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Review

Microalgae Biomass and Lipids as Feedstock for Biofuels: Sustainable Biotechnology Strategies

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Abstract: Microalgae exhibit remarkable potential as a feedstock for biofuel production compared with other sources, owing to their high areal productivity, low environmental effect, and negligible influence on food security. However, the primary obstacle to the commercialization of algae-based biofuels is the high economic cost due to the low-yield lipid content in the microalgae biomass. Maximizing biomass and lipid production is crucial to improve the economic viability of microalgae for biofuels. Identifying appropriate algal strains, particularly from indigenous environments, and developing those 'platform strains' using mutagenesis and genetic-engineering techniques is preferable. The provided discussion of conventional methods to increase microalgae's biomass and lipid productivity mostly entailed adjusting environmental (such as temperature, light, and salinity) and nutritional (such as nitrogen and phosphorus) parameters. This review illustrated a comprehensive overview of biotechnological approaches and the recent strategies to enhance the lipid productivity of microalgae. The research also emphasized the need to streamline engineering strategies with the aid of recent advancements in DNA-manipulation techniques to hinder the existing biological intricacies in lipogenesis. This review also discussed the current economic and commercialization of this algal biorefinery along with the drawbacks.

Keywords: anaerobic digestion; biogas; cost; environmental impact; nanomaterial; waste activated sludge

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1. Introduction

Fossil fuels have been regarded as the dominant source of energy, and energy consumption is increasing by 2% each year [1]. The continuous use of fossil fuel reserves combined with an ever-increasing population, industrialization, and individual energy demand is projected to worsen both economic stability and global energy security [2]. Furthermore, burning fossil fuels releases greenhouse gases (GHG) and other hazardous pollutants into the atmosphere, which contribute to global warming and environmental pollution [3]. This fact alone has prompted researchers to develop suitable substitutes for traditional fossil fuels. Recently, biofuels have drawn significant attention as potential replacements for fossil fuels because of their notable qualities, including the ability to be used directly or blended in various ratios with fossil fuel, biodegradability, overall lower emission of CO_2 and other toxic pollutants, improved ignition property, higher cetane number, higher flash point, and low sulfur content.

There are three categories of biofuels based on feedstock differences. Edible feedstock of the first generation includes soya beans, corn, wheat, rapeseed oil crops, sugarcane, maize, and sugar beet. The second generation has lignocellulosic feedstock such as switch grass and jatropha [1]. Biofuels derived from food or non-food crops are not considered

renewable or sustainable [2]. Although the production of biofuels might be a part of meeting the world's energy needs, they are reliant on the amount of available cultivable land. This would be the primary drawback since agricultural land is constrained, and the amount of land needed for their growth competes with crops grown for human consumption [3]. As a result, biofuels obtained from food or non-food crops are not advised as a replacement for fossil fuels. Third-generation biofuel is beneficial; it does not compete with food crops or accessible farmland, requires less water, and emits more CO_2 [4]. This organism's productivity is not affected by the seasons, and it is simple to harvest [1].

As the world is exploring a different fuel resource, microalgae are best referred to as a third-generation biofuel. Since microalgae have a large percentage of lipids, they are extracted and processed (esterified) into efficient transportation fuel. Microalgae have been proposed as a viable fuel feedstock due to several benefits, including increased photosynthetic efficiency and biomass production, compared with alternative sources [1]. Microalgae have many ecological and environmental adaptations. Algal lipids are often divided into neutral and polar lipids. The most significant type of lipid for biodiesel synthesis is neutral triglycerides or TAGs [2]. Microalgae use H_2O, CO_2, and light energy to transform into chemical energy and manufacture important organic components such as carbohydrates, lipids, and proteins. Under photooxidative stress or other severe environmental circumstances, microalgae can create large volumes of triacylglycerols (TAGs) used as biofuel storehouses [3]. They also generate a variety of hydrocarbons. Among all plant species, algal species have been identified to increase exponentially, synthesize a considerable amount of TAG or oil, and are consequently considered oleaginous. These algae might be used as a source of cell factories. Biofuels derived from microalgae have the potential to reduce greenhouse gas emissions because microalgae are responsible for 40% of global carbon fixation and can contain up to a maximum of 70% oil (dry weight) in some species [5]. Several methods and technologies have been investigated to stimulate fatty acid production rates in microalgae and make the process more sustainable and scalable [6]. It is important to understand that lipid production in microalgal cells goes beyond energy storage because lipids are used to build cellular membranes and are necessary for developing other biomolecules [7]. The lipid content in some of the representative algal samples is given in Figure 1. Despite the abovementioned advantages, the commercial exploitation of microalgae for biofuel production is still impractical.

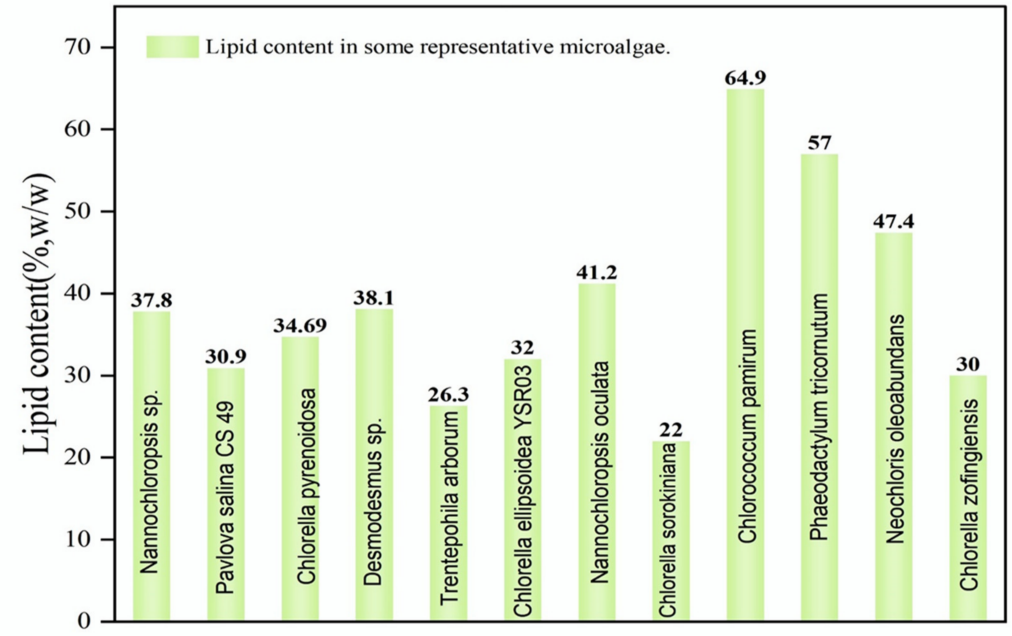

Figure 1. Lipid content in some representative microalgae [8–19].

The simultaneous overproduction of lipids and cellular biomass is thought to be one of the critical elements determining whether algal fuel production is commercially viable. However, in unfavorable circumstances, microalgae may hyper-collect lipids that substantially impair their ability to accumulate biomass and produce a final product, restricting their ability to be employed commercially [5]. Therefore, it is crucial to develop these oleaginous microalgal systems in order to produce a larger number of necessary products without depleting their cellular biomass. Microalgal metabolic engineering is one promising method for enhancing the synthesis of desired metabolites through targeted disruption of important metabolic pathways [20]. However, due to a variety of technological and biological challenges, such as the identification of important molecular alternative(s) that govern metabolic pathways, the availability of feasible molecular transformation toolkits, and regulatory challenges connected with the use of engineered algal strains, the application of metabolic reconfiguring strategies for generation of industry-suitable algal strain has not yet been developed [21,22]. Considering such factors, it is critical to eliminate the prevailing obstacles in order to use microalgae for commercial purposes.

This review aimed to highlight the advances in using various strategies to enhance the production of algal biomass and lipids for biofuel feedstock. However, this review addressed sluggish growth rate, limited biomass yield, and low lipid content. The traditional methods for increasing lipid production in microalgae include applying nutritional stress and changing environmental parameters such as temperature, light, and salinity. Newer methods including co-culturing techniques for microalgae production were also discussed. Furthermore, to increase the lipid hyper-accumulation in engineered microalgae, it is important to discover and take advantage of the critical metabolic target emphasized in this review. We also drew attention to those molecular techniques' genetic restraints and limits and stressed the necessity of establishing a viable metabolic engineering strategy to overcome these issues. Genetic engineering has been used earlier as one of the most effective stress-reducing tools. It also includes biofuel production from microalgal lipids by analyzing biotechnological approaches in lipids and with gene editing tools. This study focused on genetic–molecular and metabolic engineering approaches that could be utilized in the industry's efforts to improve microalgae as a source of biofuels, contributing to many sustainable biotechnological approaches.

1.1. Lipids in Microalgal Biomass

Microalgae produce two lipids: polar lipids as glycerophospholipids, which play a crucial role in cell structure, and non-polar lipids, such as triglycerides (TAGs), which are essential for energy storage. Long chains of fatty acids are found in structural lipids, which convert into polyunsaturated fatty acids (PUFAs), including docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [23]. These lipids play a dynamic role in optimizing biosynthetic processes and membrane fluidity and participate directly in intracellular membrane fusion events. They are also essential in signaling pathways and cellular response to environmental changes. TAGs are the most common form of storage lipids, with a high proportion of saturated and unsaturated fatty acids [24]. TAGs are efficiently catabolized and used to provide metabolic energy. They are primarily generated in light, accumulated inside cytosolic lipid bodies, and utilized when light is absent; polar lipids are synthesized. The glycosylglycerides such as mono-galactosyldiaclyglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiaclyglycerol [25] are abundant in the chloroplast, the most critical membrane lipids, and substantial numbers of phosphoglycerides. The plasma membrane and several endoplasmic membrane systems are home to these proteins [26].

1.2. Lipid Biosynthetic Pathways in Microalgal Biomass

Algae differ from other organisms in that they can fix carbon and use solar energy. The lipid metabolism of algae (Figure 2) is comparable to that of plant cells, from de nova fatty acid production to the generation of larger glycerolipids. Higher plants have distinct

organs that conduct diverse physiological functions and separate metabolic pathways. Algae, as with higher plants, convert TAG into tiny lipid particles covered in various protein molecules [27]. Most of the lipids are RabGTPases and other vesicules transport and signaling pathways. Still, a proteomics approach to algal lipid bodies has identified a significant major lipid-droplet protein (MLDP) that affects lipid droplet size. It could be a target for algal lipid content immunofluorescence imaging.

