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ORIGINAL RESEARCH ARTICLE

Microalgal Bioethanol Production for Sustainable Development: Current Status and Future Prospects

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Abstract Around the world, countries are making efforts to tackle immediate environmental concerns such as global warming and its impact on climate change, as well as the challenge of fast-depleting fossil fuel resources. Moreover, these nations strive to achieve complete elimination of greenhouse gases, considering the context of the ongoing and escalating global energy crisis. As a result, researchers are investigating bio-based feedstocks as viable and environmentally friendly alternatives for bioenergy production. Microalgae are a type of photosynthetic microorganism that could be used as a renewable energy resource. They are capable of growing in harsh environmental circumstances and on terrain that is not suitable for agriculture. Additionally, they tend to flourish in both seawater and wastewater. Microalgae exhibit superior photosynthetic efficiency and biomass productivity in comparison to their terrestrial plant counterparts. Microalgae biomass, as well as the metabolites derived from it, can be transformed into a range of biofuels, including bioethanol, biodiesel, crude oil, pyrolytic bio-oil, biomethane, biohydrogen, and jet biofuel. Nevertheless, numerous obstacles still need to be overcome to attain faster and more widespread commercial utilization of microalgae as a renewable bioenergy source for bioethanol production. To enhance the sustainability of the environment and economic feasibility of microalgal bioethanol, it is crucial to choose suitable microalgal bio jets, create methods for concentrating biomass, and utilize wet microalgal biomass for bioethanol production. All these methods and steps need to be carried out meticulously. Additionally, adopting a coordinated biorefinery approach to produce value-added products would further contribute to the identified goals. This article aims to provide an overview of the present state of research on microalgal bioethanol and its prospects.

Keywords Bioethanol · Microalgae · Sustainability · Biorefinery

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Introduction

Energy has been instrumental in the advancement of human civilizations. Countries have employed non-renewable energy sources like coal, oil, and gas to sustain their rapid development [1]. Based on the documented models, it is projected that the use of energy sources produced from fossil fuels will peak around the year 2050 and start to drop by 2075 [2, 3]. In light of the escalating energy issue, countries are currently seeking alternative and sustainable energy options. Efforts like the Paris Agreement and the Kyoto Protocol have been implemented to address the problems of climate change and global warming. However, it is predicted

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that the global temperature could increase by 3 °C, which would make it even more difficult to mitigate climate change [4]. Hence, diverse types of bioenergy sources are presently being created and utilized worldwide to address the issues posed by diminishing non-renewable energy sources derived from fossil fuels and achieving a state of zero net carbon emissions.

Throughout the past few years, there has been a consistent rise in the demand for renewable energy sources all over the world, in particular biofuels [5–8]. The emergence of this phenomenon can be attributed to the concurrent escalation in energy consumption, the exhaustion of traditional energy resources, and the imminent peril of global warming. These variables are the driving forces behind the phenomenon. Biofuels could be produced from biomass available on planet Earth. the utilization of organic material. The amount of food produced for human use has decreased, which has raised significant economic, environmental, and political problems because the initial generation of biofuels requires a sizable area of arable land [9–12]. Furthermore, the initial biofuel production method has been negatively impacting the environment. Research and development (R&D) efforts are currently shifting their focus toward second-generation biofuels. This change is something that is currently taking place. These biofuels frequently begin with non-food crops as their primary source of raw materials. This conclusion has finally been reached as a result of the inadequacy of the first generation of biofuels. As a result of the requirement for expensive and highly advanced technologies in the production of second-generation biofuels, the current endeavour has not yet proved that it is profitable [13–15]. As a consequence of this, the researchers focused their attention on the manufacture of biofuels of the third generation that were produced from microalgae. On account of their rapid growth rate, capability to absorb carbon dioxide(CO_2), exploitation of waste nutrients, and high capacity to generate storage compounds such as lipids and polysaccharides, they are currently being advocated as a feasible option for the production of biofuel [16, 17]. Furthermore, these plants thrive in extensive non-agricultural areas situated on unfavourable terrain, avoiding competition with food or feed crops [18, 19].

The level of CO_2 in the atmosphere is rising at a rate that has never been seen before, mostly as a result of human activity and the ongoing extraction and combustion of fossil fuels. It is in the best interest of our civilization to discover a set of solutions to reduce climate change and stabilize the global mean temperature. This is because this event is producing an increase in the temperature of the atmosphere and the oceans, which has relatively negative repercussions on the planet. To this point, a wide variety of technologies, including those that are physical, chemical, and biological, have been developed and put into practice to sequester and reduce CO_2 emissions [20, 21]. The use of microalgal systems presents a possibility that is both lucrative and environmentally friendly, partially supporting these aims. Microalgae are called photosynthetic creatures, and to thrive, they require CO_2, in addition to sunlight, water, and various other nutrients [22–24]. The advantages of microalgae in comparison to other alternatives for carbon mitigation include the fact that they have a carbon fixation capacity that is ten to fifty times higher than that of other plants [25].

Microalgae can also efficiently grow on salt and wastewater and do not require fertile land for their growth purposes. Because of this, they do not directly compete with the production of food. Biofuels, human nutrition, cosmetics, medicines, animal feeds, and fertilizers are some of the items that can be derived from their biomass after it has been converted into other forms.

Through the process of fermentation, bioethanol, a renewable biofuel that possesses qualities comparable to those of gasoline, is produced. To produce bioethanol, biomass sources abundant in cellulose and starch are utilized. In the present times, primary raw materials like wheat, sugar beet, and corn must be used in addition to secondary raw materials like lignocellulosic forest wastes to produce bioethanol [26–28]. Since first-generation raw materials are also used as sources of food, there is a good deal of controversy and debate surrounding their utilization for high-yield farming. Consequently, the usage of this initial version of unprocessed resources for the production of bioethanol has led to discussions over the rise in food prices and the occupation of agricultural lands [29, 30].

The utilization of second-generation feedstocks, in particular lignocellulosic resources such as waste or residual materials from forests, comes across as a feasible method to partially address the above-mentioned difficulties and provide remedies [31, 32]. When compared to feedstocks of the first generation, those of the second generation provide several benefits. The most significant are their utilization for purposes other than food production and their reduced land requirements. Consequently, the procedure of collecting, refining, and pre-processing them presents several substantial hurdles, which finally render the manufacturing of these commodities economically impossible [33–37]. Algae, a feedstock for biofuels of the third generation, provides an alternative to feedstocks of the first and second generations due to its high production, simplicity of cultivation, and convenient harvest time. The majority of their applications are in the production of biodiesel due to the high lipid content they contain. In addition, they are composed of cellulose and carry a significant amount of carbohydrates, making them suitable for direct usage in the industrial manufacture of bioethanol [38, 39]. Alternately, they could blend well with the material that is left behind after oil extraction in order to produce bioethanol. In comparison to the production of fossil fuels, the production of traditional feedstock bioethanol

is associated with greater levels of greenhouse gas emissions [40, 41]. The creation of algal bioethanol provides a solution to the above-mentioned problems. In contrast to the production of traditional feedstocks, the cultivation of algae does not require the use of fertilizer or agricultural fields. Considering the positive qualities that algae possess and the enormous amount of carbohydrates they contain, algae have the potential to considerably boost the production of ethanol [42, 43]. Utilization of bioethanol of the third generation, obtained from microalgal biomass, has the ability to serve as a fuel choice that is also environmentally sustainable. Within the realm of microorganisms, microalgae could be classified as either prokaryotic (characterized by the absence of a cell membrane and nucleus, as is the case with blue-green algae) or eukaryotic (characterized by the presence of a cell membrane and one or more nuclei, as is the case with green and red algae) [44, 45]. They tend to develop rapidly and survive in harsh environments since they have either a single-cell or a primitive multicellular structure. Microalgal cells are capable of dividing at a high rate, which exhibits a brief period during which they experience a growth increase that is twice as large. Since this is the case, they can attain significant levels of productivity with a short harvesting cycle, which typically lasts between one and ten days. In comparison to other crop feedstock, which often needs to be harvested only once or twice a year, this establishes a significant breakthrough or advancement [46, 47]. Because of the substantial amount of lipids and carbohydrates they contain, microalgae are also an excellent choice for use as raw materials in a wide variety of industrial applications. These applications include the production of food, cosmetics, medicine, and biofuel. A trend that has gained popularity in recent years is the utilization of microalgae for the manufacture of biodiesel and bioethanol. Such utilization of microalgae has been made possible through the utilization of the biorefinery process. It is to be noted that two primary production technologies are utilized in the development of microalgae [48, 49]. These technologies are open ponds and closed photobioreactors (PBRs).

The photosynthetic efficiency of microalgae is higher than that of higher plants in their natural environments. Proteins make up 30 to 50% of the microalgal biomass, while carbohydrates make up twenty to forty percent, and lipids up to 8 to 15%. The presence of a small quantity of polysaccharides in these photosynthetic organisms, which are grown under usual conditions, renders them inappropriate for the generation of bioethanol. In addition to this, they incorporate advantageous characteristics [50]. To increase the amount of bioethanol that can be produced, ongoing efforts are being deployed. Accelerating the growth rate of biomass, modifying growing circumstances to encourage higher carbohydrate content, and improving the efficiency of converting carbohydrates into ethanol are some of the specific tactics that are included in these activities [51]. Considering the benefits discussed above, microalgae have been the subject of substantial research as a potential feedstock for the production of a variety of biofuels. This category encompasses various forms of biofuel. These substances include bioethanol, biodiesel, crude oil, biojet fuels, pyrolytic bio-oil, biohydrogen, and biomethane [52, 53]. Three different approaches can be utilized in the cultivation of microalgae: phototrophic, mixotrophic, and heterotrophic. On the other hand, the production of biomass through these methods could result in high expenses, both in terms of money and energy. Therefore, the extraction of useful metabolites from microalgal biomass holds promise for the utilization of the leftover biomass in the production of biofuels [54]. This study would be useful to determine whether or not it is feasible to use the full biomass of microalgae, in addition to the numerous metabolites that it contains as raw materials for the production of biofuels. The purpose of this article is to investigate whether or not it is possible to produce bioethanol from microalgae and to evaluate its output in comparison to that of other sources of bioethanol feedstocks. The article attempts to provide a comprehensive review of the pre-treatment methods that have been developed for microalgae, as well as a comprehensive evaluation of the benefits and drawbacks associated with each specific process. An analysis is carried out to determine whether or not it is possible to generate bioethanol from microalgae, taking into account the entire process, beginning with the growing of the microalgae and ending with fermentation. The flow diagram for producing bioethanol from microalgae is shown in Fig. 1.

