated knockdown of AMP degradation enzyme namely AMP deaminase, produced effective enhancements. This approach led to increased ATP and lipid content while simultaneously improving cellular growth rates in *C. reinhardtii* [256]. However, it is essential to note that while silencing specific metabolic pathways can lead to altered growth rates, even with substantial redistribution of carbon precursors and energy towards the designated pathway; these observations underscore the paramount importance of reducing competition from alternate pathways. This strategic approach allows for the redirection of carbon precursors and cofactors towards lipogenesis, ultimately resulting in heightened lipid accumulation.

6.4. The role of reducing pathway in metabolism

The role of NADPH as the primary source of reducing power is pivotal in the context of TAG biosynthesis, primarily due to the highly reduced nature of lipids. To exemplify the substantial demand for reducing power in lipid synthesis, consider the production of a single molecule of palmitoyl-CoA from acetyl-CoA necessitates an impressive 14 molecules of NADPH [257]. In-depth lipidomic analyses involving *Chlorella* sp. and *N. salina*, cultivated within a lab-scale open pond simulation, unveiled a significant increase in acetyl-CoA levels, coinciding with a decrease in total lipid concentration. Simultaneously, biochemical analyses exposed a reduction in the enzymatic activity of malic enzyme, a change closely associated with diminished lipid content. These observations collectively underscore the critical role of NADPH in the intricate process of lipogenesis [258]. An intriguing approach to address the NADPH requirement for enhanced lipid production involved the overexpression of cytosolic malic enzyme in engineered *P. tricornutum*. This genetic manipulation led to a substantial increase in NADPH content, translating into lipid accumulation of up to 58 % of the cell dry weight [259]. Furthermore, the heterologous expression of malic enzyme from *P. tricornutum* in engineered *C. pyrenoidosa* exhibited a remarkable 3.2-fold boost in lipid content [259]. While these experiments pointed to the significance of malic enzyme in providing lipogenic NADPH in algae, the quest for understanding the mechanism behind NADPH supply for lipogenesis continue [260]. Wasylenko et al. [261] performed an exhaustive ^{13}C-metabolic flux analysis to elucidate the intricate metabolic flux distributions during lipogenesis. Their study confirmed that the oxidative pentose phosphate pathway (PPP) predominantly functions as the provider of lipogenic NADPH in the context of *Yarrowia lipolytica*.

In the intricate process of lipolysis, lipases (LIP) sequentially break down of acyl chains from the glycerol backbone of TAGs, results in the free fatty acids and glycerol face a crucial decision point. They are either recycled to replenish membrane lipids or ushered into further metabolization through β-oxidation in peroxisomes, fulfilling demanding cellular energy requirements [262]. However, the paramount importance of these lipases, our understanding within the realm of microalgae remains constrained, with only a sparse handful of candidate genes in microalgal genomes associated with this enzyme class. For instance, the discovery of LIP4 in *C. reinhardtii*, identified as a potential TAG lipase, revealed a transcript decrease (by 98.5 % within 24 h) under the limited nitrogen condition. However, these transcripts gradually increased after supplementation of nitrogen. The disruption of LIP4 activity through artificial microRNA reveals delayed TAG degradation, resulting in a remarkable more than 6-fold increase in TAG content under nitrogen limitation [263]. The classification of lipases based on substrate preference galactolipases, phospholipases, TAG lipases, DAG lipases, and monoacylglycerol (MAG) lipases-rovides a roadmap for understanding their diverse roles. In *P. tricornutum*, the identification of the gene *TGL1*, encoding a TAG lipase situated on lipid droplets (LDs), unravels intriguing insights. The microRNA knockdown of this lipase in transgenic lines results in the 2-fold enhancement in the TAG accumulation in compared to the wild type [264]. Moving to *C. reinhardtii*, where eight putative TAG lipases exhibit reduced transcription rates during nitrogen depletion. Artificial microRNA silencing of this potent lipase leads to decelerated TAG degradation under nitrogen-replete conditions, with LIP1 suggested to be a DAG lipase boasting broad substrate specificity [265]. The narrative extends to Thaps3_264297, a multifaceted lipase discovered in the diatom *T. pseudonana*. With TAG lipase, phospholipase, and acyltransferase activities in its repertoire, targeted knockdown of Thaps3_264297 in *T. pseudonana* results in a 2- to 4-fold increase in lipid yields under silicon starvation [251]. The *Chlamydomonas* genome harbors an ortholog of Thaps3_264297 [266]. In a remarkable demonstration of the power of genetic tools, CRISPR-Cas9 knockout mutants of *C. reinhardtii* lacking ELT1 (Cre01.g000300), a showcase an impressive accumulation of total lipids—up to 28.5 % more than the wild-type strain [267]. In a follow-up study employing the CRISPR-Cas9 tool, a precise disruption of the phospholipase A2 gene in *C. reinhardtii*, a pivotal enzyme governing lipid degradation, led to a noteworthy enhancement in lipid productivity, achieving an increase of up to 64.3 % compared to the original cell line [268]. This genetic manipulation demonstrates the pivotal role of phospholipase A2 in regulating lipid turnover and highlights its potential as a target for enhancing lipid production in microalgae. Plastid galactoglycerolipid degradation 1 (PGD1) plays a central role in membrane lipid turnover in *C. reinhardtii* by catalyzing the hydrolysis of acyl chains at *sn*-1 of the glycerol backbone of plastidial galactolipid MGDG. Mutants lacking PGD1 activity exhibited a 50 % reduction in TAG accumulation during nitrogen starvation, underscoring the significance of PGD1 in influencing lipid metabolism under stress conditions [265]. Within the intricate network of lipid metabolism, phospholipid: diacylglycerol acyltransferase (PDAT) emerges as a multifunctional player. In addition to transferring acyl moieties from phospholipids to DAG, PDAT harbors a conserved lipase motif (GXSXG), conferring lipase activity towards a broad range of lipids [269].

In a study strain *C. reinhardtii* showed 25 % less accumulation of TAG than the wild after silencing of pdat-1, which showed the role of PDAT in TAG biosynthesis [270]. Further investigations into the impact of overexpressing this gene on lipid accumulation will provide valuable insights into its potential as a target for manipulating lipid metabolism. The genomes of other microalgae, such as *N. oceanica* and *Chlorella* sp., also house TAG lipases [271,272]. This widespread presence of TAG lipases across diverse microalgal species underscores their evolutionary significance and suggests conserved roles in lipid metabolism. Comparative studies on these lipases could uncover shared regulatory mechanisms and offer universal strategies for enhancing lipid production. Patatin-like phospholipase domain-containing protein 3 (PNPLA3), a membrane-bound protein, has demonstrated its correlation with cytosolic phospholipase A2, inducing TAG accumulation in various organisms, including animals, plants, and microalgae. In *P. tricornutum*, the characterization and overexpression of the endogenous PNPLA3 gene resulted in significant alterations in the number and size of oil bodies, showcasing its potential for modulating lipid storage in microalgae [273]. The synthesis and expression of another PNPLA3 variant

with a site mutation in *P. tricornutum* further underscored the versatility of PNPLA3 in enhancing TAG accumulation without compromising growth and photosynthetic rates [274]. These findings highlight PNPLA3 as a promising target for genetic manipulation to boost lipid productivity in microalgae. The integration of genetic and biochemical insights from these studies provides a comprehensive framework for future strategies aimed at developing high-yield lipid-producing microalgae.

6.5. Lipid hyperaccumulation with heterologous recombineering

The challenge of slower growth rates in certain model oleaginous species, compared to their faster-growing algal counterparts, has posed a significant hurdle in the commercialization of the former [275]. To overcome this limitation, there has been a growing interest in transferring the lipogenic traits from model to fast-growing algal species that can thrive in minimal cultivation conditions. Heterologous recombineering, a promising approach, facilitates the engineering of key lipogenic genes from model oleaginous systems into the target species. Importantly, the lack of molecular data, such as complete genome sequences, does not pose a significant obstacle when studying non-model fast-growing microalgal species. This is because a comprehensive molecular toolkit and fully sequenced genomes are available for oleaginous systems [259]. It is essential to consider codon bias, which refers to an organism's preference for specific codons over others, as it plays a critical role in regulating protein expression levels and modifications within heterologous systems. Codon bias stems from sequence discrepancies within the open reading frame of a gene across diverse organisms [259]. Therefore, the utilization of a heterologous recombineering strategy emerges as a valuable tool for overcoming potential obstacles in the development of industry-suited lipogenic strains. The possible influence of heterologous recombineering in imparting desired traits to target organisms serves as a catalyst for the advancement of a microalgal toolkit and the discovery of viable target organisms. This ultimately paves the way for the successful implementation of heterologous recombineering approaches.

6.6. Transcriptomics engineering

Transcriptomics, as a pivotal field in molecular biology, plays a fundamental role in elucidating the intricate details of cellular processes. It distinguishes itself by its ability to reveal the types, structures, and functions of transcripts generated by cells under specific conditions, providing in-depth insights into their functionality. In the context of microalgae research, the application of transcriptomics emerges as a potent and indispensable tool. It serves as the guiding compass for researchers aiming to precisely pinpoint, detect, and enhance our understanding of the specific metabolic pathways and biological processes that govern these remarkable microorganisms. These insights are not only critical for basic scientific understanding but also hold substantial promise in advancing applications ranging from biofuel production to bioremediation. As such, transcriptomics is a cornerstone of modern microalgae research, paving the way for innovative breakthroughs with profound implications. Traditional metabolic engineering strategies have shown variable success when targeting specific genes in distinct metabolic pathways. This is due to the intricate nature of lipid metabolism, which requires coordinated regulation across multiple interconnected networks. Transcriptional engineering (TE) emerges as a promising alternative by introducing transcription factors (TFs) capable of orchestrating various key metabolic processes. For example, overexpressing a basic helix-loop-helix (bHLH) reported enhanced accumulation of lipid in engineered *N. salina* [276].

