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Review

Latest Expansions in Lipid Enhancement of Microalgae for Biodiesel Production: An Update

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Abstract: Research progress on sustainable and renewable biofuel has gained motion over the years, not just due to the rapid reduction of dwindling fossil fuel supplies but also due to environmental and potential energy security issues as well. Intense interest in microalgae (photosynthetic microbes) as a promising feedstock for third-generation biofuels has grown over recent years. Fuels derived from algae are now considered sustainable biofuels that are promising, renewable, and clean. Therefore, selecting the robust species of microalgae with substantial features for quality biodiesel production is the first step in the way of biofuel production. A contemporary investigation is more focused on several strategies and techniques to achieve higher biomass and triglycerides in microalgae. The improvement in lipid enhancement in microalgae species by genetic manipulation approaches, such as metabolic or genetic alteration, and the use of nanotechnology are the most recent ways of improving the production of biomass and lipids. Hence, the current review collects up-to-date approaches for microalgae lipid increase and biodiesel generation. The strategies for high biomass and high lipid yield are discussed. Additionally, various pretreatment procedures that may aid in lipid harvesting efficiency and improve lipid recovery rate are described.

Keywords: microalgae; biodiesel; genetic modification; nanoparticles; pretreatment methods

1. Introduction

Algae is the world's largest photosynthetic group that contributes most of the carbon sequestration on the globe, converting greenhouse gases into carbohydrates and lipids. These photosynthetic microbes have received high attention as potential cell factories for fatty acids (FA) and carotenoid production. Microalgae oil is used as biodiesel and has significant advantages over vegetable oils. Biodiesel acquired from microalgae is sulfur-free and releases low hydrocarbon, CO, NOx [1], and Sox emissions in contrast to traditional petroleum diesel [2,3]. However, cultivation conditions, harvesting, and cost reduc-

tion is a key barrier to its practical commercialization [4]. Biofuel production from microalgae alone does not satisfy the economic feasibility. Hence, to improve the budget and reduce the cultivation cost, the source could be utilized in many ways, such as wastewater treatment, sewage treatment, CO_2 sequestration [5]. Its co-products (protein, carbohydrates, pigments, vitamins, and antioxidants) could further be utilized in the pharmaceutical and nutraceutical industries [6,7].

Under positive growing conditions, microalgal species typically accumulate lipids between 10% and 30% of their dry weight. Some species of algae have been documented to yield greater amounts of lipids (56% in *Nannochloris* sp. 80% in *Schizochytrium* sp.). On the other hand, *Chlorella* sp. and *Scenedesmus* sp. have comparatively less lipid content but a greater growth level [8,9]. Concerning conditions required for optimum growth and lipid accumulation, algae strains have been reported to have contradictory behavior [10]. To obtain the cost-effective biodiesel cultivation of numerous low-lipid cells or a few high-lipid cells will not lead to the economically sustainable production of microalgae-derived biofuel [11]. Thus, appropriate strategies should be implemented to rectify these opposing traits so an ideal equilibrium between microalgae biomass and lipid content can be maintained [12,13]. Genetic engineering in microalgae offers a lot of possibilities to expand the procedure (Figure 1). Rapid advancements in the synthesis of DNA, tools and methods for genetic manipulation, and the accessibility of functioning genomes have expanded the potential for improved engineering in microalgae in recent years. Different environmental, nutritional, and physiological conditions have also been tried for microalgae cultivation to improve lipid production. Additionally, nanoparticles (NPs) have been widely used as an effective method to resolve barriers and technological limitations regarding the two stages [14]. The successful retrieval and recycling of NPs using economic and cost-effective technologies is a critical component of microalgae harvesting research. This review is a comprehensive study of basically two recent techniques, genetic engineering approaches and the application of nanoparticles for lipid enhancement, simultaneously using various pretreatment methods of lipid recovery to overcome bottlenecks of biodiesel production.