Microalgae

The term “microalgae” refers to the combination of microscopic algae and oxygenic photosynthetic microorganisms. Hence, the initial differentiation lies in the categorization of organisms into prokaryotes and eukaryotes. The primary differentiation between eukaryotic and prokaryotic cells lies in the existence of membrane-bound organelles in eukaryotic cells, which are absent in prokaryotes [55]. Eukaryotes purportedly obtained the latter through evolutionary processes including endosymbiosis. Eukaryotes are characterized by their bigger size, greater complexity, and ability to exist as either unicellular or multicellular organisms. In contrast, prokaryotes are simple, tiny, and consist of single cells. The existing classification systems consider several criteria, including cytological and morphological characteristics, cell wall components, and the chemical composition of storage goods. Various techniques are commonly used to identify and classify algal species. These include observing their physical characteristics under a microscope, using specific gene sequences for molecular-based classification, and more

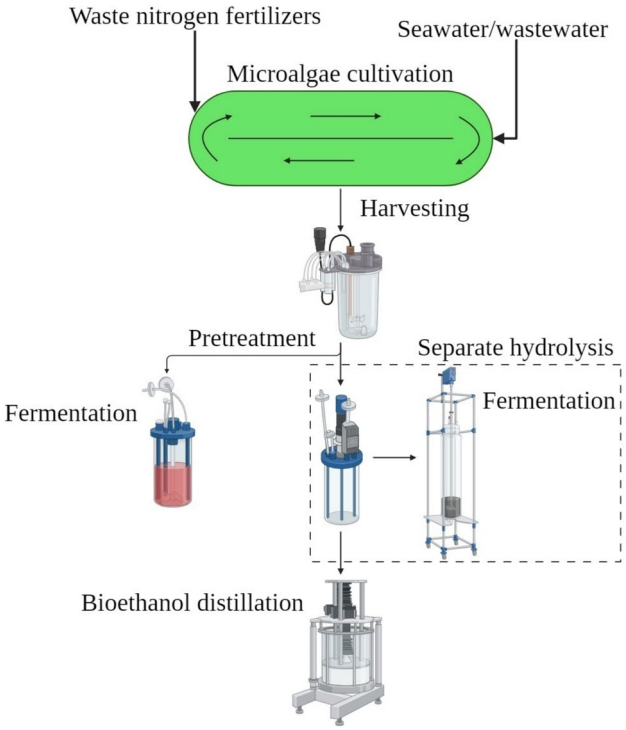

Fig. 1 The process of producing bioethanol from microalgae

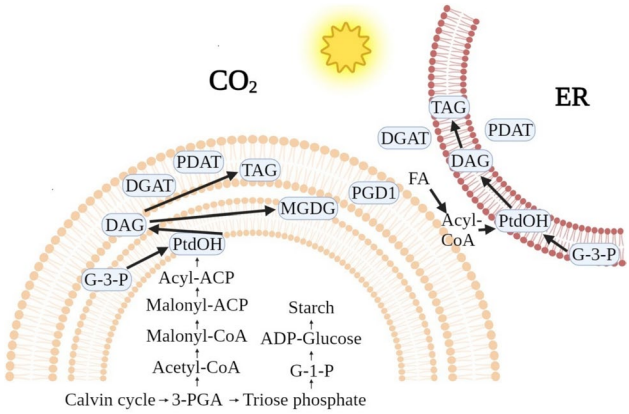

Fig. 2 The microalgal synthesis pathway for triacylglyceride (TAG)

recently, employing semi-automated or fully automated classification methods using a flow cytometer along with computational techniques [56, 57]. Irrespective of the methodologies employed to ascertain algal species, the categorization system has undergone numerous revisions through the years. Presently, there is a lack of agreement among taxonomists worldwide regarding the preference for one classification system over another. Nevertheless, the most recent classification paradigm comprises two primary domains, Prokaryota and Eukaryota, which encompass seven kingdoms: Archaebacteria, Eubacteria, Protozoa, Chromista, Fungi, Plantae, and Animalia [58].

Microalgae, a type of simple microorganism, possess chlorophyll and play a crucial role in generating about 50% of the earth's oxygen. Microalgae can undergo autotrophic or heterotrophic growth and may survive in several challenging settings, such as freshwater, saline water, high pressure, and high temperature [59, 60]. There exist various microalgae species that are of micron size, including those that are 100 µm in size. According to estimates, there are over 50,000 species of microalgae, although only a small number of species have had their biochemistry and ecophysiology studied [61]. Microalgae growing medium is diverse and dependent on the specific type of microalgae and the desired product, owing to the rapid and adaptable growth of microalgae in many settings and situations. To manufacture biodiesel, it is necessary to choose algae species that have a high concentration of lipids. Furthermore, it is necessary to optimize the environmental conditions to enhance the lipid content of microalgae. In addition, stress can be induced by reducing the temperature and manipulating the nitrogen levels in the medium. This concept applies equally to the synthesis of bioethanol. In addition, microalgae that have been genetically modified to have a higher carbohydrate content could be utilized for the generation of bioethanol [62].

Fatty acid production frequently occurs in the plastids of microalgal cells. Triacylglyceride (TAG) production takes place in the cell's endoplasmic reticulum and chloroplasts (Fig. 2). TAG, as opposed to the phospholipids found in biological membranes, does not participate in the structure of cells. On the contrary, their primary function is the storage of carbon and energy. Microalgae cells synthesize TAGs via two primary pathways: the monoacylglycerol pathway and the Kennedy pathway. In both of these pathways, the esterification process, which yields TAG, entails the amalgamation of hydroxyl groups on glycerol with acetyl-CoA. The first step in the synthesis of fatty acids is the carboxylation of acetyl-CoA. Acetyl-CoA carboxylase (ACCase) then catalyzes the creation of malonyl-CoA. To control the production of fatty acids, ACCase is essential. In microalgae cells, malonyl-CoA is initially transported to the acyl carrier protein (ACP), after which it undergoes a sequence of acyl chain-elongation processes. Several fatty acid synthase subunits catalytically create the C16 or C18 products. Two different enzymes prevent the elongation of fatty acids. First, ACP removes the acyl groups from the chloroplast acyltransferase. Next, the newly generated fatty acid is quickly moved from ACP to glycerol-3-phosphate (G-3-P). Moreover, acyl-ACP hydrolysis is catalyzed by the enzyme acyl-ACP thioesterase, which releases bound fatty acids. Transferring free fatty acids from the chloroplast results in the production of glycerides. Through esterification, the first acyl group in the Kennedy process is connected to glycerol-3-phosphate [63]. The production of phospholipids takes place during the second reaction. Prior to the

conversion into TAG, the phospholipid undergoes dephosphorylation by the phosphatase enzyme, resulting in the formation of diacylglycerol (DAG). The monoacylglycerol pathway initiates with the utilization of monoacylglycerol 2 acyltransferase (MGAT) to convert 2-monoacylglycerol (sn2-MAG) to DAG. The conversion of generated DAG to TAG occurs through the catalytic activity of acyl CoA: DAG acyltransferase, an essential procedure in the biosynthesis of TAG. Phospholipid diacylglycerol acyltransferase (PDAT) is an alternative pathway catalyst that can facilitate TAG production. Phospholipid, or galactolipid, functions as the acyl donor in this procedure. Certain plants have demonstrated the presence of PDAT activity. However, the extent to which it contributes to TAG synthesis varies among different plant species. The identification of the PDAT encoding gene in microalgae provides support for the notion that microalgae utilize a pathway for TAG synthesis that is not dependent on acetyl-CoA.

Bioethanol Production from Microalgae

Although bioethanol synthesis from microalgae has the potential to address environmental concerns such as climate change and produce biofuel, it still faces challenges in terms of attaining widespread, highly efficient production and effective commercialization. The key elements for microalgae to become a feasible source of raw material in bioethanol production are: (1) meticulous selection of microalgae species and achieving a substantial carbohydrate content through high biomass production; (2) efficient methods for harvesting; (3) suitable techniques for pretreatment; and (4) an effective fermentation process. To achieve the production of microalgal bioethanol that is both cost-effective and highly efficient, it is necessary to enhance and optimize each of the aforementioned areas. Table 1 displays the bioethanol output derived from various species of microalgae. Table 2. shows bioethanol production from different types of feedstocks.

Microalgae Cultivation

In order to enable sustainable bioethanol production, the microalgal biomass must have the ability to effectively compete with other raw materials. This can be achieved by implementing comprehensive manufacturing techniques and optimizing ideal conditions. Should the cost and availability of microalgal production decrease, it may become a more favourable option for bioethanol production. Figure 3 illustrates the initial step of the bioethanol manufacturing process, which involves cultivating microalgae in a photobioreactor. Open ponds can be utilized as an alternative to photobioreactors for the purpose of large-scale bioethanol production. Because it directly affects the desired end result, choosing the right microalgal species is crucial in microalgal manufacturing operations. For example, *Chlorella vulgaris* and *Dunaliella salina* are used to produce β-carotene [61], whereas *H. pluviaris* is used for astaxanthin production [80]. Microalgae species with a high lipid content, such as *Chlorella protothecoides*, have the potential to be chosen for biodiesel production [81]. On the other hand, microalgal species that have a high concentration of carbohydrates are better suited for the production of bioethanol. *Chlorella*, *Dunaliella*, *Chlamydomonas*, and *Scenedesmus* microalgae are reported to contribute more than 50% of carbohydrates [82].