Bai et al. [277] used computational predictions to identify transcription factors (TFs) and their binding sites, revealing a crucial regulatory hub in the lipid metabolic pathway of *Nannochloropsis oceanica*. These results help in the identification of TFs responsible for lipid regulation and their potential roles in *Nannochloropsis*. In a more recent development, overexpressed a bZIP TF, resulting in the transcriptional regulation of various key lipogenic genes, ultimately enhancing lipid production in engineered *N. salina* [278]. Nonetheless, despite mounting evidence of TFs enhancing lipogenesis through the regulation of essential genes, the precise identification of target genes and binding regions of these specific TFs remains a challenge. To tackle this challenge, [279] aimed to discover transcriptional regulators of the lipid biosynthetic pathway in *C. reinhardtii* using a combination of chromatin signature analysis and transcriptomics. This approach revealed important molecular candidates controlling lipogenesis. Specifically, it identified the transcription factor PSR1 as the first TF to be upregulated under N-starvation conditions. Overexpression of PSR1 resulted in larger cells with increased lipid content, though cell density was slightly lower compared to wild-type cells. In the pursuit of understanding lipid over accumulation in the engineered microalgae *Tisochrysis lutea* species, a comparative transcriptome analysis was conducted. This enlightening study successfully pinpointed eight genes responsible for driving this lipid overaccumulation [280]. The identification of these genes resulted from an integrated analysis that considered various factors, including positional polymorphism, differential expression levels, selection signatures, and a comprehensive exploration of putative gene functions. In a separate research endeavor, an innovative approach emerged through transcriptomics analysis, highlighting the substantial enhancement of TAGs production achieved by imposing nitrogen stress on *Neochloris oleoabundans* [281]. Beyond unveiling this effective method for boosting TAG production, the study went a step further by offering a comprehensive array of genetic engineering targets and strategies. These targets and strategies are dedicated to amplifying the production rate and augmenting the cellular content of biofuel raw material. The strain exhibited robust biomass growth when exposed to 5 % CO_2 conditions, attributed to the absence of carbonic anhydrase. An extensive transcriptomics analysis unveiled the rationale behind this heightened CO_2 tolerance, pointing to the inactivation of the carbon concentration machinery as the key factor. This distinctive characteristic carries the potential for significantly increasing the production of oil-based fuels [282].

In a notable progress, [283] examined genes that were downregulated during nitrogen starvation, identifying transcriptional regulators showing decreased expression levels under nitrogen deprivation. Using a CRISPR–Cas9 reverse-genetics approach, they successfully knocked out Zn(II)2Cys6 in *N. gaditana*. This genetic modification led to a remarkable 55 % increase in lipid accumulation in the mutants compared to the 20 % observed in wild-type cells, achieved by redirecting carbon precursors towards lipogenesis. Another notable breakthrough came from [284], who identified and overexpressed a basic leucine zipper 1 (bZIP1) TF in *N. oceanica*. This intervention led to a concurrent rise and extracellular release of lipid droplets without adversely affecting cell growth rates. Chromatin immunoprecipitation-quantitative PCR analysis uncovered bZIP1 capacity to both elevate and lower the expression of various crucial genes linked to lipid and carbohydrate biosynthesis. Moreover, the decreased expression of the UDP-glucose dehydrogenase (UGDH) gene induced alterations in cell wall composition, diverting carbon metabolic precursors towards lipid synthesis. This facilitated the excretion of surplus lipid droplets through the compromised cell wall in *N. oceanica* cells overexpressing bZIP1 [284]. In conclusion, these studies underscore the imperative need to gain a comprehensive understanding of the mechanistic roles of transcription factors and their target genes for effectively engineering complex biochemical pathways like lipogenesis, which are subject to regulation by multiple factors.

6.7. Genome editing in microalgae

Traditional genetic engineering methods have long grappled with notable limitations. These issues encompass the unpredictable

integration of target genes into the host genome, imprecise recombination, and the use of antibiotic selectable marker genes. The latter concern is particularly significant due to the potential risks associated with the release of genetically modified strains into the environment. In response to these challenges, there has been a surging demand for more precise genome engineering techniques. The emergence of sequence-specific nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the groundbreaking clustered, regularly interspaced, short palindromic repeats (CRISPR)/CAS (CRISPR-associated) system has fascinated scientists, offering powerful tools for modifying plant genomes. A plethora of studies have artfully illustrated the transformative potential of these genome engineering tools when it comes to augmenting plant traits, revolutionizing agriculture, and biotechnology. However, amidst this surge of progress, the application of these genome engineering tools in the context of microalgae has somewhat lagged. This gap in research is noteworthy, given the immense potential that harnessing microalgae holds for numerous applications, from biofuels to pharmaceuticals. Nonetheless, a select few pioneering studies have ventured into the realm of microalgal genome engineering. However, by optimizing these parameters, researchers have significantly improved the efficiency of both CRISPR/Cas9 and ZFNs for gene editing in this organism [285].

The incorporation of an engineered Cas9 with a mutated cleavage domain has paved the way for CRISPR interference (CRISPRi) technology, enabling the downregulation of phosphoenolpyruvate carboxylase (PEPC) expression and resulting in a significant increase in lipid production in *C. reinhardtii* [251]. This innovative approach extends to a system designed to concurrently express and modulate multiple genes using their respective single guide RNA (sgRNAs). CRISPR technology offers a simple and efficient way to modify genes in microalgae for increased lipid production, which is important for biodiesel. By using a modified CRISPR system (CRISPRi) with a non-cutting Cas9 protein (dCas9) and an inducible promoter, scientists can precisely control gene expression. This control allows for the fine-tuning of lipid production by adjusting the position of the targeted gene and the concentration of the inducer molecule. Overall, CRISPR technology provides a quick and straightforward method for enhancing lipid engineering in microalgae. Despite its rapid progress, ongoing improvements are necessary in gene editing technologies to address the existing challenges of low efficiency and off-target effects. ZFN and TALEN, synthetic enzymes created by combining an engineered DNA-binding domain with a DNA-cleavage domain (*FokI*) to target specific DNA sequences, have found success, albeit less frequently, in gene targeting in microalgae. While both methods have demonstrated efficacy, editing the genome with ZFN and TALEN requires more intricate programming compared to the CRISPR system. Additionally, a greater amount of whole genomic data is necessary to prevent off-target incidents by sgRNA in ZFN and TALEN approaches. It is noteworthy that CRISPR transformation offers higher efficiency in comparison, while TALEN editing may lead to mosaicism. An important distinction lies in the fact that CRISPR enables the simultaneous introduction of multiple genes in a single step, providing a significant advantage over ZFNs and TALENs, which only permit single mutations. One notable milestone comes from the work of [286], where the genome of microalgae was intricately reengineered using meganucleases and TALENs. Their target: the UDP-glucose pyrophosphorylase gene in *P. tricornutum*. The outcome was nothing short of extraordinary— a substantial surge in the accumulation of TAG, a prized commodity in the realms of biofuel and nutrition. This achievement underscores the remarkable potential of precise genome editing in microalgae. In another compelling example, TALENs were wielded to disrupt the urease gene in *P. tricornutum* [287]. The consequence was the intriguing accumulation of urea, arginine, and ornithine in the genetically modified strains. This discovery not only sheds light on the potential of microalgae in biotechnology but also accentuates the versatility of genome engineering in tailoring microalgal traits for diverse applications.

6.8. Proteomics in microalgae

Proteomics, a cornerstone of systems biology, plays a pivotal role in unraveling the intricate world of proteins and their dynamic expression patterns. It is an indispensable tool for gaining insights into how organisms respond to specific conditions, offering the potential to customize solutions that precisely cater to their needs. This approach becomes particularly invaluable when we dive deep into the proteomic landscapes within subcellular compartments, shedding light on how microalgal model organisms, like *Synechocystis* sp. and *C. reinhardtii*, adapt to environmental stresses [288,289]. One notable area of study revolves around the unicellular green microalga *C. reinhardtii*, where researchers have achieved remarkable progress in understanding the proteins responsible for the formation of oil bodies. In this context, diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine synthase (BTA1 Chlre4|70,062) emerges as a critical player, tasked with synthesizing diacylglyceryltrimethylhomo-Ser (DGTS) [290]. This lipid, primarily located in the extraplastidial membrane, is pivotal for the development of oil body membranes. Furthermore, study revealed a suite of lipid-related proteins, encompassing lipid-trafficking proteins, acyltransferases, lipases, and those involved in sterol/ergosterol metabolism. Among these, a microalgal protein, Chlre4|400,527 (GPAT9), shares structural similarities with mammalian glycerol-3-phosphate acyltransferase (MmGPAT3), a linchpin in the early stages of oil synthesis [291]. Overexpression of MmGPAT3 was found to significantly enhance oil content.

Expanding on these discoveries, researchers have put forth the hypothesis that Chlre4|415,418 functions as a LPAT1, a crucial enzyme catalyzing the synthesis of phosphatidic acid by acylating lysophosphatidic acid. The presence of LPAT (Chlre4|382,144, Chlre4|421,261, Chlre4|421,263, Chlre4|378,176; Chlre4|332,883) within oil bodies strongly suggests their role as key acyl exchange sites in microalgae [290,292]. In parallel studies, particularly focusing on diatoms like *T. pseudonana*, multifunctional lipase/phospholipase/acyltransferase enzymes have been identified [252]. This finding has sparked the idea that targeted downregulation of specific lipases involved in lipid catabolism may be a promising strategy to boost lipid accumulation. Intriguingly, this approach has minimal impacts on growth, making it a promising avenue for increasing lipid content. Additionally, extensive research has probed the influence of nitrogen availability on the accumulation of TAG in microalgae, leading to substantial changes in the proteome. Proteins central to fatty acid metabolism, including ACP, enoyl-acyl carrier protein reduction, trans-2 enoyl CoA reduction, malonyl-COA: ACP transacylase, lipid droplet surface proteins (LDSP), ACCase, and MAT (malonyl-COA acyl transferase), consistently emerge as upregulated players [293,294]. A striking example comes from a study on *P. tricornutum*, which underscores the pivotal role of malic enzyme in lipid accumulation [180]. Its overexpression resulted in a significant 2.5-fold increase in total lipid content, accounting for 57.8 % of the dry cell weight. In the pursuit of sustainable solutions, recent proteomic investigations of the microalga *N. gaditana* under industrial conditions have unveiled a remarkable transformation: the conversion of CO_2 waste from a coal plant into a valuable resource for microalgal biomass production. These products have undergone meticulous evaluation using proteomic tools, highlighting their vast potential for applications in agriculture, food production, and biomedicine. Furthermore, another study has delved into targeted microalgal proteome analysis under enhanced light conditions, harnessing advanced techniques for cell disruption and phase transfer surfactants to enable a detailed and quantitative examination of protein functions.