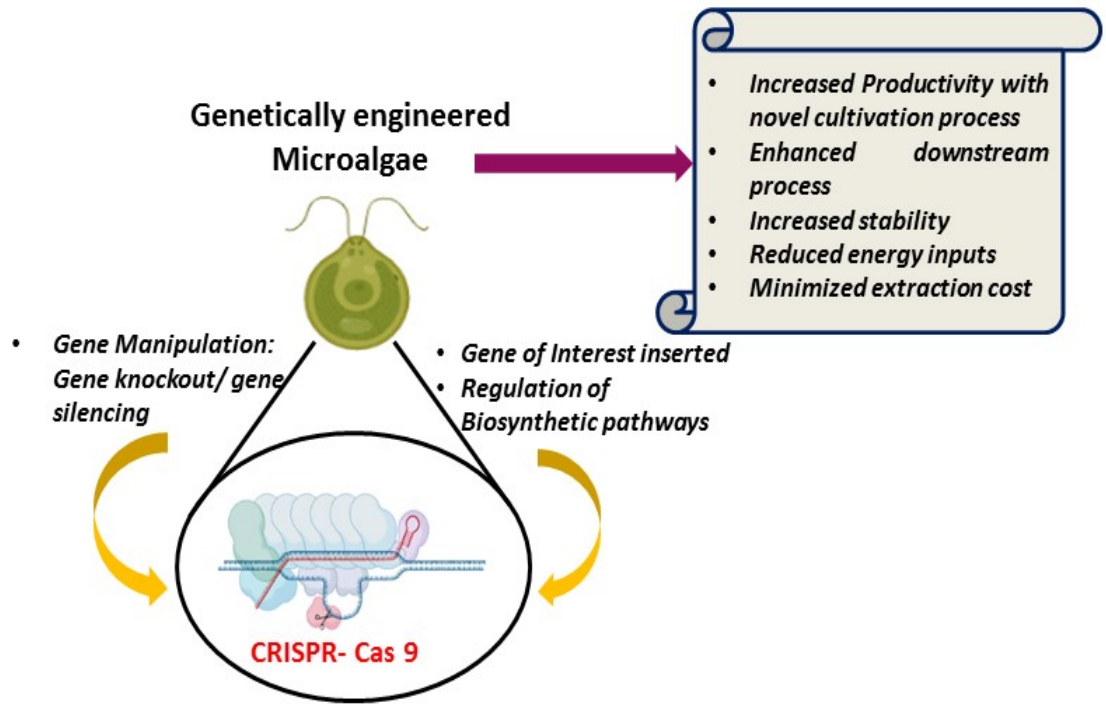

Figure 1. Genetically modified microalgae improve lipid content.

2. Genetic Engineering in Microalgae

Over the years, a balance has been pursued between increasing the lipid percent of microalgae by various methods while concurrently maintaining lipid productivity. Storage lipids in microalgae are usually neutral or triacylglycerides (TAGs) [15]. TAGs are biosynthesized in plastids, mitochondria, and the endomembrane and have esterified FA chains linked to the hydroxyl groups of the glycerol backbone. Specific genes that code for components of a metabolic pathway can be altered through genetic engineering in microalgae strains to improve the metabolites synthesis [16]. Methods such as zinc finger nucleases (ZFN), homologous recombination (HR), and transcription activator-like effector nucleases (TALEN) have been utilized to change the genetic makeup of eukaryotic cells [17,18]. However, putting these approaches into practice is time-consuming, difficult, and costly. TAG and FA synthesis involved a series of reactions driven by a variety of enzymes. Overexpression of these enzymes resulted in an increase in their function, which would positively promote lipid accumulation [19]. Genetically modified microalgae, such as *Dunaliella salina, Chlamydomonas reinhardtii*, and *Pharodactylum tricornutum*, were explored to boost FA synthesis, and consequently, lipid accumulation [20]. Some microalgae strains and overexpression of genes responsible for lipids biosynthesis are summarized in Table 1. Moreover, it has been opined that genetically engineered microalgae may change other biosynthetic pathways, which could produce toxicity and could affect other beneficial microbes and the environment. Therefore, before releasing genetically altered microalgae, it must be examined and approved by international committees [21]. A strategy such as biodiesel production in a closed photobioreactor could be promoted applied to avoid risk [22].