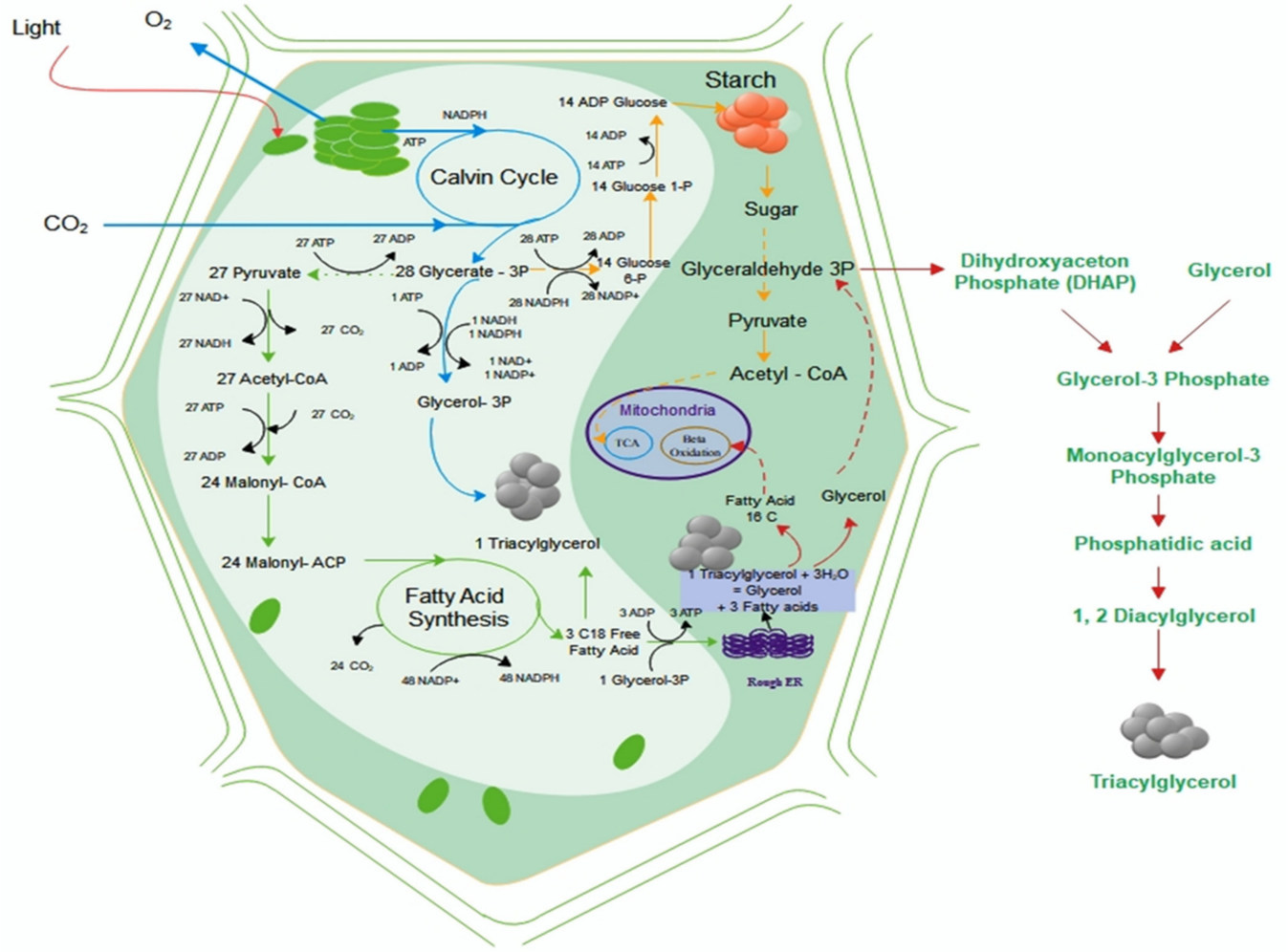

Figure 2. Metabolic pathway of lipid biosynthesis.

In a laboratory, the environmental conditions are better controlled than in open air, where the growth of terrestrial plants and algae is more promising. Growth conditions can also be modified to meet the unique requirements of genetically modified strains, resulting in significant productivity gains [28]. When many algal species are stressed, TAG is the maximum prevalent lipid found in their cells. Most algae change the lipid biosynthetic pathways to generate and accumulate. Neutral lipid, primarily in the form of TAG, acts as a carbon and energy storage form under adverse environmental or stress conditions. The TAG pathway starts with acetyl–CoA, an essential fatty acid precursor. It progresses through fatty acid biosynthesis, complex lipid assembly, and saturated heavy acid modification to TAG production and storage [29]. The TAG biosynthesis consists of three phases, each of which is regulated independently: 1. fatty acid (FA) biosynthesis, 2. the glycerol–lipid formation, and 3. packaging into lipid droplets (LDs). In plant chloroplasts, the fatty acid synthase (FAS) complex is a key factor in de novo fatty acid production. Many sequenced algae, including *Chlamydomonas reinhardtii*, have been annotated for fatty acid synthase

(FAS) enzymes. Various lipid biosynthesis genes were overexpressed or knocked out to see how they affected lipid buildup. Acetyl–CoA Carboxylase (ACCase) is the gene that codes for synthesizing fatty acid enzyme11. The carboxylation of acetyl–CoA to create malonyl–CoA is carried out by the acetyl–CoA carboxylase (ACCase). It is regarded as the rate-limiting step in plant biosynthesis of fatty acids.

As a result, the ACCase enzyme is a target to boost algal oil output. The cytosolic acyl–CoA esters are transported to the endoplasmic reticulum, elongated, modified, or utilized as membrane lipids if not stored in TAGs. Malonyl–CoA, combined with an acyl-carrier protein (ACP), is a metabolic scaffold in acid biosynthesis. PPTase transforms apo-ACP to its holo form by transferring a 40-phosphopantetheine, a flexible prosthetic group coenzyme A to a serine on the ACP. This 'arm' on holo-ACP tethers the developing fatty acid throughout and fatty acid biosynthesis via a thioester linkage [30,31]. An ACP switchblade mechanism in spinach was reported where the protein–protein interactions with enzyme FAS generated an alteration in the ACP, which promoted catalytic elongation and reduction in the fatty acid (hydrophobic core). The starting ketosynthase domain, b-ketoacyl–ACP-synthase III (KASIII), condenses acetyl–CoA with malonyl–ACP to generate a b-ketone extended 2-carbon unit on acyl–ACP. Ketoreductase (KR), dehydratase (DH), and enoyl ASI are then reduced to a saturated methylene unit. ASCII completes the cycle seven more times. Finally, a mature fatty acid attains its desired size. It may be retained in the plastid via the prokaryotic pathway, where the fatty acid is transported to glycerol-3-phosphate using an enzyme acyl transferase (AT). If not, it is sent to the cytosol via the eukaryotic pathway, which is hydrolyzed by a thioesterase domain (TE), releasing the fatty acid from the FAS to be included in membrane lipids. Fatty acids are hydrolyzed from ACP by the TE. They dispense into the envelope. They are esterified by coenzyme A by a CoA ligase enzyme, allowing the formation of fatty acyl–CoA to leave the chloroplast and become incorporated into cellular lipids such as membrane phospholipids and storage triglycerides.

The TE is a metabolic protector in lipid biosynthesis in plants, determining the carbon flux between the eukaryotic and prokaryotic lipid termination pathways. The TE is at the forefront of attempts to improve biodiesel quality. It is a usual feature to overexpress diacylglycerol acyl transferase, which catalyzes the final stage of TAG production and raises lipid levels. Hyperaccumulation of lipids has been observed in many microalgal species due to the increased production of glycerol kinase, acetyl–CoA synthase, pyruvate dehydrogenase, phosphoenolpyruvate carboxylase, and NAD(H) kinase. The synthesis of multifunctional acyltransferase/phospholipase/lipase is inhibited in *Thalassiosira pseudonana*, which causes a rise in lipid buildup without affecting growth.

On the other hand, transcriptional regulation is known to affect the system's metabolic flux since transcription factors can select many regulatory points in a metabolic pathway. Overexpression/shortfall of transcription factors that promote lipid biosynthesis gene upregulation may result in increases lipid accumulation. Knocking down a single transcription regulator ZnCys in *Nannochloropsis gaditana* resulted in a twofold rise in lipid content in one study. CRISPR/Cas9-based technology was recently used to manipulate genes in *Chorella vulgaris*, with a Cas9 fragment containing sgRNA designed on the omega-3 fatty acid desaturase (fad3) gene, which brought about a 46% (w/w) increase in lipid contented buildup [32].

1.3. Polyunsaturated Fatty Acids Biosynthesis

Essential fatty acids and polyunsaturated fatty acids are crucial constituents of the signal transduction cell membrane and lipid-storage pathways (PUFAs). PUFAs are fatty acids with 18–22 carbons in a straight chain and two or more double bonds. Saturated and monosaturated FAs are found in equal amounts in microalgal TAGs [33]. On the other hand, some oil-rich species have shown the ability to collect vast quantities of big-chain PUFA in the form of TAG. TAGs high in PUFAs are metabolically active and may serve as a storage site for specific fatty acids. Although the environmental change slows down the de novo synthesis of PUFA, PUFA-rich TAG contributes specific acyl groups to

monogalactosyldiacylglycerol (MGDG) and related lipids to enable rapid reconfiguration. *Crypthecodinium cohinii, Nannochloroposis* sp., and *Phaeodactylum tricornutum* are microalgae that may synthesize high-value PUFAs [33]. The change from acetyl–CoA to malonyl–CoA precursors is thought to be most important in chloroplastic microalgal fatty acid synthesis, followed by four condensation events that result in the creation of an acyl–ACP. The first step is converting from acetyl–CoA to malonyl–CoA via phosphorylation; by catalyzing AMP-activated kinase (AMPK), ACCase is inhibited. Then, the fatty acids are sequentially transferred from CoA to glycerol-3-phosphate (G3P) through the direct glycerol synthesis pathway, which is thought to occur in microalgal TAG production [34].

2. Enhancement of Biomass and Lipid Production from Microalgae

Microalgae are single microscopic cells, either prokaryotic (such as cyanobacteria) or eukaryotic (such as green algae). Microalgae are a valuable carbon resource for biofuels, health supplements, medicines, and cosmetics [35]. Microalgae cultivation is widely recognized as the crucial stage in "microalgae biofuel production" as the amount and composition of the feedstock are highly dependent on that particular stage. Cell growth conditions, on the other hand, are usually different from those required for lipid formation [36,37]. Additional methods, such as introducing diverse stresses throughout the biomass generation stage, have increased lipid content in algae. However, stressful environments frequently negatively affect microalgae development rates, resulting in lower product yields. As a result, achieving the best economic scenario will necessitate finding the right mix between lipid content and cell proliferation. Microalgae are exposed to stressful environments or nutrient constraints, and the lipid content of the algae can increase [38].

2.1. Conventional Methods

Lipid-engineering methods in microalgae are accomplished via traditional, genetic, and metabolic-engineering methods. One of the most appealing aspects of using microalgae as a biofuel feedstock is that the growth conditions may be tweaked to optimize biomass and lipid production. Nutrient deficiency and environmental stress, such as heavy-metal stress, salt stress, and temperature, among other things, can boost the activity of numerous proteins [39]. Metabolic factors in controlling fatty acid biosynthesis in microalgae are detailed in Figure 3. Various stresses, particularly nitrogen stress, have been linked to TAG deposition in different microalgae species.