7. Advancement in software tools for biorefinery process simulation

Recent years have seen the emergence of various methodologies for simulating the microalgae biorefinery process, representing a significant

advancement in the field. For instance, [295] undertook an extensive analysis of the different stages within a microalgae biorefinery, utilizing advanced process simulation software like SuperPro Designer (Fig. 7). Their analysis covered a range of critical processes, including chemical flocculation, filter press operations, lipid hydrolysis, and pigment extraction using a solvent. Furthermore, they explored the conversion of lipids into biodiesel, indicating the depth of their research and its practical implications. To enhance the efficiency of the planned biorefinery structure, they integrated the energy principle and employed software like Aspen Energy Analyzer. This strategic approach allowed them not only to refine the design but also to minimize energy expenses, ultimately resulting in an improved and sustainable biorefinery framework. This research exemplifies the cutting-edge developments in the field and sets a valuable precedent for future endeavors in sustainable biorefinery design.

Aspen Plus, a powerful tool for conducting comprehensive evaluations of mass and energy balances, has played a pivotal role in influential algal biorefinery studies. The study by [296], stands as a prime example, where they harnessed Aspen Plus to construct an intricate mathematical model. This model was purpose-built to conduct a thorough assessment of mass and energy balances, ascertain equipment sizes, and calculate service consumption. Moreover, their work extended to the economic aspect, taking into account the costs associated with each process unit and stage, including critical parameters like total capital investment, yearly production expenses and yearly production costs per unit of biomass generated. Similarly, [297] demonstrated the versatility of Aspen Plus by utilizing it to optimize a biodiesel production process from microalgae biomass. Their approach involved the integration of metaheuristic methods and the consideration of ten environmental variables, encompassing temperature and pressure parameters in bioreactors, as well as the configuration of distillation units, including the feed stage. Notably, their model pursued dual objectives: achieving maximum net annual income as an economic target and minimizing greenhouse gas emissions as an environmental goal. To enhance the capabilities of Aspen Plus, they further leveraged the free software Symyx Draw for creating compounds not found in the Aspen Plus database. These examples underscore the critical and indispensable role of Aspen Plus in advancing both economic and environmental optimization within the realm of complex industrial processes.

The process balancing and flow sheeting procedures are foundational elements in the design of biorefinery plants, relying on computer-assisted tools to perform steady-state mass and energy balancing, along with cost calculations. These processes are pivotal in the development of biorefinery systems, and a range of software applications have been employed in the field of bio-systems. As an illustrative example, [298] conducted an investigation to explore the potential for enhancing blending ratios and improving the quality of aviation biofuels produced from fluidized-bed catalytic cracking (FCC) of algae oil. To

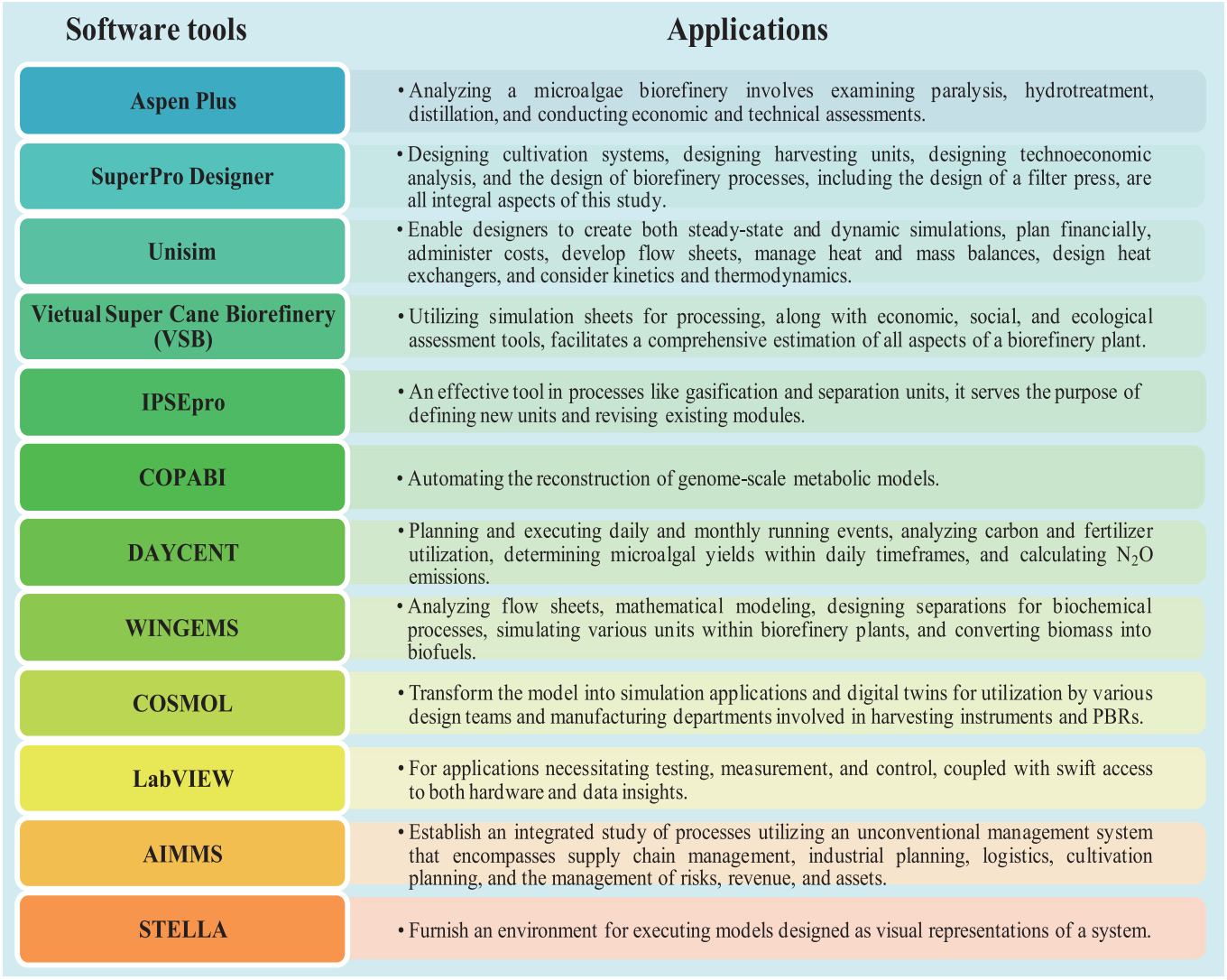

Fig. 7. The applications and utilization of general software tools in microalgae biorefinery processes.

assess the efficiency of this process, they utilized Aspen Plus for flow sheeting, mass balance, and heat balance calculations. Their study highlighted that the simulation software effectively encompassed all crucial conversion and separation processes, encompassing secondary elements like a steam reformer and furnace. They suggested that their developed simulation model could offer a foundational structure for future experimental pursuits, aiming to attain a remarkable energy efficiency of 95 % and an aviation biofuel yield of 41 %. These findings emphasize the significance of process optimization and flowchart development in advancing biorefinery technologies.

Streamlining the entire procedure with a unified simulation tool yields substantial time and cost savings [299]. Their comprehensive investigation involved not only process simulation but also a thorough techno-economic assessment. Their focus was on the extraction of a protein-rich essence comprising free amino acids and water-soluble peptides from microalgae. To accomplish this, they utilized SuperPro Designer v9.0 for simulation, encompassing all stages of the process, from cultivating and harvesting biomass to lipid extraction and the final step of spray drying. In addition, they integrated a water-based flash hydrolysis (FH) process, enabling the design of a pilot-scale continuous-flow reactor for protein extraction from microalgae discharge. Their simulation data facilitated the calculation of the minimum selling price for the protein, which was determined to be $4.31 per kilogram. Similarly, [300] contributed to the field by introducing the application of Computational Fluid Dynamics (CFD) simulation. This novel method integrated the laminar flow of air bubbles with microalgae mass transfer equations and boundary conditions. Initially, their simulations were designed to theoretically determine the best setup of a two-panel photobioreactor and adjust its operational parameters to establish an efficient dark-light cycle. In their culminating phase, they rigorously compared the outcomes of their experimental microalgae cultivation with the data derived from their simulated model. The results unequivocally validated the utility and precision of the proposed CFD simulation, highlighting its pivotal role in microalgae research and cultivation.

The [301] capitalized on the immense capabilities of SuperPro Designer® software, a versatile and indispensable tool for the design of pilot-scale systems. Their primary objective was the development of a pilot-scale CO_2 capture system that utilized microalgae for the production of high-quality biodiesel. Employing SuperPro Designer® v8.5, they meticulously executed the entire process flow sheet, commencing with the intake of vent air and traversing the various stages of biomass progression and extraction, culminating in the crucial step of bio-oil separation. This research endeavor drew extensively from practical data, enabling the team to craft intricate flow sheets, configure unit operations, and perform meticulous material balance calculations for individual process units. A significant aspect of their approach involved adopting a semi-batch process, which provided the foundation for determining the necessary quantities of feedstock, flow rates, and treatment durations. The employment of process simulation yielded valuable predictions, with estimates suggesting that the plant could effectively capture approximately 1400 kg of CO_2 on an annual basis, attaining a commendable yield of around 45 %. Concurrently, their innovative system was poised to generate an estimated quantity of roughly 200 kg of bio-oil per year. This research is emblematic of the pivotal role played by SuperPro Designer® in the development and comprehensive evaluation of pilot-scale systems that serve as sustainable and innovative solutions in the realm of biorefinery and environmental conservation.

The research of [302], undertaken using Aspen Hysys, a powerful tool for driving innovative design, finds a compelling illustration in their research. Their mission was the development of a groundbreaking microalgae biorefinery design, enriched by the inclusion of ionic liquid extraction, and they effectively harnessed the capabilities of Aspen Hysys V7.3®. However, their choice of an environmentally friendly ionic liquid, Butyl-3-methylimidazolium chloride, posed a distinctive challenge due to its departure from conventional compounds and its unavailability in the Aspen Hysys database. In response, the researchers ingeniously proposed a technique for calculating its thermodynamic and transport properties, relying on a thermodynamic model. Their extensive study encompassed the conception and simulation of a meticulous flowchart, which embraced crucial elements. These elements included a dedicated reactor for bio-oil extraction employing the ionic fluid, a sophisticated three-phase separator featuring recycled streams, and an array of heat exchanger devices aimed at energy recovery. Beyond design considerations, the research took a comprehensive approach, involving the rigorous evaluation of mass and energy balances. The insights gleaned from their work were instrumental in quantifying the volume of bio-oil extracted, contingent on the ratio between the ionic liquid and the mass of dry microalgae. Notably, their findings underscored that the efficiency of bio-oil recovery could be significantly enhanced through the strategic reduction of the operating temperature, highlighting the practical implications of their innovative biorefinery design.