Table 1. Overexpression of genes/enzymes resulted in lipid enhancement in microalgae species.

S. No.	Microalgae Species	Genes/Enzymes	Lipid Enhancement	References
1.	*Chlamydomonas reinhardtii*	ACCase Overexpression	2.4-fold increase in TAGs	[23]
2.	*Phaeodactylum tricornutum*	G6PD Overexpression	55.7% increase in lipid	[24]
3.	*P. tricornutum*	GPAT1; LPAT1 Overexpression	2.3-fold increase in TAGs in N-depletion	[25]
4.	*P. tricornutum*	G3PDH Overexpression	1.9-fold increase in neutral lipid with slight decline in growth	[26]
5.	*P. tricornutum*	G6PD Overexpression	2.7-fold increase in lipid content	[27]
6.	*Chlorella protothecoides*	ME Overexpression	2.8-fold increase in total lipid content	[28]
7.	*C. reinhardtii*	PSR1 Overexpression	Increase in starch granules, decrease in neutral lipid content	[29]
8.	*Nannochloropsis salina*	bZIP Overexpression	Improvement in growth and lipid	[30]
9.	*C. reinhardtii*	DGTA Overexpression	Enhanced saturated fatty acids	[31]
10.	*Chlorella minutissima*	GPAT; LPAAT; DGAT Overexpression	2-fold increase in lipid content	[32]
11.	*N. oceanica*	NoDGAT1A Overexpression	2.4-fold increase in TAGs accumulation	[33]
12.	*C. pyrenoidosa*	NAD(H) kinase Overexpression	1.6 times increase in lipid content	[34]
13.	*C. reinhardtii*	LPAAT Overexpression	20% increase in TAGs	[35]

14.	*T. pseudonana*	Knock-down of a multifunctional lipase/phospholipase/acetyltransferase enzyme	2.4–3.3-fold higher lipids in contrast to wild-type	[36]
15.	*Nannochloropsis oceanica*	DGAT Overexpression	69% increase in total lipids	[37]

GPAT: Glycerol-3-phosphate aceyltransferase; LPAT: Lysophosphatidic aceyltransferase; DGAT: Diacylglycerol aceyltransferase; N: Nitrogen; ME: Malic enzyme; ACCase: Acetyl-CoA carboxylase; LPAAT: Lysophosphatidic acid acyltransferase; G3PDH: Glyceraldehyde-3-phosphate dehydrogenase; G6PD: Glucose-6-phosphate dehydrogenase and DGAT: acyl-Co-A: diacylglycerols acyltransferase.

3. Regulation of Biosynthetic Pathways

The restriction of metabolic pathways that promote the storage of energy-rich compounds is another approach that leads to the increase in cellular lipid content [38]. In microalgae, starch and lipid synthesis share a common precursor [39]. When the biosynthesis of the starch pathway is blocked, carbon flux is diverted towards the lipid biosynthetic pathway, resulting in a rise in FA, consequently raising total FAs [40]. The ADP-glucose pyrophosphorylase or isoamylase genes, sta6 and sta7 mutants, were disrupted, respectively, in two separate starch-deficient *C. reinhardtii* strains [41,42]. During the period of N deprivation, these mutants accumulated higher quantities of TAGs [43]. The starchless mutant of *Chlorella pyrenoidosa* is reported to have higher polyunsaturated FAs [44].