2.1.1. Nutrient Limitation

Different dietary conditions impact microalgal metabolite content, particularly those implicated in lipid metabolism. Inorganic nutrients such as sulfur, nitrogen, carbon, phosphorous, and iron have a significant impression on microalgae cell metabolism, growth, and reproduction [40]. A lack of nutrients produces unpleasant conditions inside the cell after building additional lipids and various compounds. Researchers in industries have used this strategy to stimulate lipid production and accumulation. During the matured growth stage, the limitation of nutrients can cause environmental stress and increase lipid production [41]. This also impacts lipid productivity, which affects the biochemical pathways in the cells. Nitrogen deficiency is the most essential nutrient affecting lipid metabolism in algae out of all the studied nutrients. Many species of various algal taxa have displayed a general trend toward lipid accumulation, notably TAG, in response to nitrogen shortage. Nitrogen is an essential nutrient for microalgal development and lipid accumulation, with the link between nitrogen content and lipid production varying by species and strain [42].

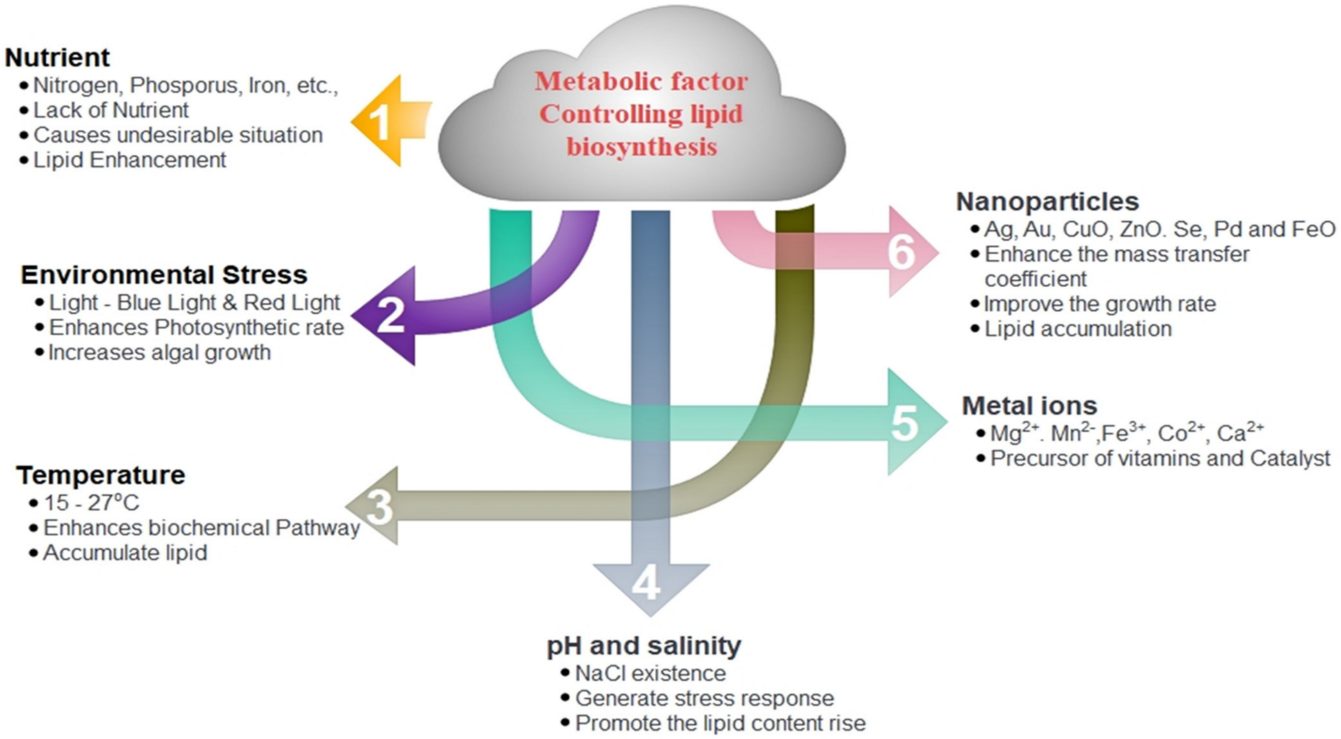

Figure 3. Metabolic factors in controlling fatty acid biosynthesis in microalgae.

Many microalgal species, including *Neochloris oleoabundans*, *Chlorella sorokiniana*, *C. vulgaris*, *Chlorella protothecoides*, and the model green alga *C.reinhardtii*, benefit from nitrogen deficiency [43]. Nitrogen limitation causes three changes in the species: the thylakoid membrane cellular content decreases, the activation of acyl hydrolase occurs, and phospholipid hydrolysis is stimulated. Nitrogen deficiency may activate diacylglycerol acyltransferase, an enzyme that converts acyl–CoA to triacylglycerides(TAG). As a result, nitrogen limitation may cause microalgal cells to produce more lipids and TAG. Silicon is an equally significant ingredient in diatoms, influencing cellular lipid metabolism [44]. Compared with silicon-replete Cyclotella cryptica cells, silicon-deficient cells contain higher amounts of neutral lipids (mainly TAG) and higher percentages of saturated fatty acid and monosaturated fatty acids. Phosphorous, in addition to nitrogen and silicon, is essential for microalgal cell development. Phosphate deprivation is more efficient than nitrogen restriction in many circumstances, such *asnkistrodesmus falcatus's* two-step technique, where lipid productivity is higher than when nitrogen restriction is used.

2.1.2. Environmental Stress

Algal growth increases biomass, where light is one of the most significant components. At the same time, the wavelength and its intensity significantly impact how these algae grow, produce, or accumulate fatty acids. Particular wavelengths of 400–500 nm and 600–700 nm on microalgae have influenced the development and maximum performance of essential enzymes in photosynthetic processes. Product generation has been confirmed. The compositions and arrangement of microalgal growth are affected by light [45]. The temperature and the other factors working together have a powerfully amplified effect on product biosynthesis. In *Isochrysis galbana*, combining temperature and light intensity greatly enhances TAG content and productivity [46].

2.1.3. Temperature

Temperature plays a vital role in microalgae growth and the accumulation of fatty acids [9,13]. Temperature directly impacts biochemical methods in algal industries, as well as photosynthesis. Every species has its maximum temperature for growth. Growths to

the optimum temperature range will exponentially boost algal multiplication [47]. Still, increases or decreases in this optimal temperature will slow or stop algal growth and metabolism [30]. High temperature significantly impacts cell activity, affecting enzymes involved in the production. *Monoraphidium* sp. SB2 showed a 7.4% increase in lipid productivity when growing at 30 °C. Microalgae biomass losses will grow at non-optimal temperatures, exclusively in outdoor cultures [39,48]. A key aspect in large-scale agriculture, particularly in open-pond culture, necessitates constant monitoring because of the dangers involved.

2.1.4. pH and Salinity

pH is a crucial environmental element that regulates cell metabolism and biomass accumulation [16]. All microalgae strains appear to have a slight pH optimum range for a high cell development rate. The pH needs of different microalgae species vary. Most algal species prefer a pH range of 6 to 8.76 [26,44]. The pH of various sources of growth medium varies. Certain algal species are susceptible to pH, and a few handfuls of algal species can sustain a wide pH range, such as *C. vulgaris* [48]. The salt concentration increases the pH of the culture media, which is highly unfavorable to algae cells.

2.1.5. Metal Ions

Metal ions can also influence microalgae growth and lipid synthesis. In a dark environment, researchers studied the effects of calcium, iron, and magnesium on lipid synthesis and biomass accumulation of a heterotrophic microalgae *Scenedesmus* sp. R-16 and discovered that the total lipid concentrations increased from 35.0% to 47.4%, respectively [49]. Under heterotrophic culture conditions, *Chlorella minutissima* UTEX2341 showed strong resistance to cadmium and the lipid and copper ions; the composition was dramatically enhanced by 93.9% and 21.1%, respectively.

2.1.6. Nanoparticles

Metallic nanoparticles with physiochemical behavior give rise to the production of versatile biomaterials, cosmetics, food processing, and optics. The potential of NPs to increase the gas–liquid mass transfer rate in fermentation is one of their most recent applications [1]. In the gas–liquid interface, the availability of NPs promotes the mass transfer coefficient, and it is assumed that increasing CO_2 concentrations via NPs will alter algal multiplication and lipid synthesis in specific microalgae [1]. Metallic NPs, CuO, Ag, Au, ZnO, Pd Se, and FeO, have been discovered to be very poisonous to various organisms.

2.2. Challenges and Limitations of Convention Methods

The attainment of lipid overproduction is necessary to foster microalgal biodiesel production, and some relevant tactics can help achieve this goal. Among all, nutrient stress is the most effective and extensively employed. Temperature, light intensity, carbon dioxide, metal stress, and salinity stress are other ways to induce lipid over-accumulation [50]. Lipids-causing tactics might be coupled to maximize lipid accumulation [51].

Microalgae is one of the organisms affected by NPs; as the NPs increase, the toxic effect of NPs is linked to the generation of reactive oxygen species (ROS) and the initiation of oxidative stress, which is realized when an optimum level of the concentration of NPs is reached. The studies show that this has contributed to a positive outlook for lipid biogenesis [52–54]. As the diversity of algae is high (67.7%), greater emphasis should be placed on genomic studies, too.

3. Biotechnological Approaches for the Improvement of Biomass and Lipid Production

The essential parameters determining the economic feasibility of algal fuel generation are biomass increment and lipid accumulation. Carefully tailoring virtual metabolic circuits in oleaginous microalgae is a viable technique for increasing algal content without

affecting cellular physiological properties [52,55]. Figure 4 details the different methods and instrumentation used for microalgal engineering.

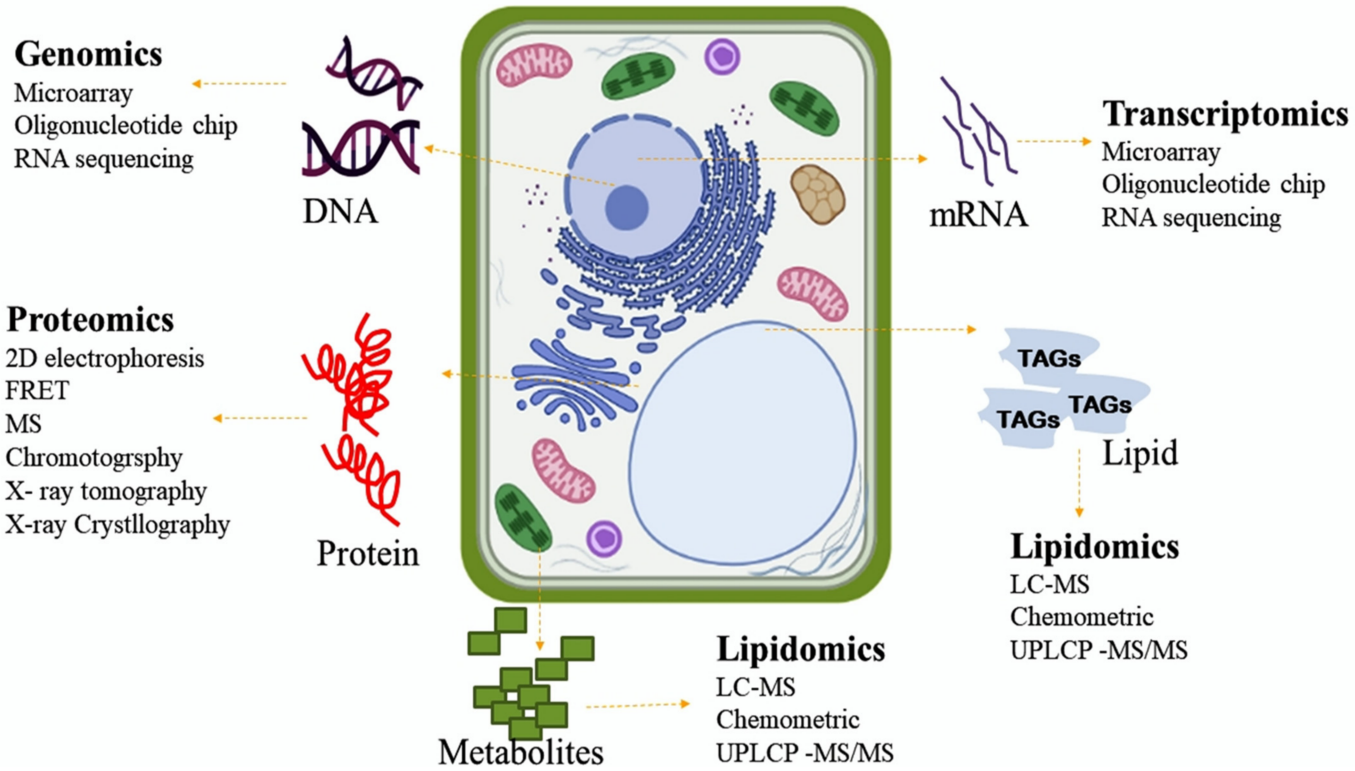

Figure 4. Different methods and instrumentation used for microalgal engineering.