The work of [303] clearly demonstrates the pivotal role played by MATLAB software in modeling microalgae cultivation. Their application of MATLAB resulted in the creation of a comprehensive mathematical model for microalgae cultivation. In the realm of microalgae cultivation, two primary systems are commonly utilized: open ponds and closed PBR, each offering unique advantages and presenting distinct drawbacks. Photobioreactors (PBRs) are available in diverse designs, encompassing bubble columns, airlift reactors, flat-plate reactors, horizontal or helical tubular reactors, and fermenter-type reactors. This study specifically honed in on the gas-liquid mass transfer rate within PBR systems, a parameter influenced by factors such as CO_2 concentration and flow rate. These variables wield a significant influence on biomass production and bubble behavior. Among the essential factors influencing the rate of mass transfer, the volumetric mass transfer coefficient (K_{La}) and gas holdup (εg) are of paramount importance. Notably, the alteration of physical attributes and operational conditions of the PBR, encompassing elements like sparger design and operational adjustments, can induce substantial modifications in K_{La} and εg, thereby directly impacting the mass transfer rate. The microalgae cultivation model designed for an open pool system exposes the interface between water and air to solar radiation. This modeling approach, grounded in equations, meticulously considers the supply of water, CO_2-enriched gas flow, and fertilizers to the open pool. Regulating the carbon consumption rate involves adjusting it according to the microalgae growth rate, which in turn reduces the flow of CO_2-rich gas to maintain CO_2 balance. In parallel, [304] harnessed a swift and dependable MATLAB code called DFBA lab to conduct dynamic flux balance analysis of bioprocess systems. This further underscores the versatile and invaluable nature of MATLAB in the realm of bioprocess modeling and analysis, highlighting its pivotal role in innovative bioprocess research.

7.1. Software for the cultivation step

Large-scale microalgae cultivation presents a multifaceted undertaking, accompanied by several notable challenges. One of the central challenges is the development and implementation of efficient growth systems and methods. These systems aim to support the growth of microalgae by harnessing solar energy and CO_2 to enhance productivity. Despite extensive research efforts in this field, achieving the creation of an entirely optimized bioreactor that can both maximize production and minimize costs remains an elusive goal [305]. To address these challenges, the current focus primarily revolves around the cultivation of microalgae in closed systems. These closed systems are designed to provide a controlled environment for microalgae growth. However, it is worth noting that many commercial operations still opt for open lake cultivation due to economic considerations, as it is a cost-effective alternative to closed systems [306]. PBRs play a pivotal role in this context, as they come in various configurations designed to optimize the

cultivation process. These arrangements are classified according to their form (tubular and flat), alignment concerning the ground (horizontal, vertical, angled, and spiral), mode of material mixing and motion (via pumps or aeration), single-phase or two-phase operation, and the construction materials employed (glass or plastic). The core principle that unites all PBRs is the reduction of their thickness, a design strategy aimed at maximizing the amount of light accessible to each individual microalgal cell [307]. The design of flat panel PBRs is significantly influenced by the imperative of achieving effective internal mixing. These systems require meticulous planning to establish an optimal mixing pattern within the reactor, ensuring a uniform distribution of nutrients and microalgae. A key design consideration for these PBRs is their deliberate emphasis on achieving a high surface-to-mass ratio (S/V) to enhance photosynthetic efficiency. Within closed systems, specific inputs such as algae, light, water, CO_2, nitrogen and phosphorus sources are essential. A critical facet of PBRs design is the careful selection of equipment for agitating and circulating the medium. The efficiency and economic viability of PBRs heavily depend on the selection of the agitator system, which determines the creation of a suitable mixing pattern. Efficient mixing serves several purposes, including preventing cell settling in the PBRs, ensuring even distribution of nutrients, facilitating oxygen removal generated during photosynthesis, regulating the supply and distribution of CO_2, and enhancing light availability within the PBRs. However, it's important to acknowledge that excessive agitation can result in cellular harm and, ultimately, cell demise [134]. Hence, choosing the mixing system and its intensity should be customized to match the distinct attributes of the microalgae employed. In recent times, endeavors have been focused on improving the photosynthetic efficacy of microalgae, especially in high-light scenarios like those experienced on sunny days. A pivotal strategy for achieving this objective involves increasing the light exposure at the surface of the reactor, a goal that can be achieved by vertically stacking the PBRs units [123]. This approach aligns with the overarching goal of optimizing the growth conditions for microalgae within these systems.

The multifaceted and promising nature of microalgae as a vital source of food, feedstock, and even fuel across various industries within the biorefinery framework underscores the essential need for a thorough comprehension of microbial systems and the implementation of effective quality control measures. In this context, [308] conducted a comprehensive exploration of diverse on-line and in-line monitoring methods for macromolecules, encompassing lipids, proteins, carbohydrates, and pigments. Their investigation also included progress in software applications, aiming to enhance microalgae production through process automation. This involved the utilization of multivariate process control (MVPC) and software sensors trained on comprehensive datasets. Their primary aim was to guarantee consistent product quality, while improving the automation and control of processes within the microalgal biorefinery. Concurrently, [309] the DayCent software was employed to assess biomass feedstock yield and greenhouse gas emissions throughout the cultivation of switchgrass and giant reed plants in the southeastern United States. This research holds substantial significance, considering that the selection of land for crop cultivation can significantly impact greenhouse gas emissions resulting from land-use conversion. Their findings indicated that states like Florida, Georgia, Mississippi, and South Carolina possessed the highest land accessibility, signifying the greatest potential for biofuel production. Furthermore, they extended the use of the DayCent software to assess the feasibility of cultivating switchgrass as a bio-ethanol crop for carbon sequestration in Europe. They explored two scenarios: exclusive cultivation in meadows covering 1.76 million hectares and cultivation in meadowlands, in addition to utilizing 5 % of currently cultivatable areas used for cereals, which collectively totaled 2.97 million hectares [310]. These research efforts collectively contribute to the advancement of sustainable practices and the utilization of microalgae in various sectors.

Leow et al. [311] introduced a dynamic biological cultivation model that combines thermochemical and biological unit processes using Monte Carlo simulation. Their study underscores the pressing need for the adoption of integrated modeling platforms to enhance the overall efficiency of microalgae biofuel systems. Moreover, it offers specific recommendations for aligning efforts in academia and industry, aimed at achieving cost-effective biofuel production from microalgae. In a similar vein, [312] employed the STELLA software to create models for tracking carbon stocks and flows in various systems, including dynamic models for brown seaweed growth. They presented a dynamic model designed specifically to analyze the carbon cycle and the movement of biogenic carbon within microalgae-based biorefinery systems. This model serves as a valuable tool for conducting comprehensive life cycle assessments (LCAs) of macroalgal biorefineries, enabling the quantification of the reduction in atmospheric CO_2 in terms of carbon. The primary aim of this model is to establish a reproducible framework that can be universally applied while accommodating variations in key parameters. Furthermore, they reported the use of software tools such as Pathway Tools and COPABI to create specialized databases encompassing genes, proteins, enzymes, and metabolites. These efforts collectively contribute to the advancement of sustainable practices and the utilization of microalgae in various sectors.

7.2. Software for the separation step

The economic viability and cost-effectiveness of producing biofuels from microalgae hinge on overcoming current challenges, with a major obstacle being the separation of microalgae from their culture medium. This separation process constitutes a substantial portion (~20–30 %) of the total production cost and presents a critical barrier to efficiently converting microalgal biomass into biofuels [313]. Cultivated microalgae typically contain significant amounts of water, necessitating the efficient removal of water from large biomass volumes. The methods for separation often involve a series of steps, including physical, chemical, and biological approaches, to achieve effective solid-liquid separation. Despite ongoing research, there is no universally accepted and cost-effective method for harvesting microalgae that is suitable for various algae species.

Microalgae cells have a negative charge and stick to organic matter, making them hard to separate from the solution. This, along with their light weight, makes harvesting them costly. Common methods to harvest microalgae include letting them settle, spinning them in a centrifuge, filtering, using ultrafiltration, or floating them to the surface. Sometimes, these methods are combined with flocculation, where particles clump together, or electrophoresis, which uses an electric field to separate them. The choice of method depends on the microalgae's properties like density and size, and the desired end products. Software tools can help improve these methods. For example, computational fluid dynamics (CFD) software, such as ANSYS Fluent or COMSOL Multiphysics, can be employed to model and optimize the design of microalgae harvesting equipment, including centrifuges, filtration systems, or flotation devices. These software tools allow researchers and engineers to simulate and analyze the flow dynamics, separation efficiency, and energy consumption of different harvesting processes, thereby improving the overall effectiveness of microalgae harvesting. Additionally, machine learning algorithms and image analysis software can automate the detection and harvesting of microalgae by analyzing images of cultures and identifying when they have reached the desired density or maturity for harvesting. This automation can reduce labor costs and increase harvesting efficiency.

7.3. Software for conversion process

The increasing need for sustainable and eco-friendly energy sourced from easily accessible raw materials has become a primary focus. As a result, a diverse range of techniques for extracting energy from biomass has been put forward. These methods can be generally classified into three primary groups: biochemical, chemical, and thermochemical

processes [314]. Within each of these categories, there is a range of specific techniques. When it comes to converting microalgae-sourced biomass, the methods include anaerobic decomposition, fermentation, and photobiological processes. These approaches primarily utilize microorganisms and enzymes to transform biomass into biofuels. Despite their environmentally friendly nature, these methods face challenges such as being time-consuming, having low conversion rates, and incurring high costs, which diminishes their attractiveness from an industrial standpoint. For instance, a chemical approach to biodiesel production involves the ester exchange or esterification of triglycerides in biomass using methanol. In contrast, thermochemical processes involve the thermal decomposition of organic matter in biomass to produce biofuels. These processes encompass gasification, pyrolysis, direct combustion, and melting, where biomass is exposed to heat generated by air, oxygen, or steam to obtain the desired products [315].