3.1. Shift of Starch Pathway to Lipid Pathway

In photosynthetic cells, starch and triglycerides are the main carbon storage components, and lipid and starch production proceed via competing metabolic processes. Microalgae change their lipid biosynthetic pathways as a carbon and energy storage form in adverse conditions (stress) to accumulate higher TAGs [45,46]. Fan et al. [47] stated carbon availability is a critical metabolic component influencing lipid production and carbon segregation among starch and lipids. TAG content was found to be much greater (up to three times against control) in starch-deficient *Dunaliella tertiolecta* mutants after N-depletion [48]. The blocking of starch biosynthesis pathways increased lipid accumulation in *Chlorella* sp. and *Chlamydomonas* is perceived in various studies [43,44,49,50]. Though the metabolic process in microalgae that controls carbon splitting from starch to lipids is still unclear, investigations are underway harnessing the mechanism behind the shift of pathways. Ho et al. [51] investigated the molecular mechanisms underlying the shift from starch to lipid biosynthesis in *Chlamydomonas* sp. JSC4 and measured the upregulation in the expression of genes encoding enzymes for lipid synthesis Acetyl-CoA carboxylase (ACCase), pyruvate decarboxylase, acetyl-CoA-synthetase, acetaldehyde dehydrogenase, and genes involved in starch degradation (starch phosphorylases).

3.2. Overexpression of Gene/Enzymes Involved in the Lipid Biosynthesis Pathway

The main enzyme involved in the precursor formation and lipid synthesis are ACCase, ATP citrate lyase (ACL), glycerol-3-phosphate (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phospholipid: diacylglycerols acyltransferase (PDAT), and acyl-Co-A: diacylglycerols acyltransferase (DGAT) [52–54]. The expression of these gene-encoding enzymes determines lipid content, and the regulation of these genes affects lipid content [55,56]. Additionally, enhanced lipid accumulation was documented due to the knock-down/overexpression of transcription factors that target the upregulation of lipid biosynthetic genes. Certain related work is also briefly summarized in Table 1. Acetyl-CoA carboxylase (ACCase) is a common and important enzyme responsible for the increase of the accumulation of lipid in microalgae. It is the first rate-limiting step in FA biosynthesis,

which accelerates TAGs synthesis. In lipid synthesis, the conversion of Acetyl-CoA to malonyl-CoA is regulated by ACCase. The overexpression of the gene (ACCase) is one of the effectively applied techniques that can be used to improve FAs in microalgae. A previous study revealed that the overexpression of ACCase affected lipid accumulation less [57]. However, coordinated overexpression with the ACCase subunit (accD) of malic enzyme (ME) was able to raise the lipid productivity of *Dunaliella salina* [58]. Likewise, overexpression of the ME enhanced *Phaeodactylum tricornutum* lipid output by 2.5-fold without adversely affecting the growth rate [59]. Genes associated with the synthesis of lipids were knocked out and overexpressed in prior work to see how they affected lipid accumulation in microalgae. ACCase encoding the FA enzyme synthesis was first over-expressed in a diatom, *Cyclotella cryptica*, by Dunahay et al. in 1996 [60]. Chimeric plasmid vectors were used for random recombinant DNA integration, and copies of multiple genes were inserted [60]. Although ACCase overexpression resulted in a 2–3-fold increase in ACCase activity, it did not affect FA synthesis [61]. *C. cryptica* has been credited with considerable genetic potential for hyper-lipid production, as compared to related diatom *Thalassiosira pseudonana*. The genomic study revealed increased expression of critical lipogenesis genes and expansion of TAG biosynthesis enzymes significantly [62]. The initial report of ACCase overexpression in *C. reinhardtii*, performed through the insertion of an overexpression vector, demonstrated that this technique can result in higher ACCase activity with better FA production [63]. However, it has been shown that the upregulation of ACCase in conjunction with malic enzymes, which catalyze the conversion of malate to pyruvate, increases lipid accumulation in *D. Salina* [58]. In *Phaeodactylum tricornutum* (PtACC2) microalgae, ACCase modification raised ACCase activity by 3.3-fold and led to a 1.77-fold rise in lipids, reaching 40.8% dry biomass [64]. Diacylglycerol acyltransferase (DGAT) overexpression, which catalyzes the last stage of TAGs biosynthesis, led to lipid enhancement [37]. The expression of multifunctional enzymes phospholipase/lipase/acyltransferase was inhibited, increasing higher lipid storage without sacrificing the growth of *T. pseudonana* [65]. It is understood that transcriptional regulation can affect the metabolomics flow of the system because transcription factors in a metabolic pathway can mark numerous regulatory points. In *N. gaditana*, knocking down a single ZnCys transcriptional regulator caused a 2-fold increase in lipid content [66]. Moreover, silencing the cht7 gene, which encodes TAG lipase, resulted in a 10-fold rise in TAGs [67].

4. CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats)

Recent advancements in gene-editing technologies, particularly CRISPR/Cas9, could result in gene alterations in commercially relevant microalgae strains (Figure 1). Genome editing involves modifying DNA in a sequence-specific manner via integrations, deletions, and insertions. In an experiment, gene manipulation was performed in *C. vulgaris* using the CRISPR/Cas9 method. For which a Cas9 fragment was constructed with engineered sgRNA in the omega-3 fatty acid desaturase (fad3) gene, resulting in a 46% (*w*/*w*) increase in lipid accumulation in the strain [68]. The Cas9 method was used to perform a target-specific knockout of the phospholipase A2 gene in *Chlamydomonas reinhardtii*. Subsequently, the mutants displayed an increased pool of diacylglycerol accompanied by a greater TAGs accumulation without extensively compensating the growth of the cells. Thus, the average lipid productivity of knockout mutants with phospholipase A2 increased up to 64.25% (80.92 $g.L^{-1}.d^{-1}$) [69]. CRISPR-Cas9 outperforms TALEN and ZFN because of its ease, adaptability, reduced price, and increased specificity [70]. Regardless of this, the most well-studied model of *C. reinhardtii* (freshwater green microalgae) had relatively little success [18,64,71]. In a recent study, a fragment of mGFP was transferred using *Agrobacterium tumefaciens* plasmid-mediated transfer to *Chlorella vulgaris* and *C. sorokiniana* FSP-E by electroporation, respectively. An increase of 67% fluorescence was observed against a wild-type strain by inverted fluorescence microscopy. Subsequently, a

plasmid-containing Cas 9 fragment with sgRNA targeting the omega-3 fatty acid desaturase (fad3) gene was created. Higher lipid accumulation (46% *w*/*w*) in *C. vulgaris* was perceived and considered as the first successful gene manipulation in *Chlorella* [68].

5. Alteration of Fatty Acid Composition

Aside from the use of genetic engineering for lipid enhancement, it is also necessary to consider lipid quality in terms of aptness as a feedstock for fuel production. The length of the carbon chain and the degree of unsaturation (double bond) in acyl composition are equally responsible for the determination of biodiesel properties. Natural microalgae synthesize a broad range of fatty acids [72]. Biodiesel with shorter chain FAs (C_{10}–C_{12}) improves cold flow properties. Therefore, the isolation of genes encoding unique shorter-chain acyl-ACP thioesterases may be beneficial and of great importance for decreasing the chain length of fatty acyl groups in oil extracted from microalgae. The first positive example of such a gene alteration attempt was demonstrated in a diatom where two genes encoding the plant-based acyl-ACP thioesterase in *P. tricornutum* were over-expressed by researchers from the Colorado School of Mines (USA) to generate a medium FA chain in the oil fraction [73].