Lipid concentration in algae can scale up to 80% dry weight. However, lipid output is still modest even in these instances [56]. Lipid concentrations between 20% and 50% in *Dunaliella*, *Porphyridium*, *Isochrysis*, *Tetraselmis*, *Nannochloropsis*, *Phaeodactylum*, *Chlorella*, and *Schizochytrium* species have been found around the world [57]. On the other hand, changing the conditions of the culture can result in increased lipid build-up. Temperature, irradiation, and, most importantly, nutrition availability have all been proven to impact algal cell lipid concentration and composition. Microalgae lipids are treated using particular techniques, creating metabolic products of other microalgae of pharmacological and nutritional significance or even utilizing algal biomass generated in wastewater treatment. Cyanobacterial and microalgae species differ in cell size, starch content, and lipid composition. Not all of them are suitable for biofuel generation because they can synthesize and gather a large quantity of starch and lipids naturally increased additionally through genome alterations.

Photo Bio Rectors (PBRs) are closed mechanical setups utilized outdoors and indoors and offer several benefits, including the ability to regulate and control critical environmental parameters, temperature, water levels, and carbon dioxide levels [58]. Moreover, PBRs permit the inevitable growth of microalgal species for a more extended period in optimal cultural conditions with a lower risk of contamination, influencing the overproduction of essential components [59]. *Dunaliella salina* and *Haematococcus pluvialis* use closed-type PBRs to produce commercial pigments (beta-carotene and astaxanthin). These reactors are used to create PUFA-containing lipids since their comparatively high cost is offset by the high-cost products. Microalgae can also be grown mixotrophically or heterotrophically, in addition to autotrophically. The Calvin–Benson cycle comprises fixed inorganic carbon sources in the existence of light. Autotrophic microalgal cultivations have several advantages, including lower costs, CO_2 mitigation in the environment, sunlight as a source of light, lower power consumption, and the ability to cultivate on low-marginal land [60].

The commercial use of cultivation in autotrophic conditions is technically constrained by reduced algal biomass build-up, lower production, insufficient light sources, and penetration. Heterotrophic cultivation appears to be the most encouraging method, as microalgae simultaneously use phototrophic and heterotrophic routes [61]. The total cells grown at specific growth rates under heterotrophic and phototrophic conditions might be utilized to calculate the specific growth rate of mixotrophically grown microalgae. Furthermore, growth in mixotrophic circumstances may be able to overcome issues with light invasion. However, there are several limitations to this type of growing system, primarily due to the possibility of contamination, which could be detrimental to algal growth.

3.1. Selection Screening and Improvement of Potent Microalgal Strains (Bioprospecting)

Algae, as a renewable resource, are produced with the right biological and technological breakthroughs without jeopardizing agricultural land and freshwater supplies or causing land degradation. The initial phase in optimizing the algal feedstock production system is selecting local oleaginous microalgae, i.e., "bio-prospecting." Lipid productivity is one of the essential factors for screening algal strains. It is well understood that choosing fast-growing oleaginous algae species will directly impact the total feedstock production process [62]. To evaluate microalgae strains for biofuel generation, bioprospecting necessitates high-throughput isolation methods. Using modern algal culture, the raw feedstocks for bioresource processing are adjusted to fit regional needs or inadequacies. Many species produce high-quality proteins, vital fatty acids, and vitamins that are used to augment local nutritional requirements. The approaches mentioned include isolation of single cells by serial dilutions, atomized cell spray, micromanipulation, and gravimetric separation. To make the isolation and characterization of algae species more accessible, new technologies such as robotics and flow cytometry, as well as new methods of sequencing and microfluidics, are being developed.

3.2. Molecular and Metabolic Engineering Approaches

3.2.1. Lipids and Fatty Acids Biosynthesis Pathway Engineering

Increasing the availability of fatty acid metabolic precursors such as acetyl–CoA and malonyl–CoA has been thought of as the primary step in lipid production, as fatty acids are the fundamental components of lipids. As the synthesis includes biochemical reactions, it is facilitated by various enzymes. Over-expression of these enzymes would increase enzyme activity, increasing lipid storage. The enzyme acetyl–CoA Carboxylase (ACCase) is most often used to promote lipid formation in microalgae [63]. The overexpression of ACCase had a lesser impact on lipid synthesis. The primary rate-limiting step in fatty acids synthesis is the stage when acetyl–CoA is carboxylated by the enzyme ACCase to form malonyl–CoA, which is a crucial carbon donor for this elongation of the acyl chain. The enzyme acts on malonyl to change into malonyl–ACP. This malonyl-ACP acts as for fatty acid biosynthesis of the substrate and catalyses the extension of acyl chains fatty acid synthesis [64,65]. The enzymes known as thioesterases hydrolyze the acyl–ACPs into free fatty acids, stopping the elongation of the fatty acid chain. To increase fatty acid synthesis, MCAT and ACCase are the primary targets for enhancing production [65]. The number of fatty acids in transgenic *Schizochytrium* improved by 11.3 percent due to overexpression of ACCase. Additionally, adequate production of MCAT in oleaginous *Nannochloropsis oceanica* caused the lipid content of modified strains to increase by 31%. It is essential to modify the fatty acid profile of lipids. As for the utilization of biodiesel, shorter-chain-length fatty acids have better fuel characteristics. The use of thioesterases to modify lipids' fatty acid chain length has been extensively studied to enhance the fatty acid [66,67].

D. salina's lipid production was successfully increased by the coordinated overexpression of malic enzyme (ME) and ACCase component. The generation of lipids by *P. tricornutum* is said to increase by 2.5 times when the malic enzyme is over-expressed without adversely affecting the growth rate. Co-expressing five yeast acyltransferases in *C. minutissima* led to one of the breakthroughs in this field since it doubled lipid synthesis

while maintaining growth rate. As for the lipid metabolism in microalgae, it is incredibly complicated, and it is obvious that suitable strains were produced before venturing into high-production biodiesel; a deeper understanding of the metabolic pathways is required [68]. The majority of the fatty acids in *P. tricornutum* have 16-carbon chains, with C16:1 accounting for roughly 50% of the total, and C16:0 for nearly 25%. The majority of the remaining fatty acids are composed of C20:5 and C14:0. Recent studies have made significant progress in carbon accumulation into primary storage molecules such as starch and lipids and identifying the major factors responsible for the well-documented triacylglycerol (TAG) accumulation produced by nitrogen shortage.

By enhancing the expression of thioesterase, an endogenous enzyme, the total fatty acid content was increased by 72% without changing the relative chain length in the diatom *P. tricornutum*. Triacylglycerols (TAGs) are considered promising precursors for biofuel synthesis because they have a high energy storage capacity and are made up of energy-dense acyl molecules [69,70]. Glycerol-3-Phosphate Acyltransferase (GPAT), Lysophosphatidate Acyltransferase (LPAAT/AGPAT), and Diacyl Glycerol Acyl Transferase (DGAT) are found in regions of the endoplasmic reticulum. The GPAT first turned the fatty acids produced by ACCase, MCAT, and FAS into lysophosphatidic acid (LPA), which was then further transformed into LPAAT (PA). The resulting PA was subsequently dephosphorylated by the enzyme Phosphatidic Acid Phosphatase (PAP) to produce diacylglycerol (DAG). The enzyme diacylglycerol was then transformed into triacylglycerol acyltransferase (DGAT), which is regarded as the major rate-limiting enzyme in TAG production. Therefore, modification of the TAG metabolic pathway is important in increasing TAG content. In modified *P. tricornutum*, GPAT overexpression increased the lipid content by a factor of up to two [71].

Due to the substrate-specific activity of overexpressed AGPAT in *P. tricornutum*, overexpression of AGPAT caused a 1.81-fold increase in TAG concentration and a striking shift in fatty acid profile [72]. The formation of plastoglobuli and cytosolic lipid droplets was seen in *P. tricornutum* after over-expression of chloroplast-localized AGPAT1 caused TAG over production with a particular elevation of C16:0. The development of algal strains used for industry depend on the significance of the chloroplast-localized acyl transferases. The TAG biogenesis and their capacity to enable excessive production of TAGs with specific fatty acid profiles have been documented. In engineered *N. oceanica*, type 2 DGAT overexpression increased lipid content by 69%. Manipulating the TAG pathway has been thought to be the key to increase algal lipid content; intricate details such as the redistribution of carbon precursors between subcellular organelles and the presence of the TAG pathway in plastids in addition to the endoplasmic reticulum have suggested the need to understand the TAG pathways and the role of subcellular organelles in regulating TAG pathways.

3.2.2. Engineering Photosynthetic Capability

Enhancing algal biomass production for biofuel generation involves maximizing photosynthesis's ability to catch the light. One method to increase the effectiveness of photosynthesis is to expand the light energy absorption range of the photosynthetically active pigments. It also illustrates the possibility of selecting microalgae with such capabilities for biofuel generation; in *Acaryochloris marina*, it was found that chlorophyll f absorbs light in the near-infrared area. Direct photodamage and reactive oxygen species formation come from the energy that cannot be dispersed, also known as photoinhibition. As they inhabit low-light situations, microalgae have evolved huge light-harvesting complexes (LHCs) to maximize light absorption [73]. In LHCs, more energy is released by heat generation and fluorescence quenching under artificial culture conditions (saturating light). As the LHC size increases, the possible cell density decreases, which also restricts light penetration into the growth medium [74]. Less fluorescence quenching is observed, increasing the photosynthetic quantum yield.