Table 1 Production of bioethanol from various types of microalgae

Microalgae	Condition	Bioethanol yield	References
Scenedesmus acuminatus CCALA 436	Wastewater and mepiquat chloride	20.32 g/L	[64]
Chlorococcum minutum	Cr3 and Cm3 media	46.97 g/L	[65]
Chlorella vulgaris	Tris-acetate-phosphate (TAP) medium	–	[66]
Chlamydomonas reinhardtii	Tap without tris base	–	[67]
Codium tomentosum	–	4 ± 0.33 g/l	[68]
Chlorella sp. *ABC-001*	–	2.80 g/L	[69]
Chlorococcum minutum	Tris-acetate-phosphate (TAP) medium	31.2 g/L	[70]
C. vulgaris ESP-31	–	98.11 g/L	[71]
Scenedesmus acuminatus	Nitrate- and phosphate-starved conditions	17.2 ± 1.2 mg $L^{-1}d^{-1}$	[72]
Trichoderma harzianum	–	22.4 g/L	[73]
Chlorella sorokiniana	Nitrate (1.0 g/L KNO3) culture medium	422.44 mg/L	[74]
Chlorella vulgaris FSP-E	–	43.9 g/L	[75]
Scenedesmus obliquus	–	8.24 g/L	[76]
Chlorella sorokiniana AK-1	10% unsterilized swine wastewater	4.2 g/L	[77]
Ulva lactuca	–	2.8 ± 0.12 mg/mL	[78]

Table 2 Bioethanol production from different types of feedstocks (adapted from Latif et al. [79])

Feedstock	Bioethanol yield (g/L)
Corn stover	21.47
Oil palm trunk	44.25
Papaya peel	0.51
Cassava starch	81.86
Galactose	96.90
Pomegranate peel	5.81
Rice husk	15.63
Rice straw	18.07
Corn starch	98.13
Pineapple	9.75
Sugarcane waste	49.77
Sugar beet molasses	79.60
Sweet sorghum	97.54
Microalgae	52.10

Microalgae can thrive in many environmental circumstances, which vary according to their species. Nevertheless, these creatures primarily rely on light and CO_2 due to their photosynthetic nature. Furthermore, nitrogen and phosphorus are essential for promoting growth and cellular functions in algae, making up approximately 10%–20% of their whole biomass. Furthermore, macronutrients such as sodium (Na), calcium (Ca), potassium (K), and magnesium (Mg), as well as micronutrients such as boron (B), cobalt (Co), iron (Fe), and zinc (Zn), play a crucial role as nutrient sources for the development of microalgae [83]. Industrial wastewater is highly suitable for microalgal cultivation. By utilizing wastewater in microalgae cultivation, we can not only recycle industrial wastewater but also decrease the expenses associated with providing nutrients for microalgae production. The development of microalgae is a crucial stage in the manufacture of bioethanol, as it requires biomass with a significant amount of carbohydrates to make bioethanol manufacturing economically viable. Consequently, by optimizing the growth conditions and subjecting the microalgae to difficulties such as light and temperature, it is possible to increase the accumulation of carbohydrates. In addition, it is worth noting that microalgae possess the capacity to endure genetic modifications that may enhance the rates at which they produce carbohydrates or accumulate starch [84]. Samiee- Zafarghandi et al. showed that a deficiency of phosphorus leads to a substantial increase in the accumulation of carbohydrates in *Chlorella* sp. [85]. Recent findings indicate that the presence of calcium and magnesium can boost the synthesis of carbohydrates and biomass in microalgae. This is attributed to the crucial role these elements play in the creation of chlorophyll and numerous enzymes.

The process of harvesting is a crucial stage in the growth of microalgae, constituting around 20–30% of the overall production expenses. It is imperative to ascertain efficient and cost-effective harvesting techniques to achieve high-yield bioethanol production at a minimal expense [86]. Efficient harvesting techniques that can be universally applied to all species of microalgae are necessary to obtain microalgae with a high dry weight. The primary phase consists of bulk harvesting, which is the process of separating the microalgal biomass from the overall suspension [87, 88]. Usually, the objective is achieved by employing flocculation, flotation, or gravity sedimentation procedures. In the second stage, the resulting algal slurry is consolidated by

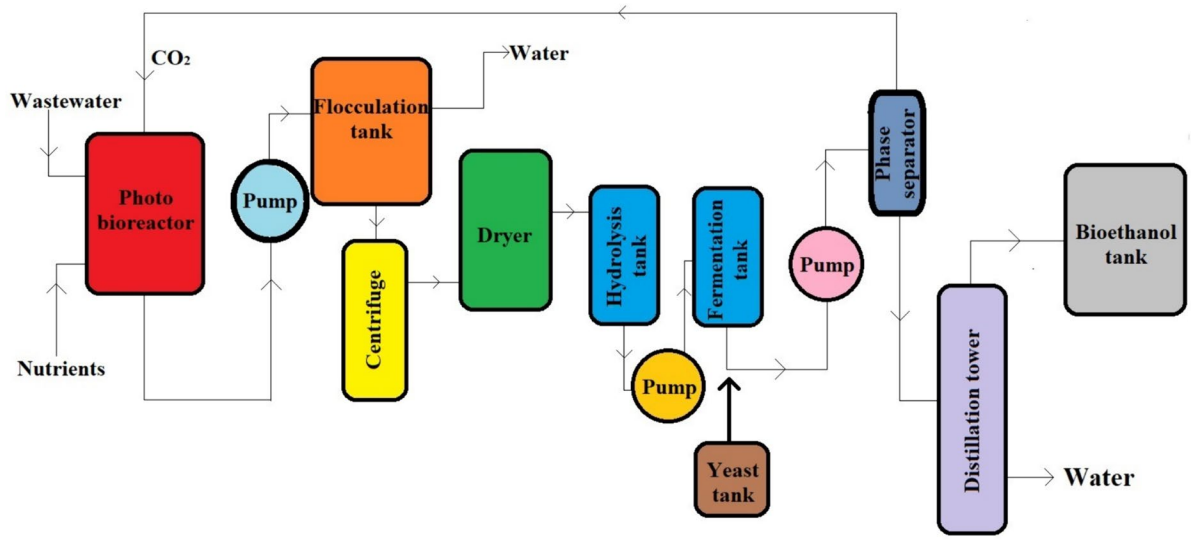

Fig. 3 Cultivation of microalgae in photobioreactor

employing centrifugation and filtering processes, which require the application of energy [89, 90]. The harvesting technique is contingent upon the characteristics of microalgae, including their size and density. One proposed approach for harvesting Spirulina microalgae is through the use of a microscreen, which is effective due to the microalgae's elongated and spiral shape [91]. Cell density below 0.3 g/L and microalgae cells smaller than 2 µm can decrease the harvesting yield [92].

Flocculation is a widely recognized method employed in the mass collection of algal suspensions. The negative charge of the microalgae serves to impede their ability to aggregate within the suspension. The flocculation process involves the neutralization of negatively charged microalgae cells, which are then precipitated from the suspension through the addition of flocculants [93]. The desired characteristics for these flocculants include affordability, ease of use, sustainability, and accessibility in tiny quantities. Inorganic and organic substances, such as aluminium sulphate ($Al_2(SO_4)_3$), ferric sulphate ($Fe_2(SO_4)_3$), and polyacrylamide, can act as flocculants [94, 95]. A considerable number of inorganic materials are present in the harvested biomass as a result of the harvesting process's use of inorganic flocculants, such as metal salts. This poses challenges when attempting to utilize these biomasses in subsequent phases. To mitigate this drawback, natural polymeric compounds like chitosan are employed as flocculants.

The drying step is essential for effectively utilizing microalgal biomass in subsequent procedures. The microalgal biomass obtained after harvesting typically consists of around 70–90% water content [96]. The predominant methods employed for extracting moisture from biomass include solar drying, freeze drying using spring freeze, and fluidized bed drying. Solar drying is the most efficient method for large-scale production compared to other techniques, which are typically costly and commonly employed in laboratory-scale systems.

Dark Fermentation for Bioethanol Production

Phototrophic microorganisms store polysaccharides and lipids in their cells throughout the day, while their main metabolic processes are photosynthetic oxygen generation and CO_2 fixation [97, 98]. Dark fermentation occurs in the absence of light, during which a significant portion of starch reserves are broken down into sugars by the action of amylase. These sugars are then converted to pyruvate through the process of glycolysis. The primary advantage of fermentation for photosynthetic organisms is the production of ATP, which is essential for powering metabolic and energy-demanding processes [99, 100]. Eukaryotic algae, such as *Chlamydomonas reinhardtii, Chlamydomonas moewusii, Chlorogonium elongatum,* and *Chlorella fusca*, have the ability to carry out intracellular starch fermentation. Eukaryotic microalgae contain starch, a type of carbohydrate polymer, which can be converted into pyruvate. Pyruvate plays a crucial role as an intermediary component [101]. Various fermentative pathways can then convert pyruvate into a variety of end products, such as acetate, ethanol, formate, glycerol, lactate, H_2, and CO_2 (Fig. 4). The ultimate outcomes differ across different species of eukaryotic algae and can also vary dramatically in response to changes in environmental factors. Pyruvate is one of the substrates utilized in fermentation processes. It functions as an energy substrate for acetyl coenzyme A (acetyl-CoA), which can be degraded and transformed into acetate in order to produce ATP [102, 103]. In Chlamydomonas, pyruvate can be transformed into ethanol as a means of preserving redox equilibrium. During the last phase, the postulated enzyme alcohol/acetaldehyde dehydrogenase (ADHI) transforms acetyl-CoA into either acetaldehyde or ethanol [104].