6. Role of Nanoparticles

6.1. In Lipid Induction

Nanotechnology is the science, engineering, and technology that is measured in nanoscale (100 nm or less) [74]. Various types of metallic nanoparticles (MNPs), ranging from 5 to 100 nm have been explored due to their diverse physical and chemical properties than the same metals on a macroscopic scale [75,76]. The exceptional physiochemical behavior of MNPs has allowed them to be approached in several ways, comprising the food industry, drug delivery, cosmetics, and synthesis of multifunctional biomaterials [77]. The ability of NPs to boost the gas–liquid mass transfer rate in fermentation is a relatively new application of NPs [78,79]. According to the concept, the presence of NPs boosts the mass transfer coefficient at the gas–liquid interface [80]. Hence, rising CO_2 concentrations may alter growth rate and lipid stimulation in some microalgae. Few MNPs, such as Au, Ag, ZnO, CuO, Pd, Se, and FeO, are highly toxic to various organisms [81–86]. The toxic effect of NPs is also observed in microalgae, and it is connected with the reactive oxygen species (ROS) generation and the stimulation of oxidative stress, which is attained when the amount of NP reaches an effective level [82–84]. Some experts stated that when microalgae are encountered with sufficient doses of NPs, they can generate oxidative stress, and consequently, increase lipid synthesis in microalgae [77,87,88]. Recently, He et al. studied the impact of carbon nanotubes (CNT), α-Fe_2O_3 and MgO NPs were tested in *Scenedesmus obliqus*. It was noticed that exposure to 5 mgL^{-1} CNT, 5 mgL^{-1} α-Fe_2O_3, and 40 mgL^{-1} MgO NPs resulted in increased lipid up to 8.9%, 39.6%, and 18.5%, respectively. Moreover, when microalgae encountered high doses of NPs, a reduction in microalgae growth and lipid enhancement was observed, owing to the high amount of ROS that induced cell death [77]. Nanomaterials have several hundred times more surface area than their corresponding macroscale material weight. The surface area is not only significantly increased, but also the elasticity, persistence, strength, and electricity are improved. The utilization of nanomaterials could increase and potentially achieve lipid extraction efficiency without harming microalgae. In the transesterification process of lipids, nanomaterials, for example, CaO and MgO NPs, have been used as biocatalyst carriers or as heterogeneous catalysts [89]. Competitive inhibition, non-competitive inhibition, and denaturation are all ways that NPs might selectively inhibit enzyme activity [89] (Figure 2). As a result, fabricating NPs with specific characteristics to bind specific enzymes or proteins could allow for the regulation of their activity. To avoid non-specific binds and aid in recognition of specific enzymes or biomolecules, functionalizing NPs by altering their enormous surface

area with organic molecules through covalent or non-covalent interactions has been suggested [90]. In this case, a substantial study is required to record and verify the molecular process of binding of a specific NP (with specified properties) with AGPase enzyme to facilitate, block, or inhibit the enzyme's activity, hence inhibiting the starch manufacturing pathway [91]. This technique will aid in overcoming the lipid production bottleneck in microalgae, resulting in increased biofuel output.

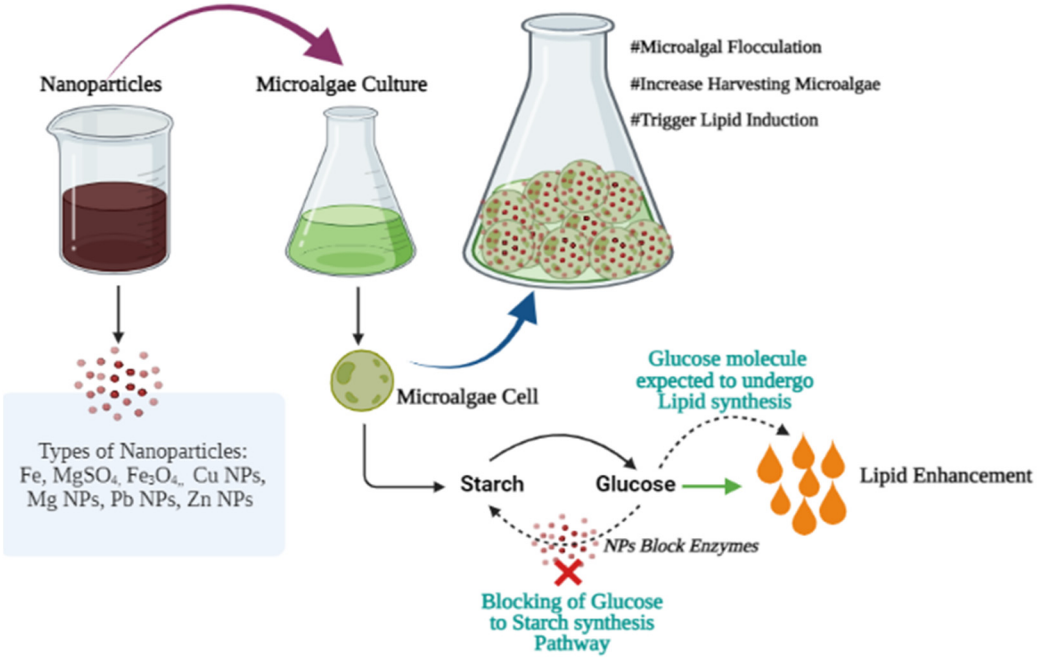

Figure 2. Nanoparticles trigger lipid synthesis and harvesting efficiency.