In industrial settings, the traditional approach to produce biofuels from microalgae involves extracting TAGs (triacylglycerols) from algal biomass, followed by various conversions like transesterification to produce biodiesel fuel. This process necessitates steps such as microalgae dewatering, drying, biomass paste dewatering, and the utilization of organic solvents for triglyceride extraction. These procedures are resource-intensive and expensive. However, an alternative method that eliminates the need for drying and organic solvents is the hydrothermal liquefaction approach. In this technique, microalgae with high moisture content are exposed to specific temperatures and pressure conditions, resulting in the breakdown of macromolecules within the microalgae into bio-oil and gases [316]. Furthermore, [317] employed. The researchers utilized Aspen Plus to model a biorefinery facility aimed at producing anhydrous ethanol by converting 1200 tons per day using a hybrid technology. Their simulation incorporated three conversion units: fermentation, gasification, and ethanol recovery. Their investigation unveiled the capability to generate approximately 36.5 million gallons of biofuel ethanol each year. Similarly, [318] utilized SuperPro Designer software to investigate the feasibility of converting sugarcane into bioethanol as an alternative feedstock within a biorefinery system. Their comprehensive simulation, employing SuperPro Designer® software, focused on the upstream components of fermentation to assess the effectiveness of bioethanol production. They conducted simulations at various efficiency levels with the goal of completely replacing oxygenates in gasoline distributed in Mexico. The findings revealed that ethanol production costs amounted to 1.34 and 1.46 USD per gallon, with the potential to produce 40.13 and 1380 million gallons per year using sugarcane bagasse.

Carmona-Garcia et al. [319] uses Aspen Plus softwere to simulate the transformation of Coffee Cut Stems (CCS) into Acetone-Butanol-Ethanol (ABE) for the computation of mass and energy balance. The process involved two key stages. Initially, all the sludge produced in the acid pretreatment unit was directed towards enzymatic saccharification. Subsequently, the liquid portion, enriched with pentoses and hexoses, served as a carbon source in the fermentation phase. This strategy aimed to mitigate the inhibitory effects on ABE fermentation by juxtaposing it with other stages, including the decontamination process. The simulation yielded an efficient production rate of 140 kg of ABE per ton of CCS. Moreover, it showcased the Minimum Processing Scale for Economic Feasibility (MPSEF) of 64.6 tons per hour of CCS. In a similar context, [320] leveraged the commercial CFD software ANSYS FLUENT® 16.0 to simulate a pilot-scale biomass conversion packed-bed bioreactor. Their primary objective was to address a significant challenge associated with Solid-State Fermentation (SSF), which holds promising potential for advancing biorefineries and bio-chemicals. SSF involves cultivating microorganisms on the surface of humidified solid substrates, with a continuous flow of gas and minimal visible water. SSF offers multiple advantages over the submerged method, such as higher product concentrations and the use of smaller bioreactors, which can translate into cost savings. Nevertheless, accurately calculating heat transfer remains a prominent challenge when applying this conversion method on a larger scale. The simulation effectively calculated heat transfer, achieving an average temperature difference of 0.07 °C between experimental and predicted values.

8. Microalgal productivity and cost of production

The productivity of microalgal biofuel is often assessed by two main parameters: biomass yield and lipid accumulation, both of which are influenced by several interrelated factors. Biomass productivity varies depending on the species of microalgae, growth conditions, and the cultivation system employed [122,321]. For instance, species like *C. vulgaris* and *Nannochloropsis* are particularly notable for their high lipid content, which can range from 20 % to 60 % of their dry weight under favorable conditions [322,323]. Furthermore, when optimized, some species can achieve biomass productivity levels of 20–50 g/m^2/day, making them promising candidates for biofuel production [324]. The choice of cultivation system plays a crucial role in determining productivity. Open pond systems, while cost-effective, typically produce lower biomass yields (5–15 g/m^2/day) due to contamination risks and environmental variability [325]. In contrast, closed PBRs offer a more controlled environment, enabling higher productivity rates of up to 50 g/m^2/day [326]. This improved performance in PBRs is largely attributed to better regulation of factors such as CO_2 availability, nutrient supply, and light intensity. Adequate provision of CO_2, nitrogen, and phosphorus is essential to sustain high growth rates, as deficiencies in these nutrients can significantly hinder productivity. On the other hand, inducing nutrient limitation or exposing cells to salinity stress can trigger lipid accumulation, although this often comes at the expense of biomass yield [11,327]. Another critical determinant of microalgal productivity is light management. Light intensity and photoperiod directly influence photosynthetic efficiency, which, in turn, affects both growth and lipid synthesis. To address the limitations of traditional lighting approaches, innovative systems such as LEDs and fiber optics are being explored to optimize light delivery in PBRs [122,321]. These advancements aim to enhance the efficiency of light utilization, thereby improving overall productivity and cost-effectiveness.

The cost of producing microalgal biofuels remains higher than fossil fuels and other biofuels, largely due to the energy-intensive nature of cultivation and downstream processes. Current estimates place the production cost of algal biofuel between $5 and $20 per gallon, significantly exceeding the price of conventional diesel or gasoline [328]. A major contributor to this high cost is cultivation, which accounts for roughly 30 %–50 % of total expenses. Among cultivation methods, open pond systems are more economical, costing around $1 per kg of biomass [329]. However, their efficiency is lower compared to closed PBRs, which, while more productive, can cost over $4 per kg due to the infrastructure and operational demands [330]. Additionally, the delivery of CO_2 and nutrients further adds to expenses, with CO_2 supply alone accounting for a significant portion of the costs. Innovative strategies such as using industrial flue gases or wastewater as nutrient sources show potential for cost reduction but are not yet widely adopted. Downstream processes also pose substantial challenges. The high water content in algal cultures makes harvesting costly, contributing an estimated 20 %–30 % to production expenses [186]. Common techniques such as centrifugation, flocculation, and membrane filtration, though effective, are energy-intensive. To address this, research is ongoing into low-energy alternatives like bio-flocculation and gravity-based settling. Lipid extraction, a key step in biofuel production, is another cost-intensive process. Conventional methods using solvents like hexane and advanced techniques such as supercritical CO_2 extraction require significant energy and resources. To mitigate these costs, biorefinery approaches are being explored, where multiple co-products, such as proteins and pigments, are extracted alongside biofuel to enhance economic viability. Finally, biofuel conversion processes, including hydrothermal liquefaction (HTL) and transesterification, are effective but energy-demanding, further inflating costs. Scaling up production from

laboratory to industrial levels adds another layer of complexity, requiring substantial capital investment in infrastructure, logistics, and energy management. Achieving economies of scale remains a challenge due to the intricate integration of diverse processes necessary for efficient production.

9. Availability of land to cultivate microalgae in large scale and how to fulfill the high demand of fuel

Microalgae cultivation requires substantial space, especially when considering the scale needed to produce biofuels; however, their unique characteristics provide several advantages for addressing land constraints. For instance, while land availability is limited by competing demands such as agriculture, urbanization, and conservation, microalgae demonstrate remarkable flexibility by thriving on non-arable lands, including deserts, saline soils, and marginal terrains unsuitable for conventional crops [331,332]. This adaptability extends to aquatic environments, where cultivation can occur in ponds, reservoirs, or even offshore systems, thereby diversifying potential locations for large-scale production. To further alleviate the need for prime agricultural land, existing infrastructure, such as abandoned agricultural lands, contaminated sites, or industrial zones, can be repurposed for microalgal cultivation [333]. Moreover, innovative approaches like closed PBRs systems or vertical cultivation setups not only optimize space utilization but also enhance productivity by maintaining controlled growth conditions. A key factor driving the appeal of microalgae is their ability to yield significantly more biomass and oil per unit area compared to terrestrial crops like soybeans or palm oil [334]. For example, while soybeans produce approximately 400 l of oil per hectare annually, some microalgal strains, under optimal conditions, can produce up to 100,000 l per hectare [335]. This remarkable productivity, combined with their ability to grow in diverse environments, makes microalgae particularly attractive for large-scale cultivation, especially in regions with abundant sunlight and water resources.

Meeting the global demand for fuel from microalgae will require a multi-pronged approach, incorporating technological advancements, resource optimization, and innovative integration methods. One effective strategy is co-locating microalgae farms with industrial or agricultural operations, which can reduce both costs and environmental impacts. For example, CO_2 emissions from power plants can be captured and used to enhance algal growth, while wastewater from industries or municipalities can serve as a nutrient source for microalgae, reducing the need for freshwater and fertilizers [336,321]. In addition to these integration methods, enhancing the lipid productivity and stress tolerance of microalgae through genetic engineering is critical for increasing yields. Advanced techniques like CRISPR-Cas9 and synthetic biology can facilitate the development of algal strains with optimized photosynthetic efficiency and tailored biochemical profiles suited for biofuel production [337,338]. Furthermore, deploying advanced cultivation systems, such as closed PBRs or hybrid systems, can improve space utilization, reduce contamination risks, and maintain consistent growth conditions. While PBRs can be costly initially, they offer better land efficiency by allowing for vertical stacking of units, which maximizes space and productivity. Government policies promoting renewable energy can further support the growth of algal biofuel infrastructure. Subsidies, tax credits, and funding for research and development are essential to scaling up production economically and making it competitive with traditional fuel sources. Moreover, international partnerships can play a key role in optimizing global production networks by pooling resources and sharing technologies [339]. Regions with abundant sunlight and marginal land, such as deserts in Africa and the Middle East, could serve as ideal hubs for microalgal cultivation, leveraging natural advantages to drive large-scale production. Together, these strategies form a comprehensive approach to meeting the increasing fuel demand through microalgal biofuels.