Transformed cells expand more quickly and are less prone to photo inhibition in high-light environments, but they do not achieve larger cell densities. These microalgae produce a large amount of carbohydrate biomass, making them ideal for use as a fourth-

generation feedstock for creating bio-hydrogen. Because of their photosynthetic efficiency, microalgae strains that have recently been genetically engineered are promising sources for a future bio-hydrogen generation [75]. Researchers are becoming interested in the potential of microRNAs for hydrogen generation. Photosynthetic microalgae are developing as a promising biomass feedstock for long-term biofuel and value-added bioproduct production. CO_2 bio-mitigation by these microalgae is considered a significant environmentally beneficial potential choice to current carbon sequestration methods [76,77]. Microalgae's poor photosynthetic output has made this technique impractical for CO_2 bio-mitigation.

Genetic Modification of NADPH Generation

Table 1 provides the genetic or metabolic approaches to enhance microalgae's lipid productivity. In *P. tricornutum* the synthesis of lipids rose to up to 55.7 percent dry weight due to over-expressing glucose-6-phosphate dehydrogenase (G6PD) from the pentose phosphate pathway. It was discovered that the G6PD was concentrated more in the chloroplast, with a high quantity of lipid bodies, pointing to the buildup of neutral lipids [78]. A technique for enhancing lipid accumulation I microalgae is to suppress lipid metabolism. For instance, selectively inhibiting a multifunctional lipase/phospholipase/acyltransferase enzyme enhanced lipid yields without impairing *T. pseudonana* growth. When nutrients are limited, photosynthesis's production of NADPH is utilized to make fatty acids, which are then used to make TAG, replenishing the supply of NADP+. In Chlorella, it has been demonstrated that the carbon flow from carbohydrates is diverted toward neutral lipid biosynthesis via pyruvate, the essential precursor for acetyl CoA [79]. Additionally, the mitochondrial TCA cycle is upregulated along with increased fatty acid degradation, which could improve the utilization of carbon skeletons for neutral lipid accumulation.

3.2.3. Genetic Transformation Approaches

Using molecular biology to genetically change microalgae is one method for lipid productivity. Researchers have recently been paying close attention to various genetic-engineering methods since they are considered innovative and extremely adjustable tools. Several bioengineering technologies have been used to modify microalgae genetically: Transcription Activator-Like Effector Nucleases (TALEN), Zinc-Finger Nucleases (ZFN), Random mutagenesis, and Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR associated with the protein 9 (CRISPR—Cas9) are all methods for changing the sequence of genes, as provided in Figure 5. Manipulation of genetic coding will result in a change in metabolic pathway flow toward the targeted chemical. Genomic editing using current technologies has been reported and confirmed in *D. salina* and *C. reinhardtii* using knockdown technology [80]. Diatoms are important in biotechnology because they have physiological characteristics that allow them to gather a large amount of lipids. The main goal of genetic manipulation is to improve the oil-related characteristics of these microalgae [81]. The discordant expression of a functional glucose carrier in the obligate photoautotrophic diatom *Phaeodactylum*, which permits microalgae to thrive on glucose without light, is one step forward. Using blue–green microalgae mosquito larvicides is another example of effective genetic modification of microalgae. Some researchers have used a critical systemic technology to generate huge algal biomass concentrations for long-term commercial uses and to change the pathway to produce more predicted high-value goods [82].

Table 1. Genetic or metabolic approach for enhanced lipid productivity in microalgae.

S.No	Microalgae	Lipid Productivity (mg L^{-1} d^{-1})	Production Process	Operational Parameters	Genetic/Metabolic Approach	Targeted Genes	Value-Added Product	Reference
1	*Chlorella minutissima*	1.37	Continuous	Temperature—25 °C–30 °C, pH—5 to 8 Mixotrophic	Molecular	Co-expression of five acyltransferases	Biodiesel and also antioxidants	[16]
2	*Chlorella sorokiniana*	0.85	Batch	Temperature—25 °C pH—6 heterotrophic	Metabolic	Ribulose-bisphosphate carboxylase and acetyl–CoA carboxylase	Biodiesel	[16]
3	*Neochloris oleoabundans*	1.13	Batch	Temperature—25 °C pH—8 Mixotrophic	Molecular	Glycerol-3-phospahte acyltransferase	Triacylglycerols Biodiesel	[43]
4	*Chlorella vulgaris*	0.91	Batch	Temperature—25 °C pH—10 Autotrophic	Molecular	Carbonic anhydrase	Biodiesel	[21]
5	*Chlorella pyrenoidosa*	1.45	Fed-Batch	Temperature—25 °C–30 °C pH—7 to 10 Mixotrophic	Molecular	NAH (H) kinase	Biodiesel and PUFA	[43]
6	*Phaeodactylum Tricornutum*	1.11	Batch	Temp—25 °C pH—8 to 10 Mixotrophic	Molecular	Pyruvate dehydrogenase	Biodiesel	[16]
7	*Chlamydomonas reinhardtii*	109	Semi-continuous	Temp—25 °C pH—5 to 10	Molecular	acetyl–CoA-synthetase	Biodiesel	[44]

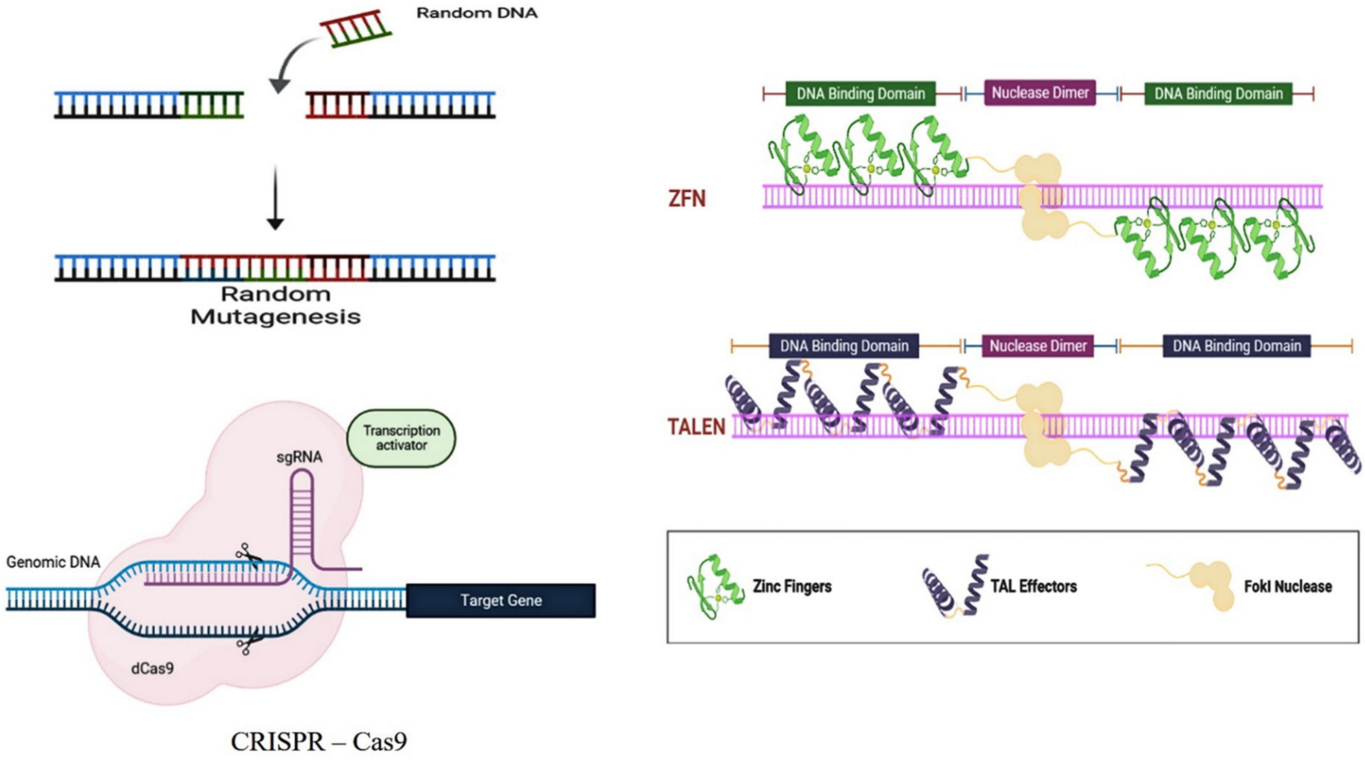

Figure 5. Various genetic transformation approaches in microalgae.

Trans-conjunction, natural and induced transformation, biolistic transformation, microinjection, electroporation (or electro permeabilization), silicon carbon whiskers method, glass beads, artificial transposon method, agrobacterium tumefaciens-mediated genetic transformation, and recombinant eukaryotic algal viruses are all examples of transformation methods used in the transformation of microalgae. The application of selected markers, such as fluorescent/biochemical markers and/or antibiotic resistance, considerably aids the efficient separation of genetic transformants. Because many microalgae have developed resistance to a wide spectrum of antibiotics, the number of medicines used to treat a single strain may be substantially smaller [83]. Enhanced gene silencing tactics in *C. reinhardtii* are one of the most significant advancements in microalgal genetics. Recently, a high output of synthetic miRNA (amiRNA) for reduced gene expression was reported, which is distinctive and secure. The use of RNAi reduced the cell response to gene expression in *C. reinhardtii*: however, synthetic silencing of comparable expression forms was widespread, resulting in varied silencing efficacy. Recently developed amiRNA techniques could be employed as a selection method in active genomics investigations of *C. reinhardtii* and other strains to lighten common metabolic pathways, such as those lined to biofuel production [84]. It intends to boost its lipid production output through metabolic and genetic engineering.

In microalgae, the main steps in lipid biogenesis are as follows. 1. carboxylation of acetyl–CoA to malonyl–CoA carboxylase (ACCase) is considered the first step in fatty acid biosynthesis, followed by 2. fatty acids production via a series of reactions catalyzed by type-II Fatty Acid Synthase (FAS), and 3. Triacylglycerol formation. The diatom *Cyclotella cryptica* was the first algae to have the ACCase gene over-expressed to increase fatty acid synthesis in stressful environments. For the metabolic engineering of diverse microorganisms, including microalgae, the following protocol is utilized: 1. Selection of the host strain and determination/formulation of nutritional requirements, 2. information on the host's characteristics is gathered, and 3. random mutation and selection are carried out. For fatty acids in microalgae, biosynthetic-manipulation approaches include lipid secretion from medium cells, over-expression of the main enzymes involved in fatty acid biosynthesis, increased accessibility of already-existing molecules such as acetyl–CoA, reduced

expression of genes involved in fatty acids catabolism by preventing lipase hydrolysis or oxidatin, and changing saturation profiles by preventing oxidation or lipase hydrolysis [71]. Despite these advances in the production of biologically active materials and valuable crops, there are at least two obstacles to microalgae genome manipulation: the growth level of microalgae and the optimal expression of external microalgae genes [85]. One of the primary steps toward producing sustainable and economically viable biofuels from microalgae is metabolic engineering through genetic alteration.