6.2. Harvesting of Microalgae

Microalgae-based biodiesel manufacturing on a large scale is one way to address energy constraints [92]. The collection of microalgae cells is another obstacle that interrupts the commercial development of algae-based biodiesel. Thus, the establishment of an efficient method of harvesting is important for the urgent need to bring about a significant reduction of the operation cost. In the photobioreactor culture procedure, the magnetic NP powder is used in the microalgae cell suspension to flocculate the cells for the even dispersal of nutrients and light throughout the reactor (Figure 2). An additional well-known technique for enhancing cell suspension is immunomagnetic identification and manipulation of microalgae cells from NP. It is difficult to separate algae from a large volume of growing medium. Various harvesting techniques have been developed using an attached culture device, such as flotation, filtration, coagulation, flocculation, centrifugation, and scratching [93,94]. Although, because of their low concentration, these traditional harvesting processes do have some limitations. Therefore, creating an effective technology to extract small algae cells from highly diluted solutions remains a challenge [95]. The harvesting of *Nannochloropsis* sp. is more difficult due to its smaller diameter compared to other microalgae [96].

Another method, magnetic separation, has been now applied due to its benefits, such as easy process, energy efficiency, quick separation, and minimum operating costs [97]. Silica coating in magnetic NPs or cationic polyelectrolyte was tested to harvest marine and freshwater microalgae [95,98]. *Chlorella ellipsoidea* and *Botryococcus braunii* were collected from freshwater using bare Fe_3O_4 NPs [97]. The evaluation of the harvesting effectiveness of *Nannochloropsis* sp. using Fe_3O_4 NPs is of great importance in contrast to freshwater

microalgae. Some essential process parameters for magnetic NPs for microalgal harvesting technologies, such as algal growth phase, harvesting temperature, and medium reusability, are yet unknown.

7. Pretreatment Methods

Pretreatment is an important step in restoring biomass composition for optimal biofuel production. It is crucial to disrupt the cell wall of microalgae to stimulate the release of inner substances, including lipids, proteins, carotenoids, and carbohydrates, into the medium [99]. Figure 3 depicts various pretreatment strategies for lipid extraction from microalgae. To solubilize the microalgae cell wall, a chemical pretreatment approach employs alkaline and acid with a heating range of 120–180°C [100]. Whereas physical pressures (solid–liquid shear forces) are utilized in mechanical pretreatment to disturb the structure of cellulose by broadening the surface area of organic material in order to depolymerize the hemicellulose that includes the algal cell wall [101]. To evade the enzymatic hydrolysis stage, acid and alkaline are used to increase the breakdown of cellulose matrix, hemicellulose depolymerization, and starch hydrolysis. By lowering the starch crystallinity and size of starch polymers, this approach causes solvation and saponification processes, resulting in the creation of openings in the cell wall that promote the discharge of internal constituents [102].