Photofermentation for Bioethanol Production

Cyanobacteria possess the capability to synthesize ethanol directly during the process of photosynthesis. The routes are referred to as the "photofermentative" or "photanol" routes [105]. Genetically engineered strains of cyanobacteria, specifically *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942*,* and *Synechococcus* sp. PCC 7002 from the Pasteur Culture Collection in Paris, has recently been developed for bioethanol production through the process of "photofermentation" [106]. The cyanobacterium *Synechococcus* sp. *PCC 7942* was genetically modified by introducing the genes for pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADHII) from *Zymomonas mobilis*. In this model system, the identical PDC/ADHII cassette was employed, as it had been previously used in Escherichia coli to illustrate the heterologous production of these genes. The Calvin-Benson cycle converts CO_2 into pyruvate in the newly established metabolic pathway, employing the photosynthesis-generated reducing power [107]. The added PDC and ADHII enzymes then aid in the conversion of pyruvate into acetaldehyde and ethanol. While the rate and volume

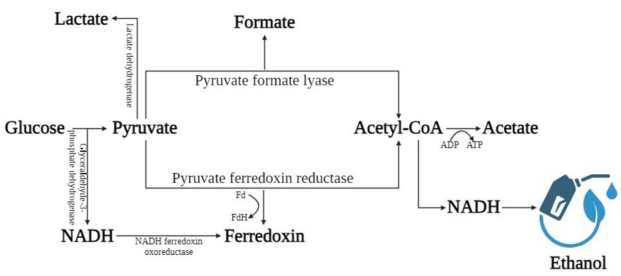

Fig. 4 Metabolic pathway of dark fermentation

of ethanol produced were not at the ideal level, the initial findings of genetic tweaks showed promise. Further investigations were carried out to understand the limitations of the topic. Ethanol suppressed the growth of *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 at concentrations of roughly 2.5 and 4.5 g/L, respectively. Proteome analysis was employed to conduct a comprehensive inquiry into the operation of the artificially introduced pathway implicated in the phototrophic metabolism of *Synechocystis* sp. PCC 6803 during ethanol production [108–110]. Additionally, cultures of *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 was subjected to systems analysis. The study discovered that the excessive drain from central metabolism caused by ethanol loss is the cause of the progressive intracellular organic carbon limitation. Additional research has conducted experiments where genetic modifications were combined with stress conditions. The findings demonstrated that the rate of ethanol generation increased when nitrogen was depleted from Synechocystis sp. PCC 6803 and certain elements of the glycogen synthesis and poly(3-hydroxybutyrate) (PHB) synthesis pathways were eliminated [111, 112]. To evaluate potential metabolic changes for the *Synechocystis* sp. PCC 6803 strain, computer-based models were created. According to the theory, under photoautotrophic circumstances, ethanol synthesis could be enhanced by deactivating NAD(P)H dehydrogenase (ndhF1) and employing ammonium as a nitrogen source [113, 114]. The ΔndhF1 mutant exhibited a substantial increase in ethanol titer compared to the wild type. The development of mutants was carried out indoors on a laboratory scale. However, the primary objective of the "photofermentation" concept was the generation of bioethanol on a wide scale in a single phase [115]. Despite the early success, notable practical challenges have already arisen during the pilot phase. The *Synechocystis* Syn-HZ24 construct was cultivated and manufactured successfully in the laboratory. However, during outdoor cultivation, *Pannonibacter phragmitetus* hindered ethanol production by outcompeting the construct and consuming the accumulated ethanol [116]. The pH-rising technique, including the use of $NaHCO_3$, was employed to decrease the population of *P. phragmitetus* and facilitate the accumulation of ethanol generated. After 10 days of culture, the ethanol concentration reached around 0.9 g/L, leading to an approximate recovery rate of 80% [117] (Fig. 5).

Chronological Development

From its inception in the 1920s, the ethanol business took nearly a century (1920–2015) to create today. It started when the cost of petroleum-based fuel increased and the need for an octane-based fuel was created as a result of environmental risks associated with leaded gasoline. Because corn was readily available and easily converted to alcohol, it was the only feedstock used to produce ethanol at first. As a result, farmers started making ethanol to increase the value of their corn. Production of ethanol-blended fuel expanded throughout the 1990s due to the finding that it significantly reduces carbon monoxide emissions and is therefore more environmentally friendly [118–120]. Various feedstocks have been employed in the production of bioethanol. This began (first generation) with crops that were consumable food staples; however, as feed vs. fuel became unbalanced, the emphasis turned to more affordable and sustainable resources. Additionally, there was a little shift in emphasis from crops to agricultural residues and finally algal biomass. First-generation ethanol was mostly made from the starches or sugars found in plants [121, 122]. Food crops are used to directly make first-generation biofuels. Three main feedstocks were used: corn, wheat, and sugarcane. Even now, first-generation biofuel is sold commercially and provides advantages for CO_2. First-generation feedstocks provide the majority of the commercially accessible biofuel generated. First-generation biofuels like fatty acid methyl ester (FAME) or biodiesel, maize ethanol, or sugar alcohol are made from feedstocks like vegetable oil, corn sugar, etc. [16, 123, 124]. The primary issue with this crop is that it is a staple in many rich and developing nations, which has increased food prices globally and even caused hunger. When sugarcane is utilized as feedstock, a similar issue arises. Fertilizers and insecticides must be used during the cultivation of maize and sugarcane, which is expensive and contaminates the land and water. Environmental risks presented yet another challenge to this kind of production [125, 126].

The "plant biomass" employed in the manufacturing of bioethanol in the second generation was significantly less expensive, more plentiful, and did not cause conflicts with food. In order to prevent conflicts between food and fuel, second-generation ethanol production technologies were created with an emphasis on agricultural residues and forest wastes, which primarily consist of various forms of lignocellulosic material [127, 128]. Although the second-generation bioethanol production techniques were a bit inexperienced

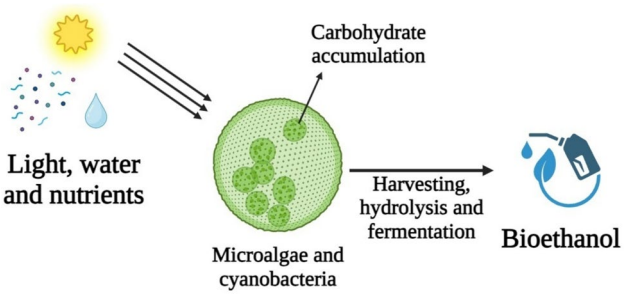

Fig. 5 Schematic diagram of photo fermentation

at first, they eventually became viable for a small number of producers thanks to improvements in bioprocess strategies, cost reduction, and the availability of sustainable resources [27, 127]. The main issues with the manufacturing of second-generation bioethanol were energy consumption and sugar deterioration during pretreatment procedures, which in turn added to the entire process's expense. The absence of effective microbes for the simultaneous fermentation of C5 and C6 into bioethanol constituted a significant additional barrier. Once more, the cost of the enzymes used in the saccharification process increased the total cost of production [129, 130].

For the synthesis of third-generation bioethanol, high-carbon embedded biomass was utilized. The creation of third-generation biofuels, particularly those derived from macro- or microalgae, is currently the subject of increased research due to its significant potential for biomass conversion. Algal biofuels are regarded by many scientists as the most promising substitute for first- and second-generation biofuels. Typically, the fermentation of algal polysaccharides such as starch, sugar, and cellulose is the foundation for the synthesis of bioethanol from macroalgae. They can therefore be regarded as feedstock for the synthesis of bioethanol [19, 121]. The main disadvantage of algal biorefining was that it required further improved pretreatment as a requirement and did not directly provide fermentable sugars. In order to produce biofuels using microalgae, third-generation bioethanol production required the development of a cost-effective technology. Alganol Biofuels Inc. created a method that uses specific photobioreactors to use sunlight-trapping microalgal cells as a miniature biorefinery for the generation of ethanol [121, 131].

Thus far, we have primarily concentrated on using sugar obtained from plants to create hydrocarbons, such as ethanol and biodiesel. Now, more recent and innovative methods, like introducing specific genes into *E. coli* to break down cellulosic biomass and produce an abundant and affordable supply of sugar, have a good chance of succeeding because this particular microorganism is highly researched and adapts well to genetic modifications [16]. Furthermore, *E. coli* grows 100 times quicker than most agricultural microorganisms and three times faster than yeast. The main issue with *E. coli* is that its maximal sugar content is just 10%. This means that commercialization will be challenging unless we can get a yield of about 70–90%. Since the metabolic pathway is well established, this problem can be handled by increasing sugar production utilizing metabolic engineering approaches [132, 133]. By manipulating biomass crops to function as effective "carbon capture" devices that take CO_2 out of the atmosphere and store it in their branches, trunks, and leaves, fourth-generation production methods are being used. Then, these biomasses rich in carbon are transformed into fuels. The important findings made by two research groups in creating trees that store a substantial amount more CO_2 than typical trees have created new opportunities for the production of less expensive fermentable sugar.

Microalgae Pretreatment for Bioethanol Production

The primary constituents of carbohydrates in microalgae include glycogen, starch, and cellulose. Starch and glycogen are essential substrates for the production of bioethanol from microalgae [38]. In contrast to the lignocellulose found in terrestrial plants, the cellulose found in the cell wall has a unique composition that makes it a good resource for the synthesis of bioethanol. Because microalgae don't contain lignin, less pretreatment is required to extract biodegradable organic compounds [26]. For the production of ethanol, lignin is difficult to metabolize; therefore, hydrolysis is required to convert it to glucose. Following the processing of biomass, the glucose obtained can be subjected to fermentation using either yeast or microbes in order to produce bioethanol. To extract the chemicals that are contained within the cells, they must be physically, chemically, or enzymatically disintegrated prior to fermentation [134, 135]. Several pre-treatment techniques can be seen in Fig. 6. Different treatment methods can be seen in Table 3.