3.2.4. In Silico Metabolic Engineering Tools

Secondary metabolite studies on steroids, lectins, fatty acids, carotenoids, polyketides, polysaccharides, and toxins are performed under microalgal metabolomics. Plenty of new bioinformatic tools are increasingly becoming available, allowing researchers to define gene regulatory networks, predict metabolic shift results, and functionally annotate genomes of various algae species. In a metabolic design that compromises over 1000 genes across ten different cellular compartments, metabolic networks with detail in silico models were utilized to recognize rate-limiting stages in starch metabolism and to anticipate the light-induced metabolic reaction to various wavelengths. In metabolomics studies, NMR-based analysis plays an important role, as a recent study using NMR found that changing the f/2 nutrients medium and daily average solar output improved the synthesis of amphidinols and other biological compounds such as fatty acids, oxylipins, carotenoids, and other compounds from the microalga *Amphidinium carterae* [86].

Metabolic flux analysis (MFA) is a sophisticated approach that uses a radio-labeled tracer and stoichiometric constraints to provide insights into flux distribution in microorganisms. MFA also detects intracellular flow changes in the context of changed cellular responses, such as metabolite overproduction. Previously, methods for balancing variability around cellular metabolites in the stoichiometric network were utilized for MFA, but the fundamental restriction was the determination of variability in parallel or reversible processes. For C-MFA investigations, cells are currently fed a tagged substrate or a combination of substrates labeled at one or more carbon atoms. In recent years, MFA has progressed by incorporating new experimental and measuring techniques, computational technologies, mathematical data assessment methods, and algorithms. Most of these enhancements necessitate extensive abilities in using NMR techniques and computational and statistical expertise [87]. Despite advances in MFA investigation, the analysis of microalgae's internal metabolic/physiological activity remains challenging, and the entire process must be significantly accelerated to provide more pragmatic results. Overall, the accessibility of metabolic replica and in silico techniques for recognizing critical lipid metabolism residues can play a significant role in establishing features and providing necessary information. Wet lab investigations must, without a doubt, validate the insights obtained by in silico metabolic engineering on microalgal lipid metabolism.

A reliable genome-scale model (GEM), which is typically built using stoichiometric techniques based on annotated sequences, can assess the system-wide effects of environmental and genetic perturbations under the various situations of an organism. By assuming it is in a pseudo-steady state, constraint-based methods such as flux balance analysis (FBA) can be used to analyze a genome-scale biochemical network that needs the mass/charge balances of the metabolites in biochemical pathways and the stoichiometry matrix (S) of metabolic reactions. Genome-scale metabolic modeling is currently being investigated as a promising method for the investigation of metabolic pathways in the fields of chemical, disease, and environmental research. In general, biochemical reaction knowledge (S matrix), species-specific information from genomic annotations, and high-throughput experimental data from publically accessible databases such as EcoCyc, KEGG, BRENDA, and BKM-react are needed for metabolic model reconstruction. These days, metabolic models can be accessed by importing them into an FBA software program such as COBRA Toolbox. These models are available in systems biology markup language (SMBL). The newly rebuilt model uses gap-filling techniques to increase accuracy, which boosts connection to the

point that the model can imitate phenotypes. In silico experiments can be performed to predict flux distribution in various metabolic pathways and phenotypic behavior under various user-interested settings after building an improved functional model. Additionally, this model may be used to calculate knockout lethality or growth rates, which can then be further evaluated using experimental data to pinpoint potential gene targets for increasing strain efficiency.

3.3. Transcriptional Regulations

It was recently proposed that metabolic pathway regulation should be investigated in the circumstances of the entire cell, rather than at the pathway level. The use of regulatory elements such as transcriptional factors (TFs) to regulate the quantity or activity of various enzymes in creating desired products has piqued interest. TFs are proteins that bring protein DNA and protein interaction after recognizing certain DNA sequences to regulate DNA transcription. They have been divided into 50 groups based on their DNA-binding domains and conserved structure [88]. They can bind with transcription factors such as the enzyme DNA polymerase and activate them to increase the transcription rate of certain groups of genes. They can also operate as repressors or cause minor alterations in a metabolite synthesis without completely suppressing it. A single metabolic pathway is usually regulated by a group of transcription factors. The production of transcriptional factor (TF) that can regulate several metabolic nodes in referred to as transcriptional engineering (TE). Though TFE studies in microalgae are calm in their infancy, multiple TFs were found to induce the excess-production of beneficial metabolites in various species, and different TFs for lipid biosynthesis control have been identified in mammals, plants, and microbes [89,90]. TFE may be able to use these findings to improve microalgal lipid production. Leucine Zips (zip) TFs regulate stress responses and metabolic activities, including lipid synthesis. They naturally tend to build up an excessive amount of lipids. Transcription factors garner more attention as essential regulators of metabolic pathways to increase the yield of high-value compounds or boost the production of foreign proteins in microalgae. In *N. salina*, overexpression of NsbZIP1, a transcription factor containing the basic leucine zipper, led to increased growth rate and fat content [91]. About 70 TF genes implicated in nitrogen-deprivation control were discovered in *C. reinharditii* utilising a combined omics (transcriptomic, proteomic, and metabolomic) investigation. Many genes involved in lipid biosynthesis are thought to be regulated by Dof-type transcriptional factors that bind directly with DNA in their promoter regions [92]. The author discovered that a Dolf-type TF (GmDol4) dramatically boosted Arabidopsis lipid production and that the acetyl–coenzyme A carboxylase was involved. In microalgae *Chlorella ellipsoidea*, a combination of overexpression of GmSolf4 and mixotrophic culture conditions revealed that lipid content rose from 46.4% to 52.9% with no effect and no growth rate. Furthermore, the study revealed that in transgenic *C. ellipsoidea*, the expression of 754 genes was up-regulated. In contrast, the expression of 322 genes was down-regulated, suggesting that GmDolf4 may regulate genes involved in fatty acid, lipid carbohydrate, and protein metabolism. Some of the thoroughly researched DNA-binding proteins are zinc-finger protein transcription factors (ZFP TF), which often have multiple fingers lined in tandem. The zinc-finger domain is found in the proteomes of many different animals, and it allows diverse proteins to combine or interact with DNA, RNA, or other proteins [93,94].

Zinc-finger proteins are further divided into categories per the amount and order of Cys and His residues that bind the Zinc ion. C2H2-type zinc-finger proteins are one of the largest families of TFs in plants, with 176 members in Arabidopsis thaliana alone. Overexpression of a ZFP TF that binds an aDNA sequence inside the promoter region of a therapeutic protein from a mammalian production cell line resulted in enhanced production of therapeutic protein [95]. The ZFP TF increased the protein production of CHO cells by more than 100%. ZFP-mediated increases in protein production were increased by up to 500% using expression vectors with up to 10 ZFP binding sites. Plants such as *Arabidopsis. thaliana* have been investigated to discover how MYB and bHLH transcription

factors affect flavonoid production, specifically anthocyanin and seed coat tannin. The buildup of anthocyanin was observed when the genes R and C1 encode a BhlH and an MYB protein ectopically expressed in typically non-pigmented cell lines [96]. This happens to be the result of a coordinated reaction to TFs in the form of global structural gene expression. In Arabidopsis, over-expression of MYB resulted in a considerable increase in flavonoid production. SebHLH protein, a member of the bHLH family of transcription factors, has been shown to have a vital role in the transcriptional control of genes involved in the production, storage, and gathering of lipids during the development of seeds in plants [97–102]. CHT7 is a transcription factor that regulates quiescence and proliferation in both nutrient-depleted and nutrient-rich environments, and *C. reinhardtii* mutation of CHT7 can increase starvation-induced TAG buildup without limiting biomass yield. The TAR1-deficient *C. reinhradtii* mutant had a more dramatic cell division arrest, resulting in a 100%-greater TAG production. The high-level TFs influence the low-level TFs in a pyramidal structure of TF classification. As various species have different TFs for lipid regulation, the TFs that regulate lipid production are low-level TFs. Analyzing the transcriptomics of a target microalga under a controlled environment that permits and prevents the production of metabolites of our choice is a popular technique for TF discovery. The TF method is a hope of technology that has the potential to be an innovation that allows for cost-effective microalgal oil production [103,104]. However, still it is in its infancy and finding TFs that regulate microalgal lipid biosynthesis will be the first step.

3.4. Gene Editing Tools for the Development of Biofuel from Microalgae

Integration of systems approaches is required to achieve an algal variety by producing 'designer algae', which can cater to our need for a sustainable biofuel feedstock.

3.4.1. CRIPSR Associated Lipid

The Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated CRISPR/Cas system is a modern developed gene-editing tool that has led and been helpful in the fields of molecular biology and biotechnology. The RNAi mechanism developed in *C. elegans* by Fire and Mello has been widely used to silence any gene sequence. It begins with an overview of double-stranded RNA entering into a cell; then, it is shown to increase into small-interfering RNA (siRNAs) by enzymatic degradation in the RNAi mechanism. The lipid-producing enzyme is targeted to increase lipid synthesis through a genetic-engineering approach [105–112]. Genes involved in synthesizing acetyl–CoA are a good target for genetic alterations as a biofuel production goal. Synthetic circuits are required to synthesize lipids in microalgal cells. Those synthetic circuits are transcription factors that function as a starting point for genetic alteration to increase lipid synthesis. The CRISPR/Cas9 method for targeted gene modification was initially established in *C. reinhardtii*, employing Cas9 expression and single-guide RNA according to reports (sgRNA) (3.4.1.24). Using *Chlamydomonas* as a model, it was also discovered that the composition of Cas9 ribonucleoproteins and sgRNA could be used to achieve the target efficiency of CRISPR/Cas9. Chlorella and Chlamydomonas have been used in most gene-editing experiments in microalgae for lipid enhancement. Other microalgae, such as *Scenedesmus*, *P. tricornutum, D. salina*, and *N. oceanica*, are used as model organisms for the synthetic pathway in *C. reinhardtii*; over 30 nuclear genes were silenced the RNAi system was shown to be a valuable tool for analyzing microalgae genetics. Regarding gene editing, the CRISPR/Cas9 technique is a valuable and efficient tool. It is feasible to double the lipid synthesis using algae by altering the genes, leading to a third-generation biofuel [113,114]. CRISPR/Cas9 might be a viable new source of energy.

3.4.2. CRISPRi Technology

The CRISPR interference (CRISPRi) method employs a nuclease-deficient Cas9 (or dead Cas9) that lacks the capacity to break DNA and instead serves as a DNA-binding complex for gene interference rather than gene regulations (3.4.1.24). Without damaging

the dsDNA, the CRISPRi process allows gene editing. CRISPRi was initially used on *C. reinhardtii* CC400 in 2017 to increase lipid synthesis by efficiently repressing the CrPEPC1 gene [115–117]. CRISPRi has been used to suppress several genes in *Synechicystis* sp. PCC 6803. Some researchers used CRISPR and CRISPRi systems to increase succinate synthesis in *Synechococcus. elongatus* PCC 7942. A CRISPRi-mediated CrPEPC1 strain produced a high lipid yield of 28.5 percent compared with the wild type of CrPEPC1 [118].