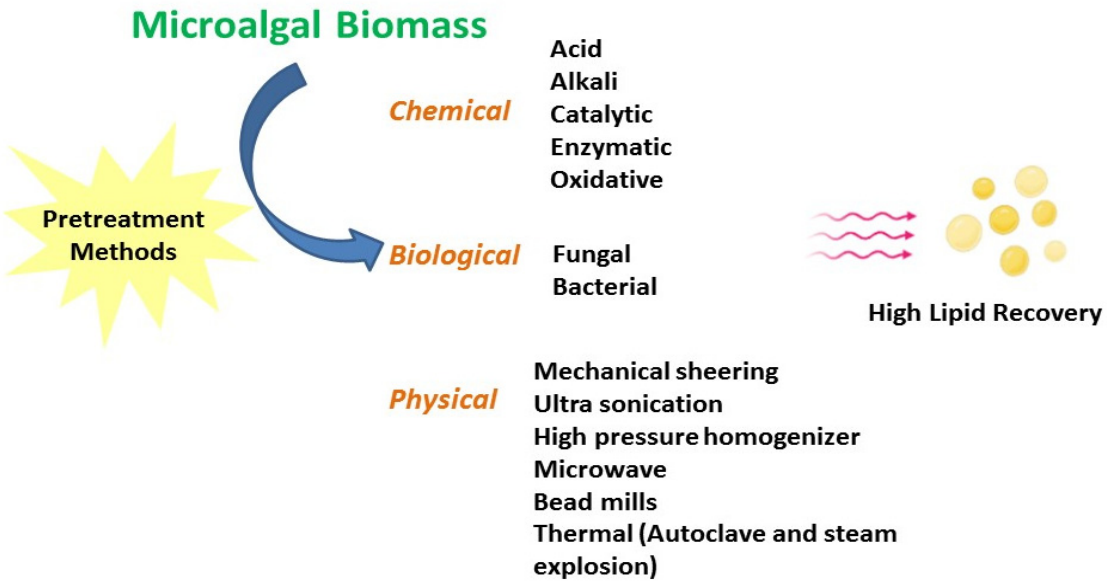

Figure 3. Different pretreatment methods for high lipid recovery.

Sert et al. [103] investigated the effects of concentration of the solution, duration of pretreatment, and temperature and discovered that 60 min of acid pretreatment (1 N H_2SO_4) at 100°C yielded the highest bioethanol content (18.52%). This amount was three times higher than the alkaline pretreatment. The chemical pretreatment, on the other hand, is caustic, poisonous, and creates inhibitory chemicals that might bring contamination downstream [104]. The natural ability of enzymes and some microorganisms to break the constituent of the microalgae cell wall is exploited in enzyme-based treatment (biological pretreatment) [99]. By boosting the release of intracellular components, hydrolytic enzymes are used to hydrolyze the cell wall of microalgae, resulting in a quicker and more successful recovery [105]. Hydrolytic bacteria are also used in the pretreatment method; the algicidal capacity of these bacteria utilized for microalgae pretreatment is imperative. This is because the bacteria's algicidal molecule causes autolysis in microalgae cells, resulting in the liberation of extracellular components [106]. Bai et al. [107] and Muoz et al. [108] stated that the pretreatment of *C. vulgaris* with *Flammeovirga yaeyamensis* and *Bacillus*

thuringiensis increased the lipid recovery rate by 44.3% and 100%, respectively. Another study found that employing *Bacillus licheniformis* caused considerable cell wall breakdown in *Chlorella* sp. within 60 h [109]. In this condition, pretreatment with hydrolytic bacteria was found to be more successful than enzyme pretreatment, as enzymes drop their capability to function with time. The pure culture system, which is used to pretreat enzymes and bacteria, has several difficulties, including long pretreatment duration, pretreatment in open conditions, and the maintenance of pure culture [110]. In addition, to guarantee appropriate biomass degradation during the pretreatment process, microbial consortia should have cellulose and hemicellulose degradation capacity [111]. The microbial consortium's synergistic metabolism caught some curiosity; thus, further investigation for bioprocessing technology is still needed.

8. Conclusions

Microalgae-based biodiesel is yet to be marketed, the reason being that the total cost of processing is twice that of fuels based on petroleum. For the growth of microalgae, culture maintenance, biomass production, lipid yield, extraction, and later conversion to biodiesel, each step needs high effort and strategy to get cost-effective biodiesel production compared to fossil fuels. The current review is focused on the promising metabolic engineering innovations that enable enhanced TAG production. The article outlines the genetic engineering methodologies, NPs applications, and several pretreatment methods that have been explored to increase TAGs synthesis in microalgae species, resulting in an economically viable energy production strategy. Where NP's amendment triggers lipid production, on the other hand, pretreatment sustains the high lipid recovery rate. Thus, the concept of combining various technologies supporting biomass and lipid enhancement is a viable strategy for biodiesel production from microalgae.

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