Physical Pretreatment

Physical pre-treatments are frequently applied to promote the breakdown of carbohydrates and cell disintegration. A variety of energy-intensive techniques have been tested on several microalgae species to disturb their cellular structure, such as agitation, forceful mixing, bead milling, high-pressure homogenization, steam autoclaving, ultrasonication, and microwave therapy. These techniques are often combined with freezing, air-drying, and grinding processes [136, 137]. Operational variables that impact the process's efficiency include the capacity of the system, the amount of activity, the length of the processing, the concentration, and the state of the biomass (dry or wet). The ultimate determination of suitable techniques for cell disintegration primarily relies on the physiology and cell wall properties of the chosen microalgae [87, 138]. Cell disruption can be accomplished through the application of tangential force, such as strong mixing (vortexing), which creates shear stress on the cell wall. Bead milling is employed to disrupt cells by subjecting them to abrasion induced by solid beads moving at high velocities. While this procedure is often effective, it necessitates a relatively large amount of energy and generates a substantial

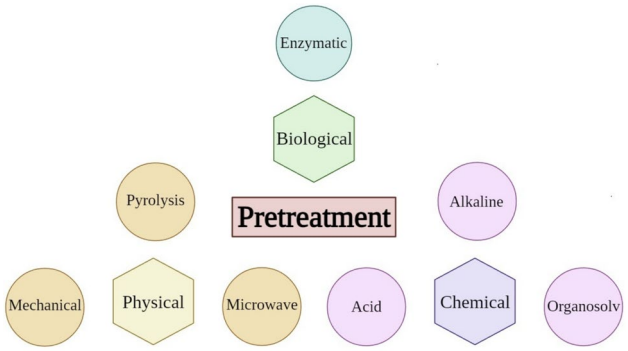

Fig. 6 Microalgae pretreatment techniques

quantity of heat. This approach is highly suitable for microalgae such as chlorella and other chlorophyta that have a resilient cellulosic cell wall [139, 140].

High-pressure homogenization is a straightforward method for breaking microalgal cells with strong cell walls. This process relies on the application of pressure and the features of the cell suspension. Agitation is an uncomplicated and energy-efficient method for breaking apart filamentous organisms [141, 142]. Arthrosporic cells are susceptible to physical agitation-induced rupture due to the fragility of their non-cellulosic cell wall, which is particularly susceptible to glycogen release into the surrounding medium upon stirring. After 24 h of treatment with α-amylase or glucoamylase, glucose was discharged into the medium [143]. Autoclaving is a more aggressive and efficient technique for cell disruption compared to prior gentle methods, as it produces high-pressure steam at temperatures ranging from 110 to 160 °C for around 15 to 60 min. This method is effective for both cell breakdown and improving the hydrolysis process since it enables the production of fermentable sugars without the need for acid treatment [144, 145]. In comparison to the untreated biomass, the red microalga *Gelidium amansii* recovered 40–55% of the solid biomass, 16–33% of the galactose biomass, and 82% of the glucose biomass after autoclaving for 20–80 min. In contrast to acid treatment, the glucose concentration exhibited constancy irrespective of the treatment duration [146]. The glucose content showed a proportional increase, but the galactose concentration exhibited a reduction as the treatment duration was extended. Acid pretreatment can impede the proliferation of microorganisms and result in environmental contamination. Ultrasonication is a high-frequency technique that causes cell disruption via two basic mechanisms: shock-wave propagation and cavitation. Strong shear pressures caused by shock waves cause jet streams to form in the surrounding media, which causes cell disintegration [147, 148]. Ultrasonic treatment was employed in *Chlorella* cultures at different power levels (ranging from 600 to 1000 W) and for varying durations (ranging from 30 to 90 min). The highest glucose yield, around 37 g per 100 g of dry weight, was achieved by applying 1000 W of power for 80 min. After this point, the glucose output began to decline significantly [149, 150].

By employing an electromagnetic field between 300 and 300 GHz, microwave therapy induces heating and vibration in biomass in a non-contact manner. This strategy features swift processing speed, remarkable disruption efficiency, and marginally increased energy consumption [151]. Microwave treatment can create unstable bonds within the carbon-chain structures, which in turn can modify the quality of the goods. Microwave-assisted hydrothermal extraction was employed to synthesize sulfated polysaccharides from Ulva spp. and *Monostroma latissimum* [152, 153].

Table 3 Different treatment methods with their description

Pretreatment method	Description
Physical pretreatment	Mechanical processes such as milling, grinding, or size reduction to break down lignocellulosic structure and increase surface area
Chemical pretreatment	Treatment with acids, alkalis, or solvents to dissolve or degrade lignin and hemicellulose, making cellulose more accessible
Biological pretreatment	Utilization of microorganisms or enzymes to degrade lignin and hemicellulose, typically under controlled environmental conditions
Steam explosion	Treatment with high-pressure steam followed by rapid decompression to disrupt biomass structure and increase accessibility
Organosolv	Treatment with organic solvents such as ethanol or acetone at elevated temperatures to dissolve lignin and hemicellulose
Ozonolysis	Treatment with ozone gas to selectively degrade lignin, leaving cellulose and hemicellulose intact
Ionic liquid	Dissolution of biomass in ionic liquids, which are salts in liquid state, to effectively separate lignin from cellulose and hemicellulose
Microwave-assisted	Application of microwave energy to enhance pretreatment processes by promoting rapid heating and biomass breakdown

Chemical Pretreatment

The simultaneous cleavage of carbohydrates and disintegration of cell walls can be achieved through the use of chemical pre-treatments, such as acids and alkaline. The primary benefit of acid hydrolysis is its rapidity, simplicity, and cost-effectiveness in comparison to other hydrolysis techniques [154, 155]. Conversely, the presence of acidity can cause carbohydrates to break down into undesirable chemicals that hinder the fermentation process. In addition, elevated levels of acid can impede the fermentation process due to the development of salts following the neutralization of the combination. Acid pre-treatments involve the use of acid as a catalyst to enhance the accessibility of cellulose to enzymes. The processes can be categorized into two groups: those that utilize concentrated acid and those that utilize diluted acid. The utilization of concentrated acid is comparatively less favorable than that of diluted acid on account of the potential for apparatus corrosion and the formation of a substantial quantity of inhibitory components [36, 156]. Acids such as sulfuric, hydrochloric, nitric, and phosphoric acids are commonly employed in various pretreatment processes. Reduced concentration acid solutions are employed at room temperature to convert lignocellulosic structures into water-soluble sugars. In modern times, biomass is commonly subjected to dilute sulfuric acid treatment, mostly to break down hemicelluloses and make enzymatic hydrolysis easier. Sulfuric acid, when diluted, breaks down biomass into hemicelluloses. These hemicelluloses are then further broken down into xylose and other sugars. Xylose can be further converted into furfural. The hazardous compound furfural, present in the process of ethanol synthesis, is extracted by the process of distillation [156, 157].

These procedures are conducted under lower temperature and pressure conditions in comparison to alternative methods. In contrast to acid pre-treatments, lignin removal had no discernible effect on the other constituents. However, there are limitations, such as the fact that certain alkaline materials can turn into salts that cannot be recovered. Additionally, hemicelluloses and cellulose have less solubility in this pre-treatment procedure than they do in acid pre-treatment [158]. Alkaline pre-treatment increases surface area, reduces the concentration of lignin and hemicelluloses in biomass, and makes it easier for water molecules to separate the two. Ammonia, calcium hydroxide, potassium hydroxide, and sodium hydroxide are the most common catalysts used in this process [159, 160]. The effects of alkaline pretreatments vary depending on the type of biomass. Reducing sugar yields in coastal bermudagrass decreases as the alkaline concentration increases during pretreatment [161].

A technique called "organosolv pre-treatment" makes use of organic solvents such as ethylene glycol, methanol, acetone, and ethanol. In addition to solvents, catalysts can also be added to the process. Ammonia, sodium hydroxide, sulfuric acid, and hydrochloric acid are the catalysts used in the process [162, 163]. Furthermore, alongside the breakdown of lignin and hemicellulose connections, it is feasible to obtain uncontaminated and superior-grade lignin as a secondary output. Removing lignin increases the available surface area and improves the ability of enzymes to reach cellulose. After undergoing the pre-treatment procedure, we obtain cellulosic fibers, solid lignin, and a liquid solution that contains hemicellulose sugars [164]. This approach is associated with several drawbacks, such as oxidation, volatilization, and increased danger during high-pressure processes. In addition, it is necessary to recover solvents to address the creation of substantial quantities of furfural and soluble phenols, as well as to minimize operational expenses.

Biological Pretreatment

Biological pretreatment is a more environmentally friendly procedure; however, it is more expensive than chemical treatment. It yields a significantly high amount of glucose without generating any inhibiting byproducts. On the other hand, efficiency depends on several variables that must be adjusted, including temperature, pH, and enzyme concentration. When it comes to producing bioethanol, utilizing microorganisms in pretreatments is regarded as environmentally favourable due to the absence of chemical usage, lower energy requirements, no need for corrosion-resistant and pressure-resistant reactors, and less inhibitor creation [27, 28]. These microorganisms can partially break down lignin, hemicelluloses, and cellulose. Although it has advantages, the long processing time, huge production area, and necessity for continual control of microbe development are limitations for commercial manufacturing [135, 165].