3.4.3. ZFN-Mediated Lipid Enhancement

Zinc-finger protein (ZFN) recognizes DNA/protein. The design is very challenging. The targeting efficiency is low and variable. However, the cost is much too expensive. Traditional genome-editing technologies, such as Zinc-Finger Nucleases (ZFNs), used to modify the genomes of microalgae's nuclear, mitochondrial, and chloroplast genomes are comprehensively reviewed (Ng, n.d.). Engineered nucleases such as ZFN, TAL effector endonuclease (TALEN), and clustered regularly interspaced palindromic sequences (CRISPR)/Cas9 have substantially aided genome editing [119–123]. In *C. reinhardtii*, ZFN-mediated gene editing was employed to boost lipid synthesis [2].

3.5. Co-Culturing Techniques

3.5.1. Microalgae and Microalgae

Coculturing of microalgae is performed by cultivating a microalga alongside other microalgal strains, which has been shown to increase the production of lipids. As per a study carried out by ref. [124], depending on the cultivation technique, a binary culture of *Chlorella* sp. *U4341* with *Monoraphidium* sp. *FXY-10* had different results in lipid productivity. For a binary culture, the primary cell count for *Chlorella* sp. and *Monoraphidium* sp. were 3.41 10^{-6} cells mL1 and 4.26 10^{-6} mL1. This improves the lipid synthesis by binary culture with two microalgal strains, as explained by symbiosis, where the production yield of lipid is interconnected under co-culturing conditions. The superiority of the microalgae–microalgae binary culture over monoculture is owing to the culture being dense and having a faster growth rate. The method also aids in bioremediation and facilitates bio-flocculation for the increased production of lipid and biomass gathering [125–130]. Excessive exopolysaccharide (EPS) accumulation can hinder mass transport, preventing the nutrient level that eventually dissolves the CO_2, which will be inaccessible to microbes. Hence, this co-culture technique should be selected cautiously, and large-scale applications are limited.

3.5.2. Microalgae and Bacteria

The consortia between bacteria and microalgae are much too complicated. Some bacteria can produce hormones that encourage algal development. When *C. vulgaris* and *Azospirillum brasilense* were immobilized in alginate beads, the number of algae, colors in algal cells, and the size of microalgal colonies all rose dramatically [131]. Bacteria serve as an exogenous supply for specific chemicals such as biotin, thiamine, and cobalamin, clarifying the relationship between algae and bacteria. In this co-culturing technique, a vitamin-based symbiotic relationship was recorded that connects the green alga *Lobomonas rostrate* and the *Mesorhizobium loti* and between the *C. reinhardtii bacterium* and *Sinorhizobium meliloti 1021*. It was discovered that bacteria-produced indole -3- acetic acid enhanced the interaction between the bacteria and microalgae by promoting growth and lipid productivity [132]. After tuning the parameters for the oleaginous microalgal strain, the co-culture technique of *Ankistrodesmus* sp. and Rhizobium strain 1022 demonstrated a nearly 30% increase in overall weight gain and lipid output, with the lipid biomass reaching 112 mg L^{-1} d^{-1} [3].

In comparison to algal monoculture, Santos et al. [131] performed coculturing for the *C. reinhardtii* stain CC849 with B. japonicum, which recorded an increased microalgal growth to the extent of 3.9 × 107 cm^{-3}, resulting in a 26% increase in microalgal biomass yield which is 14-fold higher in hydrogen production. The rise in the respiration rate of the binary culture complex, which may have resulted in excess O_2 utilization and the gradual

establishment of an anaerobic environment in the system, was responsible for the increased growth and lipid output. Higher Fe-hydrogenase activity was induced by the anaerobic environment, resulting in increased H_2 generation [133–136]. The reduction of chemical-oxygen demand (COD) is successful with this consortium. Again, it was discovered that a co-culture of microalgae and bacteria that produces bioflocculant outperformed a bioflocculant-free bacterial companion in terms of COD removal and concurrent complex chemical breakdown [137]. The abovementioned technique also ensured that residual nutrients and biomass from downstream processing were recycled and could be used for additional algae biomass, lowering energy expenditures.

3.5.3. Microalgae and Fungi

This binary culture system has leveraged the synergistic interaction between microalgae and fungi for growth and lipid increase. The fungi partner eats the accumulated carbon by the microalgae in the medium via photosynthesis. In contrast, the fungi protect the microalgae and serve as a habitat for mineral nutrients owing to the fungi's water-retaining ability. Lichen constitutes an excellent synergistic association among microalgae and fungi. Binary culturing coprophilous fungi, such as *Byssochlamys* sp. *F52* with *Cladosporium* sp. *F1*, minimizes sugar production. That pathway can then be diverted toward lipid biosynthesis under a controlled and specific environment [138]. It is channeled to co-culturing with oleaginous microalgal strains to obtain enhanced lipid yield. Lichen, as previously mentioned, constitutes one of the most effective algae–fungi symbiotic partnerships, which has the extra benefit of degrading cellulose and lignin. Due to its increased enzyme activity, white-rot fungus revealed a considerable delignification pattern. The issue of lipid-yield augmentation is addressed by interconnecting lignin degradation with lipid fermentation via a co-culturing technique, which significantly minimizes the running cost for lipid extraction because pretreatment processes are reduced [139–143]. Few filamentous fungi have the potential to self-pelletize, which is an algae fungus cultured together that has been proven to provide microalgae bio-flocculation. The fungal partner in the binary culture system's enhanced flocculating advantage also solves one of the biggest hurdles in algal biofuel commercialization: the incompetence to harvest microalgal cells on a scale-up system due to the dilute algal culture. It is stated that the co-cultivation of microalgae with fungus is a more promising idea for increasing the biomass and lipid productiveness for biofuel generation and bioremediation [144]. Furthermore, molecular research is necessary to investigate the deeper aspects of the interactions between microorganisms to commercialize the entire process, from microalgal culture to biofuel production.

3.5.4. Microalgae and Yeast

Microalgae characterized by releasing more oxygen and other organic exudates inside the system encourage co-culture aerobic yeast development. In turn, the yeasts release CO_2 through organic compound fermentation, prompting microalgae growth and lipid synthesis. In a mixed culture environment, parameters such as O_2–CO_2 equilibrium, pH balance, dissolved oxygen, and substrate exchange significantly impact the symbiotic relationship and synergistic effects on cell development [145–147]. In comparison with monocultures, the co-culture system for *Spirulins plstrndid and Rhodotorula. glutinis* for higher biomass and lipid escalation resulted in an appreciable increase in total biomass and total lipid accumulation, with a concurrent decrease in COD of 73% and nitrogen consumption of 35%. As ventilated with CO_2-supplemented air from its heterotrophically grown culture, *C. protothecoides* showed an increase in lipid yield and cell growth of more than 55% for both biomass and lipid yield when compared with the bioreactor supplied with air. Co-culturing *C. vulgaris* and *vulgaris TISTR 8261* with *T. spathulate* produced the best biomass in a huge-percent-mass fraction lipid content of dried cells out of all the combinations investigated [148,149]. The overall lipid output was significantly increased in the co-culture system for the oleaginous yeast *R. glutinis* and *C. vulgaris*. The co-culture technique permits waste remediation, bio-flocculation, and other processes in addition to biofuel production.

Along with microalgae-fungi binary cultures, microalgae-yeast co-cultivation has proven to be one of the most promising strategies for improving biomass and lipid output [150]. The proper implementation of the experiment also necessitates parameter adjustments and deep molecular analysis to understand better the microorganisms' interrelationships.

4. Biotechnologically Enhanced Lipids as the Substrate for Biodiesel Production

Microalgal biotechnology looks to have a lot of potential for making biodiesel since heterotrophic growth and genetic engineering techniques can significantly boost microalgae's lipid content [151,152]. Due to their high oil output and relatively little land required for their growth, microalgae appear to be one of the most encouraging feedstocks for producing significant amounts of lipids that may then be used to create sustainable biodiesel to replace fossil fuel. They expand rapidly, and some species are highly oil-rich. They can quadruple their biomass in 3.5 h during the exponential growth phase. However, it takes them 24 h to double [153]. In certain species, that includes *Botryococcus, Nannochloropsis*, and *Schizochytrium*; the oil content of microalgae is as high as 80% by weight of the dry biomass. Microalgae can generate a wide variety of lipids.

Long-carbon-chain lipids are suitable for the manufacture of biodiesel. Palmitoleic (16:1), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids make up the majority of the unsaturated fatty acids found in microalgae oils. Low concentrations of saturated fatty acids such as palmitic (16:0) and stearic (18:0) acids are also detected [154]. Microalgae have high lipid contents, yet their photosynthetic activity makes them more dependent on land than other oleaginous microorganisms. Daily and seasonal fluctuations also impact their growth because they need sunlight. Additionally, they require low-density cultures, which necessitate large amounts of water, and the manufacturing cost will rise. Compared with many terrestrial plants, microalgae offer many advantages when it comes to biodiesel generation. Choosing appropriate microalgal species, scaleup culture systems, biomass harvesting, fatty acid extraction, and transesterification methods are among the first steps in biodiesel manufacturing using microalgae as a feedstock [155]. Technology advancement and process optimization are required to lower total manufacturing costs. In microalgae, genetic engineering may also be used to manipulate lipid production. Many methods have proven their potential for cost reduction, such as sequestering CO_2 from factory facilities as a carbon source, utilizing wastewater as a fertilizer source, and maximizing the value of by-products. Microalgae create microbial lipids, and lipid yields are enhanced by providing optimum amounts of carbon sources [156,157]. As a result, utilizing low-cost organic chemicals found in organic waste with oleaginous microbe production might be a potential way to achieve commercial scale-up.

5. Integrated Approaches (Microalgae Lipid and Pigment Production)

5.1. Lipids in Microalgae

Polar and neutral lipids are divided into two classes. They are not soluble in water but are insoluble in the majority of organic solvents. Phospholipids and glycolipids are examples of polar lipids, while acyl glycerides and fatty acids are some examples of neutral lipids. Polar lipids are used in cell membranes, and microalgae use neutral lipids as an energy source [158]. However, fatty acid components such as pigments found in microalgae are not synthesized into biodiesel. Because of this, reduced fatty acid synthesis is implied by increased pigment production. This indicates that even though microalgae generate high lipid content, a high biodiesel output is not necessarily the result. Accordingly, the lipid content proportion per microalgae's dry weight can range from 1.5 percent to 75 percent. A particular microalga can produce varying lipids depending on the growth medium and method [159]. For example, *Chlorella vulgaris* had a lipid level that ranged from 12 to 26 percent, while *Botryococcus braunii* had a lipid content that ranged from 14 to 75 percent. Unicellular such as *Auxeno chlorella protothecoides, C.vulgaris, C.reinhardti,* and *D. salina* help manufacture biodiesel. Some algal species and lipid productivity are detailed in Table 2.

Table 2. Various types of inducers in microalgae to enhance biomass productivity and lipid yields.