10. Necessary equipment converting microalgae to fuel

Converting microalgae into biofuel requires a series of specialized equipment and processes to extract the lipids (oils) and other useful components, followed by their conversion into usable fuel forms such as biodiesel, bioethanol, or biogas. This transformation involves several stages, including cultivation, harvesting, extraction, and conversion. The first step in microalgae biofuel production is cultivating the microalgae, with two primary cultivation systems being open ponds and closed PBRs. Large, shallow raceway ponds are commonly used for algae cultivation, where paddlewheels or airlift pumps are essential for ensuring circulation and mixing of the culture [122,321]. This prevents algal cells from settling while enhancing nutrient uptake. In contrast, closed systems, such as horizontal, vertical, or tubular PBRs, offer better control over growth conditions, utilizing transparent materials to maximize light penetration while minimizing contamination. Additionally, CO_2 injectors and temperature control systems are integrated into these systems to optimize growth. Once the microalgae reach optimal growth, harvesting is necessary to concentrate the biomass. This process involves separating the microalgae from the culture medium, using various methods. One common method is centrifugation, where high-speed centrifuges spin the algae culture to separate the microalgal biomass from the water. While centrifugation is effective, it can be energy-intensive. Alternatively, flocculation is used to add chemicals or adjust pH levels, causing the algae to clump together, which make it easier to separate the biomass through settling or filtration. For more energy-efficient harvesting, microfiltration or ultrafiltration membranes can be employed, which filter the algae from the culture medium more efficiently than centrifugation. By linking these stages, the process of cultivating, harvesting, and extracting the biofuels from microalgae is optimized, ensuring that the maximum amount of biomass is captured and ready for conversion into fuel.

The next step in the process is to extract the lipids from the harvested algae, which are then converted into biofuels. Common lipid extraction methods include using solvents such as hexane to dissolve the lipids from the algae [340]. Afterward, the solvent is evaporated, leaving behind the extracted oil. To ensure the extraction process is efficient and sustainable, solvent recovery systems are employed to recycle and reuse the solvents. Alternatively, a more environmentally friendly approach involves using mechanical presses, such as screw presses or expellers, to squeeze the oil from the algal biomass. Although this method is less efficient than solvent extraction, it is simpler and more sustainable. Once the lipids are extracted, they must be converted into usable biofuels. For biodiesel production, the extracted oil undergoes a process called transesterification, where it reacts with methanol or ethanol in the presence of a catalyst, usually sodium hydroxide or potassium hydroxide [341]. This reaction is carried out in batch reactors or continuous-flow reactors, depending on the scale of production. Alternatively, the lipids can be subjected to hydrothermal liquefaction (HTL), a method that involves heating the algae biomass under high pressure and temperature to break down the lipids into bio-oil, similar to petroleum [342]. The necessary equipment for HTL includes high-pressure reactors and heat exchangers to maintain the required conditions. For converting carbohydrates in algae to bioethanol, fermentation tanks are used where yeast or bacteria are added to convert the sugars into alcohol [343]. This process is crucial for producing ethanol from the carbohydrate content of the algae. After the conversion processes, biofuels often require further purification to remove impurities and enhance fuel quality. This is achieved through equipment such as distillation columns, filtering units, and vacuum dryers. Additionally, biofuels may undergo upgrading processes like hydrotreating, which improves their combustion properties, making them more suitable for use in engines and other fuel-dependent systems.

11. Impact of microalgae on environment and life cycle assessments (LCAs)

Microalgae are unicellular organisms that can grow in a variety of environments, including freshwater, saltwater, and even wastewater, using sunlight, CO_2, and nutrients like nitrogen and phosphorus [321,344]. Their high photosynthetic efficiency allows them to absorb large amounts of CO_2 from the atmosphere, making them a potential tool for carbon sequestration and mitigating climate change. Unlike terrestrial plants, microalgae do not require arable land or fresh water for cultivation, which reduces competition with food crops and alleviates pressure on land and water resources [333]. One of the most significant environmental benefits of microalgae is their ability to produce biofuels such as biodiesel, bioethanol, and biogas. These biofuels can serve as sustainable alternatives to petroleum-based fuels, leading to reduced greenhouse gas emissions and a lower carbon footprint. Microalgae also offer the potential to treat wastewater by removing nutrients and contaminants, thus contributing to the reduction of water pollution. However, the environmental impact of microalgal cultivation is not without challenges. Large-scale cultivation requires significant energy inputs, especially when it comes to harvesting and processing microalgal biomass [345]. In some cases, the use of fertilizers and other chemicals to boost growth can lead to nutrient pollution, which, if not properly managed, could offset the environmental benefits. Moreover, the land and water required for industrial-scale microalgal cultivation could also result in unintended ecological consequences, such as habitat disruption or water consumption issues in arid regions.

LCA is a comprehensive method for evaluating the environmental impacts of a product or process from cradle to grave, including raw material extraction, production, transportation, use, and disposal [346,347]. In the context of microalgae, LCA helps to assess their sustainability by considering factors such as energy consumption, water usage, carbon emissions, and resource depletion throughout their entire life cycle. LCA studies of microalgae-based biofuels have shown that the overall environmental impact is highly dependent on the cultivation method, energy sources, and downstream processing techniques used. For example, open pond systems are generally less energy-intensive than photobioreactors but may have a higher risk of contamination and lower biomass yields [346,348]. Similarly, the type of energy used to power the cultivation and processing systems plays a significant role in determining the carbon footprint of the final product. Biofuels produced using renewable energy sources have a much lower environmental impact compared to those produced with fossil fuels. Another critical factor in LCA is the use of nutrients. The source of nitrogen and phosphorus for microalgal growth is essential in determining the overall sustainability. If synthetic fertilizers are used, the environmental benefits may be diminished due to the energy-intensive production of these chemicals and the potential for nutrient runoff, which could lead to eutrophication in nearby water bodies [346].

12. Biohydrogen production from microalgae

Biohydrogen production from microalgae represents an innovative and sustainable approach to clean energy generation, leveraging the natural photosynthetic capabilities of these microorganisms. Among the various microalgae species such as *C. reinhardtii, Synechocystis* sp., and *Synechoccus* sp. are particularly promising due to their ability to produce hydrogen under specific conditions [344,349]. This potential stems from their capacity to generate biohydrogen through two primary pathways: biophotolysis and fermentation. In direct biophotolysis, water molecules are split into oxygen and protons using light energy during photosynthesis. The protons are then converted into hydrogen gas by hydrogenase enzymes. However, a major limitation of this process is the oxygen sensitivity of hydrogenase enzymes, which reduces their activity in the presence of the oxygen produced during photosynthesis [344]. To overcome this challenge, an alternative pathway, indirect biophotolysis, has been explored. In indirect biophotolysis, microalgae first fix carbon dioxide into organic compounds during photosynthesis. These compounds are subsequently metabolized under anaerobic conditions to produce hydrogen. Unlike direct biophotolysis, this method decouples hydrogen production from oxygen evolution, thus mitigating the inhibitory effects of oxygen on hydrogenase enzymes [350]. Another approach, fermentation, occurs in dark, anaerobic environments, where microalgae metabolize stored carbohydrates into hydrogen and organic acids. While this pathway is independent of light, its yields are generally lower compared to photolytic methods [351]. Several factors significantly influence the efficiency of biohydrogen production in microalgae. For instance, optimized light intensity and wavelengths, particularly blue and red light, are crucial for enhancing photosynthetic activity and hydrogenase expression [352]. Additionally, nutrient deprivation, such as limiting sulfur or nitrogen, can trigger physiological stress in microalgae. This stress inhibits oxygen evolution and activates the hydrogenase pathway, thereby increasing hydrogen yield. Maintaining strict anaerobic conditions is equally essential for sustaining hydrogenase activity, with strategies like sulfur deprivation or the addition of reducing agents helping to achieve this. Recent advancements in synthetic biology have further bolstered the potential of microalgae for biohydrogen production. Genetic modifications can improve hydrogenase stability, enhance photosynthetic efficiency, and redirect metabolic pathways towards hydrogen production [353]. These innovations address many of the limitations associated with natural systems and open new possibilities for scaling up production. By integrating these insights, the development of biohydrogen production from microalgae can be significantly optimized. The interplay of pathways such as biophotolysis and fermentation, along with external factors like light conditions, nutrient availability, and anaerobic environments, highlights the intricate balance required for efficient hydrogen production. Advances in genetic engineering and synthetic biology further underscore the transformative potential of this renewable energy source, paving the way for a sustainable future.

13. Feasibility study on the production of biofuel from microalgae, financial and non-financial aspects

The production of biofuel from micro algae has gained significant attention due to its potential to provide a sustainable and renewable energy source. A feasibility study involves evaluating both financial and non-financial factors to determine its viability (Table 3).

The feasibility of microalgal biofuel production hinges on balancing financial and non-financial factors. While high costs and technical challenges remain, the potential for environmental sustainability, coupled with revenue from co-products and government support, strengthens its prospects. Continued innovation and policy-driven incentives are essential for making microalgal biofuels a viable alternative to fossil fuels.

14. GE algal strains in progress of biosafety

The utilization of GE algal strains for commercial purposes introduces various challenges, as demonstrated by the potential of microalgal genetic engineering in closed laboratory environments. Despite this potential, the evaluation of GE strains in open cultivation systems remains largely unexplored. This knowledge gap not only hinders the widespread application of GE algal systems but also fuels an ongoing debate, particularly in the agricultural sector. In a study by [362], an analysis of critical factors related to the cultivation of GE algal strains was conducted, revealing a stronger conviction among people regarding the benefits of GE algal-derived fuels compared to other fuel sources. This underscores the importance of addressing public perceptions and preferences in the discourse surrounding GE algal applications. Furthermore, there was a consensus emerging from the survey results, emphasizing the necessity of implementing closed algal cultivation

Table 3
Feasibility study of microalgal biofuel production based on financial and non-financial aspects.