Enzymatic hydrolysis refers to the process of breaking down cellulose through the action of cellulase enzymes. The compounds obtained by hydrolysis are reducing sugars, specifically glucose. The cost of enzymatic hydrolysis is lower than that of acid or alkaline hydrolysis because it is conducted under mild conditions, with a pH of 4.8 and a temperature of 45–50 °C [166]. Bacteria and fungi can manufacture cellulase enzymes for use in hydrolysis. These bacteria can exhibit aerobic, anaerobic, mesophilic, or thermophilic characteristics. Some examples of bacteria that produce cellulase are *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, Acetovibrio, Microbispora*, and *Streptomyces*. *Trichoderma, Penicillium, Fusarium, Phanerochaete, Humicola*, and *Schizophillum* sp. are fungi known for their ability to produce cellulase, a type of enzyme that breaks down cellulose [167, 168]. Even though anaerobic bacteria exist that show great specific activity in the manufacture of cellulase, these bacteria are

not suitable for large-scale industrial production. The three enzymes that makeup cellulase are β-glucosidase, endoglucanase, and exoglucanase. Endoglucanase targets areas of cellulose fibers with reduced crystallinity, while exoglucanase eliminates cellulose units from liberated chains in conjunction with endoglucanase, ultimately breaking down the molecule [169, 170]. Glucose is produced when B-glucosidase catalyzes the breakdown of cellulose units. Certain factors related to the enzymes and substrates involved can affect the process of enzymatic hydrolysis. Parameters connected to the substrate have a direct impact on the process of enzymatic hydrolysis [171, 172]. The enzymatic conversion is influenced by these interrelated characteristics. The cellulose's degree of polymerization and crystallinity, its accessibility, the quantity of lignin and hemicelluloses, and the size of the pores are the variables that can be determined.

The degree of polymerization and crystallinity of the cellulose determine the rates of hydrolysis of biomass. The degree of crystallinity is directly correlated with the degree of polymerization. Enzymes called cellulase are able to degrade the inflexible cellulose structure. Endoglucanase enzymes cleave the inner sites of cellulose chains during enzymatic hydrolysis, reducing the degree of polymerization of the cellulosic component [173, 174]. The rate of hydrolysis is significantly influenced by the substrate's accessibility. The accessibility of the substrate directly affects the rate of hydrolysis because an increased surface area makes it more vulnerable to enzyme attack. Because of their complex structures, lignin and hemicellulose pose difficulties for hydrolysis in lignocellulosic materials [175]. Lignin, functioning similarly to cement, serves as a physical barrier that hinders the hydrolysis of the digestible components of cellulose and impedes enzyme access to cellulose. Consequently, they diminish the effectiveness of hydrolysis. The elimination of hemicellulose increases the size of the pores and allows enzymes to easily access cellulose, enabling effective hydrolysis [176, 177]. The substrate's pore size is a limiting element in the enzymatic hydrolysis process. Within several lignocellulosic materials, the external surface area is comparatively lower than the interior area. Consequently, this circumstance leads to the entrapment of cellulase enzymes within the material's pores. To enhance the rate of hydrolysis, it is necessary to augment the porosity of the biomass.

Factors Affecting Bioethanol Production

Salinity

Salinity has the potential to influence a variety of metabolic processes that are integral to the development and functioning of microalgae. Increased concentrations of salt will impede the ability of microalgae to absorb water and nutrients, consequently impeding their development and ultimately leading to their demise. Salinity stress can be categorized into three separate types: ionic stress, osmotic stress, and oxidative stress. Ionic stress occurs when there is a disruption in the balance of ions in the body. The competition between NaC and KC caused by salinity stress depletes KC in the cytoplasm [178]. In addition, reactive oxygen species and oxidative stress are also unbalanced by salinity stress. Lipid synthesis often increases in microalgal cells under conditions of oxidative stress. Salt stress during microalgae culture is an efficient way to reduce pollution, contamination, and competition from other microorganisms and invasive species [179]. Oversalinity can affect the structure and growth of microalgal cells [180]. Hence, it is necessary to establish an optimal range of salinity.

Temperature

The impact of temperature on microalgal growth and lipid accumulation is comparable to that of light intensity. As the temperature increases, both variables grow exponentially and may finally reach a maximum value. Nevertheless, depending on the particular variety of microalgae, there are differences in the ideal temperature for getting the maximum output. Around 25 °C is when the lipid concentration in chlorella peaks, and as the temperature drops, it drastically declines. Scenedesmus obliquus exhibits an increase in lipid content from 18 to 40% as the temperature rises from 20 to 27.5 °C [18]. Nevertheless, the rise in temperature does not necessarily result in an increase in lipid content.

Light

Light is necessary for the growth of microalgae. High light intensity promotes lipid formation in microalgal cells by facilitating the storage of surplus photosynthetic products and their subsequent conversion into chemical energy. Nevertheless, the optimal light intensity for attaining maximum lipid production varies among microalgae species due to their distinct light utilization efficiencies [181]. Saturated or low light intensities inhibit the proliferation of microalgae. Low light intensity has an adverse effect on microalgae proliferation and lipid accumulation, specifically below the compensation point [182]. Conversely, after the compensation point is attained, the productivity of microalgae improves with higher light intensity, and the maximum efficiency of photosynthesis is achieved at the saturation point. Hence, augmenting the intensity of light positively influences the promotion of lipid synthesis in microalgae. However, an excessive amount of light can cause photoinhibition, which in turn decreases the production of lipids in microalgae.

Nutrient

Utilizing nutrient deprivation is a cost-effective and environmentally sustainable approach to efficiently enhancing lipid production in microalgae. At present, nutritional deprivation has been demonstrated as the most efficient and widely used method for inducing fat synthesis. Among the several nutritional deprivations, nitrogen (N), phosphorus (P), and sulfur (S) deficiencies are the most commonly utilized techniques for inducing lipid production in microalgae. Microalgae produce substantial quantities of lipids during nitrogen stress, whereas the amino acid concentration experiences a large drop [183, 184]. Scenedesmus exhibited a significant increase in lipid content, rising from 10 to 29.5% when subjected to phosphorus stress during growth. The presence of stress can impact the allocation of carbon within microalgae, thereby facilitating the synthesis of lipids. With the exception of nitrogen (N), phosphorus (P), and sulfur (S) deficiencies, the exploitation of nutritional elements is a highly effective method to boost lipid production in the development of microalgae [185, 186]. This is due to the culture medium's capacity to deprive every component. The creation of lipids, proteins, and carbohydrates allows for the use of carbon that is taken during photosynthesis. A crucial element in the process of protein synthesis is nitrogen. When the quantity of nitrogen is limited, it has a negative impact on cell division, photosynthesis, and the growth rate of microalgae cells [187]. The distribution of carbon in microalgae is similarly impacted by nutrient limitations. In photosynthesis, carbon is fixed at a rate 7–10 times faster than nitrogen, which is digested when there is a sufficient supply of the element nitrogen (N). This is adequate to manufacture biological components including proteins, DNA, and pigments that include nitrogen [188].

pH

Under some circumstances, the pH value of the surrounding environment serves as a critical and thorough indicator of the metabolic processes of microalgae. Additionally, it has an impact on the culture medium's relative concentration and dynamic forms of inorganic carbon sources. Consequently, pH significantly influences the cellular proliferation and lipid storage of microalgae. Moheimani conducted a study to examine how the pH value affects the production of lipids in *Tetraselmis suecica* and *Chlorella* sp. [189]. At pH 7.5, *T. suecica* was able to reach a maximum lipid productivity of 92 ± 13.1 mg/l/d, while Chlorella sp. was able to produce 99 ± 17.2 mg/l/d at pH 7.0 [189]. Qiu et al. examined how different pH levels affect the production of lipids in a particular strain of *Chlorella sorokiniana*. Through the introduction of CO_2 during the feeding process, the pH value was modified, resulting in the identification of an ideal pH of 6.0 for the accumulation of lipids. Furthermore, the biodiesel produced at pH levels of 6.5, 7.0, and 7.5 met the requirements of the diesel standard in terms of cetane numbers [190].

Metabolic Engineering and Synthetic Biology for Enhanced Bioethanol Production

Certain natural strains of microalgae possess inherent biological pathways for the production of biofuel compounds. Figure 7 provides a diagrammatic representation of lipid metabolic pathways in microalgae. The primary metabolic routes of microalgal lipids consist mostly of the de novo biosynthesis pathway for fatty acids and the synthesis route for TAG. The process of synthesizing fatty acids from scratch takes place within the chloroplasts of microalgae. Acetyl-CoA is the primary compound implicated in the synthesis of fresh fatty acids. Malonyl-CoA is formed from it, resulting in the synthesis of saturated fatty acids. These fatty acids then undergo an additional process of desaturation and elongation to form unsaturated fatty acids. This entire process is facilitated by complex enzymes called fatty acid synthases [191, 192]. TAG synthesis in the endoplasmic reticulum is hypothesized to occur via three consecutive acyl group transfers from acyl-CoA to glycerol-3-phosphate [193, 194]. The industrial and commercial development of microalgal biofuels is impeded by the restricted natural supply. In the realm of biotechnology innovation, metabolic engineering is an indispensable field of study involving the modification of metabolic pathways to induce the synthesis of particular biofuel molecules.

Triacylglycerol Synthesis Pathway

Microalgae contain the enzyme G3PDH, which catalyzes the conversion of DHAP to glycerol-3-phosphate in the cytoplasm. The transformation of glycerol-3-phosphate into lysophosphatidic acid is then catalyzed by GPAT. Through catalysis, lysophosphatidic acid is changed into phosphatidic acid by LPAAT. Phosphatidic acid is enzymatically converted by PAP into diacylglycerol, which is further converted by DGAT in the endoplasmic reticulum into TAG [195]. *Chlorella minutissima* cells exhibited a twofold increase in lipid production when G3PDH, GPAT, LPAAT, PAP, and DGAT genes from *Saccharomyces cerevisiae* and/or *Yarrowia lipolytica* were upregulated concurrently, in contrast to the wild type [195].