S.No	Microalgae	Culture System	Biomass Productivity	Lipid Productivity	Lipid Content	Type of Inducer	Reference
1	*Chlorella vulgaris*	Algal bloom hydrolysate	436 mg $L^{-1}d^{-1}$	188 mg L^{-1} d^{-1}	53%	Nitrogen starvation	[92]
2	*Monoraphidium dybowskii LB50*	BG-11 medium	80.56 mg $L^{-1}d^{-1}$	31.12 mg $L^{-1}d^{-1}$	44.4%	Nitrogen starvation	[147]
3	*Chlorella sorokiniana*	Defined medium	300 mg $L^{-1}d^{-1}$	502 mg $L^{-1}d^{-1}$	~25% AFDW lipids	IAA cytokinin kinetin (K)	[90]
4	*Tetraselmis* sp.	Charcoal -filtered seawater with nutrient enrichment	201 mg $L^{-1}d^{-1}$	85.5 mg $L^{-1}d^{-1}$	45%	Salinity + Nitrogen	[91]
5	*Para chlorella*	$\frac{1}{2}$ SS nutrient medium	409-1291mg $L^{-1}d^{-1}$	161–604 mg $L^{-1}d^{-1}$	66%	Nutrient sulphur deprivation	[93]
6	*Chlorococcum* sp.	BG-11	175 mg $L^{-1}d^{-1}$	2.0–19.3 mg $L^{-1}d^{-1}$	56%	Nitrogen starvation	[94]
7	*Nannochloroposis* sp.	BG-11 medium(+ glucose or acetate)	90–145 mg $L^{-1}d^{-1}$	324 mg $L^{-1}d^{-1}$	18.16–25.49%	Salinity	[95]

Their lipid output is higher. Cyanophyta and Chlorophyta are considered for use in the manufacturing of biodiesel production [160]. Some create poisonous and bio-accumulative chemicals, such as the carcinogen microcystin.

5.2. Pigments in Microalgae

Microalgae are categorized based on their pigmentation, such as green algae including chlorophyll; blue and green algae have phycocyanin, red algae have phycoerythrin, and brown algae contain fucoxanthin. Greenish pigments containing a porphyrin ring are called chlorophylls [161]. Chlorophyll is the most prevalent pigment. It is found in almost every photosynthetic organism. It acts as the electron donor in the electron transport chain. Chlorophyll in microalgae ranges from 0.5 percent to 1.0 percent. Chlorophyllins control body odor for geriatric patients and dietary supplements [162–167]. Chlorophyll can indeed be used medicinally as an anti-inflammatory and wound-healing component. Phycobilin is water soluble and simple to extract and purify. Phycobilin is often utilized in industrial and immunology laboratories as it has absorption properties. It is often used as a fluorescent dye for microscopy, immunoassays, and markers in molecular biology. Carotenoids are pigments found in all algae and act as photoprotectors against oxidative damage. Some of the carotenoids from microalgae include carotenoids, astaxanthin, cantaxanthin, zeaxanthin, and lutein, which are commercially important. These pigments are used in the pharmaceutical, cosmetic, and food industries and are expensive. An oxidized form of carotenoid with a high capacity for oxidation is astaxanthin. It helps prevent and cure a range of ailments, including cancer, diabetes, metabolic syndrome, chronic inflammatory diseases, and gastrointestinal illnesses. The apoptosis rate in prostate cancer cells increased in β-carotene from *D. salina* as compared with synthetic carotene [4].

6. Economic and Commercialization Feasibility

As microalgae are lignocellulosic biomasses, they have been identified as one of the most adaptable raw materials for biorefinery systems. Hence, the bio-refinery technique will be required to isolate several segments of the cell to obtain non-biofuel lipids and other

possible value-adding products [168,169]. The entire process must be addressed to ensure a successful biorefinery, from selecting the suitable feedstock to the separation methods employed to the end-product's final form. However, modern technologies involving nanotechnology that produce pre-treatments to the cells or as fuel additives can reduce costs. Another method is to use mild cell disruption and extraction procedures that do not injure or denature macromolecules in the cell. Still, the approaches must be low-cost and energy efficient. Microalgae have potential properties such as high lipid yields and the ability to use low-quality soil and water and integrate with CO_2 sources. This simple attribute has attracted a lot of effort into improving the processing process of the production of microalgae, which could be utilized to yield biofuels and even other costly products [170]. The advancements and scale-up of the commercial possibilities of microalgae-based feedstock to fuel transformation have recently been the focus of attention. Microalgae biomass composition and conversion efficiency are crucial criteria for successful biorefineries, although environmental considerations also contribute. For a transitional stage from the laboratory scale to commercial production, feedstock and final productivity are the major factors taken into account in the techno-economic analysis (TEA) of the biofuel production process [27]. The right feedstocks are crucial since the breakdown of biomass to produce fermentable sugars affects the end cost. According to annual operating costs in biofuel facilities, raw feedstock and facility-dependent costs (insurance, maintenance, and overhead) are the biggest cost drivers [46]. According to a TEA comparing the entire production costs needed to produce ethanol, butanol, and isobutanol, feedstock contributed the majority (around 32%) of the whole cost [9]. Lignocellulosic biomass is a plentiful and affordable renewable resource that can produce biofuel in various industrial facilities [70].

According to estimates, 349.47 million t/year of maize stover, 354.36 million t/year of poplar, and 398.38 million t/year of switchgrass may each generate an average of 30 million gallons of fermentable sugars each year [61]. The price of breakdown procedures using acids, ionic liquids (IL), and enzymes is high in addition to the cost of the raw biomass itself. The price of the enzymes varies depending on the initial feedstock load. Based on saccharification and fermentation yields, enzyme cost contribution ranged from \$0.68 to \$1.47/gal when using acid-treated poplar as the feedstock for ethanol production [92]. The economic potential of IL-based processes in "water-wash" (WW) and "one-pot" (OP) was compared using a TEA, with the price of IL fixed at \$0.75/kg with 99.6% recovery and \$10.14/kg enzyme price. While both methods showed equivalent economic performance at greater feedstock loadings, the study concluded that OP was more cost-effective at a minimum ethanol selling price of \$4.5/gallon [18]. Algal biomass has also been demonstrated to be an economically viable feedstock source for biodiesel or ethanol production, with selling costs below \$5 per gallon and \$2.95 per gallon, respectively [123]. Several businesses, including Sapphire Energy, Algenol, and Seambiotic, produce bioethanol at commercial scale from algal biomass with an annual output of 1 billion gallons and costs of 85 cents/L.

Challenges such as raw material use, H_2O and land use, energy consumed, and emission of greenhouse gas from process-related energy utilization must all be considered as the scale-up for the biorefinery approach, which will also be environmentally sustainable [171,172]. The primary assessment tool for estimating the cost and determining the economic feasibility of algae biofuel is Techno-Economic Analysis (TEA) [173–177]. Numerous TEAs have been conducted on the viability of various methods for producing algae biofuels. Fuel prices range from \$0.44 per liter to \$8.76 per liter. Cost estimations are frequently based on literature, independently developed small-scale trials that have not been empirically validated, and numerous assumptions about growth rate, nutritional requirements, lipid production, and energy requirement.

Life Cycle Assessment (LCA) has become critical for maximizing the creation of biofuels from microalgae [15]. To reduce the environmental impact, questions have been raised about the varieties of microalgae suited for specific products, the sites for microalgae culture, and the change of process parameters. Various elements of microalgae biofuel and coproducts production have been studied in LCA studies, including energy, water

utilization, and environmental implications. The present cost of production per liter of biofuel produced from microalgae is still costlier than that of conventional fossil fuel, but, in the near future, this will become inverted; that is, an improved product can reduce the cost in the system and conversion techniques. Despite microalgae's potential as a fuel source, numerous modifications are needed to make the system more economically viable and sustainable [128].

7. Future Aspects

Altered microalgae as a feedstock have great potential, but also have a lot of drawbacks, such as varying cultural growth conditions, less productivity, and huge investment and management requirements [51]. Using phospholipase A2, the lipid yield of *C. reinhardtii* was increased by 64.25 percent. Single-gene overexpression or deletion has been the focus of the current genetic engineering work on microalgae lipid synthesis. However, it is unknown if this is the superior approach to improving lipid accumulation while maintaining cells [139]. The analysis of several metabolic engineering approaches for microalgae lipid increase indicated that multigene engineering is more favorable than single-gene manipulation. On the other hand, multi-gene alterations are still in their infant stages [16]. New species with increased fatty acid yield and quality are generated through molecular breeding due to the improvements in this unique scientific field. Furthermore, innovative genetic engineering strategies could improve photosynthetic efficiency in microalgae domestication [2,155]. In addition to changing the genome of unique microalgae strains, metabolic-engineering techniques promise to enhance biofuel conversion efficiency. The application of the carbon partitioning approach, combined with a higher inhibitory light threshold, has considerably improved microalgae growth and lipid productivity [34,77]. Using biomass from wastewater treatment in high-rate algae ponds has also been suggested to lower microalgae biodiesel production costs. When addressing energy accumulation in terms of both the lipid energy content and the light energy required, lipid synthesis from microalgae cultured in treated wastewater demonstrates low net energy recovery [117]. Microalgae cultivation is crucial to the biofuel supply chain and the cost of biofuel. Runoff from fertilizers and pesticides from conventional framing can contaminate surface water and cause algae growth in lakes and streams, undermining the environment and the social sustainability components of the entire production. These environmental challenges are the biggest priority for the future of microalgae-based fuel. A reasonable basis has been formed among the current research efforts in this field based on a detailed review of technologically feasibility and growth cost optimization, which will help as the first step for future research and development. Availability of land, production infrastructure, harvesting equipment, downstream processing, and costs (fixed and variable) should all be included when calculating operation costs [37,59,80]. Using sweet sorghum as a carbon source for microalgae cultivation is a new strategy. It is anticipated that sweet sorghum cultivation will cost between $0.027 and $0.48 [65,142]. The cellular structure and dynamics of microalgae have been targeted and modified using mutagenesis or transgenesis techniques, depending on the intended outcome [63,145]. External stress can stimulate metabolic variations in certain microalgae, which could teach the biomass with additional metabolites. Still, researchers are limited by the genetically modified organism's high environmental sensitivity when exposed to environmental factors, which could result in new metabolic pathways. Since the integrated approach provides superior processing of the various stages, such as breaking down microalgae biomass, separating and extracting metabolites, and synthesizing biofuels, developing an integrated biorefinery is the best way to develop an integrated biorefinery effective and economic strategy for microalgae-based biofuel production. Furthermore, advancements in integrated biorefinery pretreatment operations, such as biomass harvesting and drying, could result in significant energy and cost reductions. The integrated biorefinery can use active bio-components of microalgae other than lipids, such as proteins and carbohydrates, to convert them into value-added by-products, demonstrating that the increased economics gained from using high-value

added by-products from the microalgae-based alteration process can potentially help to address labor- and income-inequality issues [32,71,125]. TEA and LCA should be used to investigate problems associated with microalgae-based fuel and commercialization, despite the fact that LCA and TEA can only be carried out on pilot-scale projects using mixed-biomass feedstock in a combined closed-loop biorefinery with a multi-product retrieval scheme, limiting the potential charge and prediction of the challenges in scale-up commercial production. Microalgae typically contain harvested and used bioproducts, such as colors, vitamins, and antioxidants [51]. More research is required to enhance the potentiality of scaling up the existing processes and successfully executing industrial modules in the production of relevant microalgae sp. However, producing microalgae biomass has substantial challenges. The currently frequently utilized technology, self-flocculation or bio-flocculation, is reported to be efficient while remaining quite economical.

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