Parameters	Financial Aspects	Merits	Demerits	References
Production costs	Biofuel production from microalgae involves expenses for cultivation, harvesting, extraction, and processing. Key costs include photobioreactor construction, nutrients, CO_2 supplementation, and energy inputs. While high initial investment costs pose challenges, advancements in technology and large-scale operations can reduce costs.	Potential for high-value biofuel market.	High initial investment and production costs.	[354,355]
Economic returns	The economic viability depends on achieving competitive yields of biofuels like biodiesel, bioethanol, or biohydrogen. Co-products, such as pigments, proteins, and bioplastics, can enhance profitability by diversifying revenue streams.	Job creation and economic growth.	Uncertain profitability due to fluctuating oil prices.	[356]
Market factors	Factors like fluctuating fossil fuel prices, biofuel demand, and government subsidies for renewable energy impact economic feasibility. Policies incentivizing biofuels, such as tax credits or grants, can improve cost-competitiveness.	Opportunities for government subsidies and incentives.	High operational and maintenance expenses.	[357,358]
Infrastructure requirements	Establishing efficient harvesting and refining systems, such as centrifugation and hydrothermal liquefaction, requires additional investment. However, integrating with existing biorefineries can offset some costs.	Revenue from byproducts (e.g., proteins, pigments).	Long payback period for return on investment.	[359,360]
	Non-Financial Aspects			
Environmental impact	Microalgae cultivation sequesters CO_2, mitigating greenhouse gas emissions. It also avoids competition with arable land and freshwater when grown in saline or wastewater. However, energy-intensive processes can offset these benefits if not optimized.	Reduces greenhouse gas emissions.	Land and water resource competition.	[314]
Technical feasibility	Strain selection, optimized growth conditions, and process scalability are critical. While lab-scale experiments show promise, transitioning to commercial-scale operations requires overcoming challenges like maintaining stable productivity under varying conditions.	Sustainable and renewable energy source.	Technological challenges in large-scale production.	[361]
Social acceptance	Public perception of biofuels as a sustainable alternative and support from policymakers are essential. Raising awareness about the benefits of microalgal biofuels can improve adoption rates.	Enhances energy security.	Environmental concerns like nutrient runoff.	[362]
Sustainability considerations	Using non-arable land, recycling nutrients, and employing renewable energy sources for cultivation enhance sustainability. However, ethical concerns regarding energy-food competition and potential ecological risks need careful management.	Supports circular economy (waste utilization).	Genetic modification concerns in engineered strains.	[363]

systems with stringent security parameters and thorough ecological risk assessments. The release of GE algal strains into natural environments poses potential ecological risks and benefits. On the positive side, enhanced biofuel production from genetically modified strains could alleviate the pressure on traditional crops and reduce the environmental impact associated with conventional biofuel production. However, concerns exist regarding the unintended consequences of introducing modified organisms into ecosystems. These include potential ecological disruptions, such as competition with native species, gene flow to wild populations, and alterations to nutrient cycling dynamics. Striking a balance between maximizing biofuel yields and minimizing ecological risks is crucial for the sustainable deployment of GE algal strains. Several case studies and field trials have been conducted to assess the ecological impact of GE algal strains. For instance, experiments involving the release of modified strains into controlled environments, such as enclosed ponds or bioreactors, have provided insights into their ecological behavior and interactions with other organisms. Additionally, researchers have utilized modeling approaches to simulate the potential spread and persistence of genetically modified traits in natural ecosystems. These studies contribute valuable data to our understanding of the ecological consequences and inform the development of responsible deployment strategies for GE algal biofuel strains. Building on this, a previous study showcased the successful cultivation of engineered diatom *P. tricornutum* in both closed systems (photobioreactor system and mini raceway pond) and open outdoor fields. The engineered diatom, overexpressing elongase (*Elo5*), exhibited comparable production levels of essential fatty acids, EPA, and DHA in both closed and outdoor environments [364]. Similarly, an engineered strain of *Acutodesmus dimorphus*, overexpressing specific genes, was grown in an outdoor field recognized by the US Environmental Protection Agency (EPA) without any apparent negative ecological impact over a 50-day cultivation period [365]. This suggests that GE algal strains can be cultivated outdoors without out competing native algal strains, bridging the gap between closed and open cultivation systems. While these positive reports indicate the feasibility of cultivating engineered strains for commercial purposes in both closed and outdoor systems, persistent challenges, especially those related to potential ecological risks in outdoor settings, need to be addressed. As the field of GE algal biofuel production progresses, future research should focus on refining genetic engineering strategies to minimize ecological risks while maximizing biofuel yields. Moreover, comprehensive ecological impact assessments, including long-term monitoring and ecosystem modeling, are essential for evaluating the sustainability of these technologies. Collaboration between scientists, policymakers, and environmental stakeholders is crucial to develop robust regulatory frameworks that address the potential ecological implications of genetically modified algal strains. By navigating these challenges, the development of GE algal biofuel strains has the potential to contribute significantly to a sustainable and environmentally responsible energy future.

GE microalgae offer significant advantages, such as enhanced lipid accumulation, faster growth rates, and increased stress tolerance, making them promising candidates for biofuel production. However, their release into natural ecosystems raises concerns about potential disruptions to local biodiversity, gene transfer to wild populations, and other unintended ecological consequences [110]. To address these risks, strict containment strategies and regulatory frameworks must be implemented. Beyond ecological concerns, public acceptance remains a major hurdle. Skepticism surrounding genetic modification, environmental safety, and ethical implications can hinder widespread adoption. Overcoming these challenges requires transparency in research, adherence to regulatory standards, and effective communication about both the benefits and risks associated with GE microalgae [366]. Public education and stakeholder engagement play a crucial role in dispelling misconceptions and fostering trust. Ultimately, the successful implementation of GE microalgae in biofuel production hinges on balancing technological advancements with ecological responsibility and societal

concerns.

15. Artificial intelligence (AI) in optimizing microalgal cultivation systems

Microalgae are a promising source of high-value products, but their production and harvesting are complicated by factors like light intensity, temperature, CO_2 concentrations, pH, and nutrient levels, which vary across species. To address this, computational modeling and control techniques can optimize production and harvesting processes. AI plays a key role in predicting, optimizing, monitoring, and controlling parameters such as light, temperature, and nutrients. This enhances bioproduct yield predictions, optimizes biomass production, and allows for autonomous control, improving efficiency and reducing labor costs.

15.1. Biomass and bioproducts simulation

Considerable research has been devoted to AI-based modeling of microalgal production and harvesting, with standalone artificial neural network (ANN) models frequently used to predict biomass and bioproduct yields [367]. However, these studies typically rely on the trial-and-error approach to determine the architecture and hyperparameters of ANN models, which is both time-consuming and computationally demanding. To address these limitations, several studies have adopted a hybrid modeling approach that integrates metaheuristic optimization algorithms, such as genetic algorithms (GA), to optimize ANN architecture and hyperparameters more efficiently [368]. Moreover, GA has also been combined with ANN models to identify the optimal combination of process parameters that can maximize microalgal production [369]. Despite these advancements, challenges remain with ANN models, including issues of interpretability, overfitting, and poor generalization performance. These limitations must be carefully considered and resolved to ensure the successful application of ANN models in microalgal production and harvesting processes.

Researchers have conducted comparative analyses of various AI algorithms to model microalgae production and harvesting, aiming to identify the most accurate predictive model. Notably, tree-based models like random forest (RF), boosted regression tree (BRT), and decision tree (DT) have been evaluated alongside other models, including artificial neural networks (ANN), convolutional neural networks (CNN), long short-term memory networks (LSTM), k-nearest neighbors (kNN), RF, ANN, BRT, support vector regression (SVR), logistic regression (LR), and naïve Bayes [370,371]. Tree-based methods are straightforward to implement, requiring minimal data preparation, and are proficient at handling missing predictors while allowing for easy assessment of feature importance. However, these models are limited in their capacity for incremental learning, detecting hidden patterns, generalizing to new data, managing unstructured data, and capturing complex relationships in high-dimensional datasets. Consequently, studies have employed deep learning (DL) techniques, specifically CNN and LSTM, which are subsets of machine learning (ML) [372,373,374]. These DL models feature intricate architectures with multiple layers and advanced node interactions, enabling them to autonomously learn complex patterns from data, making them highly effective for predicting microalgal biomass and bioproducts in scenarios involving high-dimensional or unstructured data.

15.2. Optimization of cultivation systems

The conventional approach to optimizing microalgae production and harvesting is labor-intensive, time-consuming, and overlooks complex parameter interactions. AI-driven metaheuristic algorithms, such as genetic algorithms (GA) and particle swarm optimization (PSO), efficiently explore multiple factors simultaneously, accelerating optimization and enhancing biomass yield and harvesting efficiency. Metaheuristic optimization algorithms have been applied to improve algal biomass growth, bioproduct production, and flocculation efficiency [375]. A study optimizing *Nannochloropsis* sp. cultivation used RSM and GA to refine environmental and nutrient conditions [376]. RSM improved biomass, lipid, and EPA productivity by 16.92 %, 30.40 %, and 23.85 %, respectively, over the f/2 medium. GA further enhanced these values by 22 %, 37 %, and 41 %, respectively, demonstrating its advantage in exploring solution spaces without requiring predefined assumptions.

16. Future perspectives with microalgal biofuel industry

Continued innovation, research, and collaboration will be absolutely essential to harness the full potential of algal biofuels and integrate them seamlessly into the renewable energy landscape. The future of novel approaches in cost-reduction and scale-up of algal biofuel production holds great promise, with several key developments on the horizon. Table 4 holds some key future perspectives for the microalgal biofuel industry. Firstly, genetic engineering and synthetic biology will play a pivotal role in refining algal strains for optimal lipid production while addressing ethical, regulatory, and ecological concerns [377]. Researchers are striving to create genetically modified algae that are not only high-yielding in lipid production but also environmentally safe, with built-in containment mechanisms to prevent unintended environmental release. These strains will serve as the foundation for more productive and ecologically responsible algal biofuel production.

Moreover, advancements in cultivation techniques will further refine and optimize large-scale algal production systems. Integrated monitoring and control systems, driven by real-time data analytics and AI, will become increasingly sophisticated, enabling dynamic adjustments to environmental conditions and minimizing the risk of contamination [378]. Innovations in closed-loop PBR_S and modular cultivation systems will provide greater control over environmental parameters, resulting in more consistent and efficient algal growth. These advancements will be instrumental in achieving higher yields, reduced resource consumption, and increased scalability in algal biofuel production [379]. Efforts to address the challenge of energy-efficient harvesting and dewatering will yield transformative solutions. Research into novel separation techniques, such as magnetic harvesting, acoustic-based methods, and microfluidics, promises to significantly reduce energy consumption and costs associated with traditional harvesting approaches [345]. Additionally, the development of cost-effective and environmentally friendly dewatering methods, such as bioflocculation, electrocoagulation, and membrane filtration, will contribute to streamlining the overall production process. These advancements will improve the economic viability of algal biofuel production and diminish its environmental impact.

Furthermore, the future of algal biofuel production will see major advancements in lipid extraction methods. Ultrasound-assisted extraction, enzymatic treatments, and the utilization of environmentally friendly solvents will become more refined and scalable, ensuring efficient lipid recovery while minimizing energy consumption and environmental impact [380]. Researchers will continue to explore novel techniques, such as electroporation and mechanical cell disruption, to enhance lipid extraction efficiency further. These developments will contribute to higher biofuel yields and improved overall process sustainability. Incorporating renewable energy sources into algal biofuel production facilities will become more commonplace and economically viable. Advancements in energy storage technologies will help mitigate the intermittent nature of renewable energy sources, ensuring a stable and cost-effective energy supply for the various stages of algal cultivation and processing [381]. Additionally, the integration of solar tracking systems, advanced wind turbine designs, and emerging technologies such as wave and tidal energy will enhance energy capture and efficiency. These developments will reduce the carbon footprint of algal biofuel production and contribute to its long-term sustainability. Collaboration between academia, industry, and government institutions

Table 4
Some key future directions for the microalgal biofuel industry, along with their advantages and challenges.