When compared to other genes involved in TAG production, the genes producing GPAT and DGAT may be more efficient in increasing lipid buildup during the process. Zulu et al. carried out a study in which they successfully introduced DGAT from yeast and oleosin (a protein important for

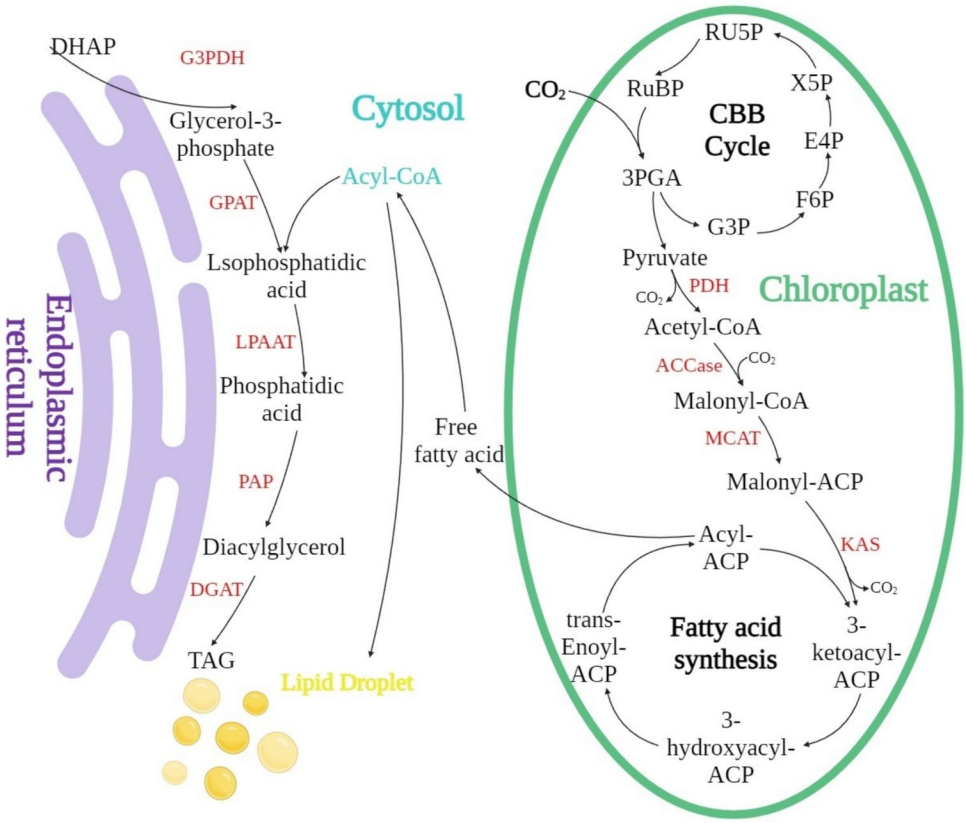

Fig. 7 The diagram illustrates the metabolic pathways responsible for lipid synthesis in microalgae. 3PGA is an abbreviation for 3-phosphoglycerate. ACCase denotes acetyl-CoA carboxylase. DGAT is an acronym for diacylglycerol acyltransferase; DHAP is the acronym for dihydroxyacetone phosphate; and E4P is an abbreviation for erythrose-4-phosphate. F6P refers to fructose-6-phosphate. FAT is an acronym for fatty acyl-ACP thioesterase. G3P is a shortened form of the term glyceraldehyde-3-phosphate. G3PDH is an abbreviation for glycerol-3-phosphate dehydrogenase, while GPAT is an abbreviation for glycerol-3-phosphate acyltransferase. KAS is an abbreviation for 3-ketoacyl-ACP synthase. LPAAT is an abbreviation for lysophosphatidic acid acyltransferase. MCAT refers to malonyl-CoA: acyl carrier protein transacylase. PAP stands for phosphatidic acid phosphatase. PDH refers to the pyruvate dehydrogenase complex. Ru5P stands for ribulose-5-phosphate. RuBP refers to ribulose-1,5-bisphosphate. TAG stands for triacylglycerols. X5P refers to xylulose-5-phosphate (adapted from Zhu et al. [195])

stabilizing lipid droplets) from *Phaeodactylum tricornutum* plants. As a result, the TAG concentration in cells of the wild strain increased by a factor of 3.6. In comparison to traditional single gene building methods [196], Niu et al. discovered that by increasing the expression of the same GPAT gene, *P. tricornutum* was able to produce double the quantity of neutral lipids compared to cells with normal gene expression [197]. Chen et al. used a DGAT gene from *Chlamydomonas reinhardtii* to genetically modify a strain of Scenedesmus obliquus. A 40-L cylindrical photobioreactor was used to successfully cultivate the modified version. This genetically modified strain's lipid content came out to be 12.3% of the dry cell weight, which is 128% more than the lipid content of cells from the wild strain [198]. If necessary, the promising strategy of metabolic engineering can be employed to enhance the production of essential enzymes involved in the pathway for triacylglycerol (TAG) synthesis in microalgae. Furthermore, it is logical to enhance the quality of the lipids produced, in addition to employing metabolic engineering to increase their number in microalgae. Efforts have been made to enhance the quality of lipids by modifying the degree of unsaturation of fatty acids and the length of their carbon chains.

Pathway for de novo Fatty Acid Biosynthesis

Malonyl-CoA: The transformation of malonyl-CoA into malonyl acyl carrier protein is catalysed by acyl carrier protein transacylase (MCAT). The first and most important step in the creation of fatty acids is this enzymatic reaction, which is followed by a sequence of reduction-dehydration-reduction processes. It has been demonstrated that overexpressing MCAT causes fatty acid buildup to rise [186]. For example, the MCAT gene in *Schizochytrium* was artificially enhanced, leading to a total lipid production of 110.5 g/l during fed-batch cultivation. The lipid output in cells of the

wild strain was surpassed by a 39.6% increase. Moreover, the increased expression of MCAT resulted in an enhancement in the production of polyunsaturated fatty acid [199]. By means of MCAT overexpression, the lipid content of the oleaginous microalga *Nannochloropsis oceanica* increased to 42.9% of the dried cell weight. This led to a 36.0 percent increase in lipid content in comparison to wild-type cells [198]. The conversion of acetyl-CoA to malonyl-CoA is catalyzed by ACCase, which enables malonyl-CoA to enter the fatty acid biosynthesis pathway. A multitude of studies have provided evidence that up-regulating ACCase expression facilitates the fatty acid biosynthesis pathway [200]. Genetic engineering has been extensively used to enhance the production of fatty acid-based biofuels by increasing the expression of ACCase in model microorganisms including *Escherichia coli* and *Saccharomyces cerevisiae* [201].

Glucose-6-phosphate dehydrogenase (G6PD) plays a crucial role in the pentose phosphate pathway by facilitating the production of NADPH, an enzyme that is vital for maintaining redox equilibrium and the balance of reducing agents. Xue et al. engineered a genetically modified *Phaeodactylum tricornutum* strain in which G6PD expression was increased [202]. This led to higher levels of both G6PD mRNA and enzyme activity, as a result of enhanced NADPH production [195]. Enhancing lipid formation in microalgae can be significantly improved by increasing the declining power supply and overexpressing G6PD. This implies that G6PD has the potential to be a valuable focus for metabolic engineering to enhance microalgal lipid synthesis. Overexpressing the malic enzyme has been shown to have a vital function in enhancing the production of neutral lipids in *P. tricornutum* by supplying more NADPH [48].

Techno-Economic Analysis

An earlier investigation recorded the Techno-Economic Analysis (TEA) pertaining to the generation of bioethanol from microalgae cultivated in an open raceway pond measuring 3.94 hectares in diameter [203]. The entire direct cost consists of the aggregate amount spent on equipment, including its acquisition, installation, and commissioning. Additionally, the total direct cost includes expenditures for piping, electrical, and instrumentation. The indirect costs that contributed to the capital expenditures (Capex) were derived from engineering, contingency, fees, and miscellaneous items. The primary expense of operational expenditure (Opex) stemmed from the unit processes of microalgae cultivation, extraction, hydrolysis, fermentation, and distillation. In addition to operational costs, the salvage value of the bioethanol facility was another factor that contributed to the Opex costs. Assuming a 20-year duration for the project, the remaining value is included in the Opex as a depreciation cost. The cost of producing bioethanol can vary from 1.3 to 19.4 US dollars per gallon. The cost discrepancy is attributed to the selection of different case study situations for the TEA investigations. Additional assistance through subsidies, tax credits, and a compulsory policy for blending bioethanol could decrease the cost of microalgal bioethanol. While gaseous biofuels possess greater calorific values compared to liquid fuels, the storage and transportation of gaseous fuels pose significant challenges. Bioethanol possesses the lowest higher heating value (HHV) in comparison to other fuels. As a result, it can be utilized as an additive in gasoline fuel to elevate the octane value [204]. This research demonstrates that the primary focus of TEA studies is the production of biomass that is rich in carbohydrates or lipids, with the intention of using it to produce biofuels. The economic feasibility of microalgal biofuels is compromised by the negative effects of cultivating lipid- and carbohydrate-rich biomass, including longer cultivation times, lower biomass productivity, and increased costs for harvesting and processing [205].