Parameters	Explanations	Advantages	Challenges	References
Advanced strain development	Future research is expected to focus on identifying and developing high-performance microalgal strains through advanced breeding techniques and genetic engineering. Tailoring strains for specific environments, stress resistance, and increased lipid content will be essential for optimizing biofuel production.	Enhances lipid productivity and stress resistance, optimizing biofuel yield.	High research costs and potential ecological risks of genetically modified strains.	[377,384]
Precision cultivation systems	The future of microalgal biofuels lies in the development of precision cultivation systems that integrate automation, AI, and real-time monitoring. This will enable more efficient use of resources, minimize energy consumption, and enhance overall productivity.	Improves resource efficiency and enhances biofuel productivity.	High initial investment and maintenance costs for advanced systems.	[378,379]
Integration with other industries	Microalgal biofuel production is likely to become an integral part of broader industrial ecosystems. Integration with other industries, such as wastewater treatment, agriculture, and aquaculture, can create synergies, reduce costs, and enhance overall sustainability.	Promotes cost reduction and sustainability through industrial integration.	Requires complex infrastructure and coordination between industries.	[355,385]
Circular economy practices	The industry is expected to adopt circular economy practices, where waste and by-products from microalgal biofuel production are utilized for additional value-added products. This includes the development of biorefineries that extract multiple products from microalgae, such as biofuels, animal feed, and nutraceuticals.	Maximizes resource utilization and enhances economic viability through biorefineries.	Requires significant investment and technological advancements for efficient implementation.	[333,383]
Emergence of co-products	Beyond biofuels, the industry will likely witness the emergence of various co-products derived from microalgae. These may include high-value compounds such as omega-3 fatty acids, pigments, and proteins, contributing to diversified revenue streams.	Diversifies revenue streams by producing high-value co-products.	Requires additional processing and infrastructure, increasing production complexity.	[81,386]
Green extraction methods	Future research will focus on developing environmentally friendly and sustainable methods for extracting lipids and other valuable compounds from microalgae. Green extraction methods aim to reduce the ecological footprint of the industry and minimize the use of hazardous solvents.	Reduces environmental impact by minimizing hazardous solvent use.	May involve higher costs and lower efficiency compared to conventional extraction methods.	[380,387]
Biofuel blending and infrastructure integration	As microalgal biofuels become more established, the future involves blending them with conventional fuels to create biofuel blends. The integration of microalgal biofuels into existing fuel infrastructure will be a crucial step towards achieving wider market acceptance.	Facilitates market adoption by integrating with existing fuel infrastructure.	Compatibility issues with current engines and fuel distribution systems.	[388,389]
Techno-economic advancements	Techno-economic analyses will play a vital role in determining the feasibility and competitiveness of microalgal biofuel production. Future advancements will focus on optimizing production pathways, reducing costs, and increasing overall economic viability.	Enhances economic viability by identifying cost-effective production strategies.	Uncertainties in market dynamics and policy support may affect feasibility.	[390,392]
Global collaboration and standardization	The future of the microalgal biofuel industry involves increased collaboration among researchers, industry stakeholders, and policymakers on a global scale. Standardizing protocols, regulatory frameworks, and best practices will facilitate the growth of a cohesive and sustainable industry.	Promotes industry growth through standardized regulations and best practices.	Coordination challenges and regulatory delays may slow implementation.	[393,394]
Public engagement and education	Building public awareness and acceptance is crucial for the success of the microalgal biofuel industry. Future efforts will involve extensive public engagement, education programs, and transparent communication to address concerns and foster positive perceptions.	Enhances public support and market adoption through education and awareness.	Requires significant time and resources to overcome misinformation and skepticism.	[395,396]
Emerging markets and policy support	The industry's growth will be influenced by emerging markets and the support of progressive policies. Governments and regulatory bodies are expected to play a pivotal role in creating an enabling environment through incentives, subsidies, and clear regulatory frameworks.	Encourages industry expansion through government support and financial incentives.	Dependence on policy stability, which may change with political shifts.	[397,398]
International research collaborations	International collaborations in research and development will continue to drive innovation in the microalgal biofuel industry. Shared knowledge, resources, and expertise will accelerate progress and contribute to solving common challenges.	Accelerates innovation by leveraging shared knowledge and resources.	Differences in regulations and priorities may hinder effective collaboration.	[394,399]
Climate change mitigation	The role of microalgal biofuels in climate change mitigation is likely to gain prominence. The industry can contribute to reducing carbon emissions by providing a sustainable alternative to fossil fuels.	Reduces carbon emissions by offering a sustainable alternative to fossil fuels.	Scaling up production to meaningful levels remains a major challenge.	[389,400]
Educational and career opportunities	As the industry expands, there will be increased opportunities for education and careers in microalgal biofuel research, development, and production. Academic institutions and training programs will play a crucial role in preparing professionals for the evolving needs of the industry.	Creates new career opportunities and fosters expertise in a growing industry.	Requires substantial investment in education and specialized training programs.	[333,389]

(*continued on next page*)

Table 4 (*continued*)

Parameters	Explanations	Advantages	Challenges	References
Evolving consumer preferences	As sustainability becomes a key consideration for consumers, microalgal biofuels may gain popularity as a cleaner and more environmentally friendly energy source. Future market dynamics will be influenced by evolving consumer preferences.	Increases demand for eco-friendly energy solutions driven by consumer preferences.	Market adoption may be slow due to higher costs compared to conventional fuels.	[389,401]

will continue to be the driving force behind research and development efforts in algal biofuel production [382]. Public-private partnerships, research consortia, and government-funded initiatives will support large-scale pilot projects and commercialization efforts, facilitating the transition from lab-scale research to industrial-scale production. Regulatory frameworks will evolve to accommodate novel algal strains and production methods, providing a clear path for commercialization while addressing safety and environmental concerns. Interdisciplinary collaborations will lead to holistic solutions, where biologists, engineers, chemists, and environmental scientists work together to tackle the multifaceted challenges of algal biofuel production.

Additionally, the development of circular economy approaches in algal biofuel production will gain prominence. By utilizing waste streams and byproducts from other industries as nutrient sources or co-culturing partners, algal cultivation systems can become more resource-efficient and sustainable [383]. Efforts to utilize carbon dioxide emissions from industrial processes as a feedstock for algae will not only reduce greenhouse gas emissions but also provide a cost-effective carbon source for algal growth. These circular economy practices will enhance the overall sustainability and economic viability of algal biofuel production. As the technology matures and scales up, economies of scale will be realized, leading to reduced production costs and increased competitiveness with traditional fossil fuels [333]. Algal biofuels will find applications beyond transportation fuels, including feedstock for chemical industries and even as a source of high-value products such as pharmaceuticals and nutraceuticals. The versatility of algae as a biomass source and the ability to tailor their composition for specific applications will open up new markets and revenue streams, further incentivizing investment and innovation in the field. With concerted efforts from researchers, industry leaders, and policymakers, algal biofuels have the potential to play a significant role in addressing our energy needs while reducing the environmental impact of energy production. These efforts will not only contribute to a more sustainable future but also drive economic growth and job creation in the emerging biofuel industry.

17. Conclusions

This study delves into the latest breakthroughs in biotechnology and bioengineering, aiming to revolutionize the landscape of microalgal biofuel production for a sustainable and eco-friendly biofuel revolution. Microalgae have attracted interest as a prospective biofuel source because of their fast growth rates and elevated lipid content. Overcoming challenges related to scalability, productivity, and cost-effectiveness has been a focal point, and recent advancements in biotechnological and bioengineering strategies have played a pivotal role in addressing these issues. The integration of cutting-edge techniques, including genetic and metabolic engineering, has substantially improved microalgal lipid yields. These approaches offer precise control over the biochemical pathways involved in lipid production, enhancing the efficiency of microalgal biofuel synthesis. By optimizing cultivation processes and improving strain performance, researchers are paving the way for a more economically viable and environmentally sustainable microalgal biofuel industry. The study emphasizes the eco-friendly nature of microalgal biofuel production, presenting it as a promising alternative to traditional fossil fuels. The reduced environmental impact, coupled with the potential for large-scale production, positions microalgae as a key player in the quest for sustainable energy sources. The biofuel revolution heralded by these recent advancements underscores the industry's potential to contribute significantly to global energy needs while mitigating the detrimental effects of conventional fuel sources on the environment.

To enhance lipid accumulation, biomass productivity, and stress tolerance, researchers should focus on genetic engineering, metabolic pathway optimization, and adaptive strain selection. In this regard, integrating synthetic biology, CRISPR-based modifications, and omics technologies can further accelerate the development of high-yield microalgal strains, making large-scale biofuel production more viable. At the same time, policymakers play a crucial role in facilitating funding, establishing regulatory frameworks, and fostering industry collaborations to support the commercialization of these advancements. By implementing policies that promote renewable energy incentives, carbon credit programs, and infrastructure development, governments can create a conducive environment for investment in microalgae-based biofuels. Moreover, interdisciplinary collaboration between biotechnologists, environmental scientists, and energy experts is essential to bridge the gap between research and commercialization. By fostering a synergistic approach between science and policy, stakeholders can unlock the full potential of microalgal biofuels, ultimately reducing reliance on fossil fuels and advancing global energy sustainability.

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CRediT authorship contribution statement

Chaoqun Zhang: Writing – review & editing, Formal analysis. **Rahul Prasad Singh:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Priya Yadav:** Writing – review & editing. **Indrajeet Kumar:** Writing – original draft. **Amit Kaushik:** Writing – review & editing, Formal analysis. **Rajib Roychowdhury:** Writing – review & editing. **Mustansar Mubeen:** Writing – review & editing. **Sandeep Kumar Singh:** Writing – review & editing. **Ajay Kumar:** Writing – review & editing, Data curation, Conceptualization. **Jie Wang:** Writing – review & editing, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors have declared no conflicts of interest.

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Data availability

No data was used for the research described in the article.

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