Life Cycle Assessment

Life Cycle Assessment (LCA) is a methodology employed to assess the feasibility of bioethanol production from microalgae as well as to analyse the environmental impacts during the entire production process. This study assesses the economic feasibility of the several processing methods used in the synthesis of bioethanol. Various growing techniques were employed to assess the viability of implementing a biorefinery system for commercial purposes. Converting the oil-free biomass and lipids into bioethanol would result in a higher ratio of non-renewable energy to greenhouse gas emissions (GHG). To enhance the sustainability of the process, it is imperative to optimize growth conditions, improve extraction procedures, and promote the reuse of co-products. Greenhouse gas emissions from various technologies were analyzed and modeled using SimaPro software [37]. The integrated techniques demonstrate a reduction in greenhouse gas (GHG) emissions, with a growth phase of algae contributing 0.03 GHG (kilograms of CO2 equivalent per megajoule) and accounting for 50% of the total emissions. The conventional extraction paths have a greenhouse gas (GHG) emission rate of 94% and a fossil fuel consumption rate of 84% [10]. Additionally, these pathways require an upgrade of the extraction process. The base scenario exhibits greater magnitudes of consequences in comparison to bioethanol, while the future case demonstrates improved efficiency with reduced impacts, resulting in outcomes that are quite similar to those of petroleum [16]. The life cycle commences with the culture of microalgae followed by the dewatering of microalgae, the extraction of lipids, the conversion of oil, and the recovery of the final product and co-product. Both

the raw materials and emissions resulting from production were considered.

Commercialization

Microalgae are increasingly gaining popularity in several industries, particularly in the field of biofuels. It takes a well-managed supply chain to maximize the profitability of algae biofuel. Bioethanol produced from microalgae is likely to prove to be a competitive fuel alternative to petroleum-based fuels. Biofuels such as diesel, gasoline, and bioethanol can be produced from microalgae and used directly for electricity generation or as a means of transportation. Because of its higher-octane number (108), a wider range of flammability, quicker flame propagation, and higher heat of vaporization, bioethanol is more efficient than gasoline. These characteristics allow for a shorter combustion period and a higher compression ratio [206]. In June 2006, PetroSun founded Algae Biofuels in Australia and the United States to investigate the production of hydrogen, ethanol, methanol, biodiesel, and methane from microalgae. The business also provided feedstock to BioAlternatives, a different company, with an annual capacity of half or up to 150 million gallons [207]. Furthermore, in the United States, Algenol, a company that started making bioethanol from algae in 2006, produced a significant volume of 8000 gallons of liquid biofuel per acre annually. They achieved this by utilizing algal feedstocks, sunlight, CO_2, and saltwater [207]. In 2007, Sapphire Energy, Inc. was founded in California, USA, with an investment of more than $100 million. The production of 100,000 gallons of ethanol annually that satisfies fuel-grade requirements was its main goal [208]. By the year 2030, it is projected that the algal biofuel market will experience significant growth and capture 75% of the market share, establishing its dominance. Different bioethanol plants around the world can be seen in Table 4.

Advantages

Bioethanol that is created from algae is considered to be the third generation of biofuels. The use of this energy is considered to be the most effective fuel since it has the potential to reduce consumption and demand for non-renewable energy sources, while simultaneously reducing greenhouse gas emissions that are responsible for the phenomenon of global warming. It is generally accepted that the production of biomass from microalgae can make a substantial contribution to the generation of clean energy for the environment. Furthermore, when it comes to the synthesis of bioethanol from algae, it is essential to note that some limitations must be taken into consideration. Comparatively speaking, the growth rates of microalgae are substantially higher than those of terrestrial crops. It has been stated that the oil output per unit area from algae is expected to range from 20,000 to 80,000 per acre in 2017 [210]. This is seven to thirty-one times more than the yield of palm oil, a crop that is considered to be the next most prolific crop. Furthermore, in contrast to other crops that produce oil, algae do not require the use of potable water or dry soil to be cultivated. This is a significant benefit. Furthermore, they do not compete with one another for the resources that are allocated to the production of food. On the other hand, to cultivate these algae on a large scale, a significant amount of land and water are required. Furthermore, the algae can remove nitrogen from wastewater and separate CO_2 from the environment. However, it is possible that the concentration of CO_2 in the atmosphere is not sufficiently raised to drive the rapid expansion of algae. To attain a high level of sustained and high production of algae in a controlled environment, there is a need for highly effective systems that can deliver a huge quantity of vital nutrients. These nutrients include sulfur, iron, nitrogen, and CO_2.

Challenges

Despite the potential demonstrated by microalgae in bioethanol production, there are still significant economic and sustainability challenges that hinder its adoption as a viable fuel in the market. Hence, it is imperative to effectively implement advanced technologies and discoveries in order to overcome the challenges associated with microalgal bioethanol. Microalgae have been found to produce a higher yield of bioethanol, namely 15,000 gallons per acre, compared to

Table 4 Bioethanol plants around the globe (adapted from IEA Bioenergy [209])

S.no	Company	Location
1	Anhui BBCA Biochemical	Bengbu, China
2	ArcelorMittal	Ghent, Belgium
3	ARD	Pomacle, France
4	AustroCel Hallein	Hallein, Austria
5	Biomaterial in Tokyo Co., Ltd	Kawasaki-shi, Japan
6	Borregaard AS	Sarpsborg, Norway
7	Chempolis Ltd	Oulu, Finland
8	Clariant	Straubing, Germany
9	Domsjoe Fabriker	Ornskoldsvik, Sweden
10	Enerkem	Westbury, Canada
11	GranBio	Sao Miguel, Brazil
12	Indian Glycol & DBT-ICT	Kashipur, India
13	BioCentury Research Farm	Boone, USA
14	Crescentino Biorefinery	Crescentino, Italy

other plants and feedstocks that grow on land and have a lignocellulosic composition [211]. The generation of bioethanol from marine microalgae has lately gained attention as a means to decrease the consumption of freshwater. Microalgal cell disintegration for bioethanol production poses a challenge. However, the application of biological pretreatment approaches to microalgae has shown encouraging results in terms of bioethanol yields. While the resistant composition of the cell wall is a challenge to efficient biological treatment, additional study is necessary for enhancing the biological pretreatment of microalgal cells. Biological pretreatment is regarded as a more environmentally sustainable method in comparison to certain thermo-mechanical pretreatment approaches, according to a study [212]. In addition, recent studies have focused on the advancement of transgenic microalgae through the use of synthetic biology and recombinant DNA technology. This involves the modification of individual microalgae or cyanobacteria to produce and release bioethanol, a desirable biofuel product [213]. To address the issue of high process costs, researchers are working on developing a method called consolidated bioprocessing. This method entails utilizing fungal enzymes during the initial stage of processing to facilitate the concurrent breakdown of carbohydrates into sugars and their subsequent conversion into alcohol within a single vessel [214].

Future Perspective

The use of bioethanol has gained popularity worldwide due to the detrimental effects of fossil fuel consumption on the environment and oil supplies. Nevertheless, a significant obstacle lies in the lack of uniformity among systems for commercializing bioethanol production, necessitating further research in this area. Furthermore, the commercialization process of bioethanol derived from microalgae is beset by challenges including inadequate government support, the substantial initial investment required for facilities, and the inadequate application of relevant policies. At present, researchers are emphasizing the improvement of algal bioethanol technologies and are actively pursuing a more sophisticated transgenic algae variant to achieve consistent results [38]. The term "fourth-generation algal biofuels" or "photosynthetic biofuels" refers to the utilization of synthetic biology techniques in algae and cyanobacteria for fuel production. Most of the research on fourth-generation biofuels focuses on the development of photobiological solar fuels, which depend on unicellular algae and cyanobacteria for synthesis. The technique entails the direct utilization of photosynthesis to produce fuels and chemicals. This is accomplished by metabolic engineering, wherein a solitary photosynthetic bacterium functions as a catalyst and processor to produce and release readily usable products with exceptional efficiency in photosynthesis [215, 216]. Utilizing bioengineering techniques and recombinant DNA, the production of biofuels from phytoplankton of the fourth generation is accomplished through the direct manipulation of cellular metabolism. This is achieved by introducing, deleting, and/or modifying the metabolic networks of algae to boost biofuel production. This major methodology offers a higher level of efficiency in the production of biofuels and enhances economic sustainability by reducing the costs associated with biomass separation and processing, in comparison to the conventional approach [38, 217]. However, fourth-generation biofuel is purported to have a carbon-negative impact, as it sequesters a greater amount of waste CO_2 than it generates. Research on fourth-generation algae biofuels is limited, and there is insufficient knowledge of the metabolic engineering aspects of their technical performance.

Conclusion

In recent years, microalgae have become a significant source of raw materials for the manufacturing of biofuels. Currently, there is extensive research focused on enhancing the biodegradability of simple sugars using pretreatment approaches. The main objective is to increase bioethanol yield while minimizing economic and environmental expenses. Concerns associated with chemical pretreatments include the disposal of waste produced during reactions and the contamination of the environment by solvents and products. While employing enzymes in pretreatment may lead to a higher ethanol yield in comparison to alternative methods, the principal drawback of this strategy is the elevated ethanol production expense caused by the costly enzymes. Concerns such as the selection of suitable fermentative microorganisms, the optimization of pretreatment methods to disrupt the cell structure, the choice of microalgae strains, and the financial implications of expanding the operations remained obstacles to the production of this eco-friendly energy. We give preference to algae strains that produce commercial biofuels and have a high biomass output as well as high levels of carbohydrates and lipids. To tackle this issue, it may be necessary to conduct extensive research in the fields of biotechnology, genetic modification, and metabolic engineering. The objective of this study is to employ evolutionary engineering methods to enhance the synthesis pathway of lipids and cellulose in microalgae while also improving the tolerance of fermentative bacteria to increase ethanol output. An impediment to the expansion of the commercial value of algal bioethanol is the inadequate implementation of efficient technologies and discoveries. Economic

and sustainability challenges impede the commercial production of bioethanol from microalgae for use in the fuel market. While the potential for bioethanol generation from microalgae is promising and genuine, it is crucial to prioritize the development of economically viable, globally compatible, sustainable, and eco-friendly biofuels.

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Author Contribution SP and CP: both have equal contribution, Conceptualization, methodology, validation, investigation, writing-original draft, writing-review and editing, visualization. RCK and ASM: investigation, writing-review and editing, Visualization. SR and SM: writing-review and editing, writing-review, visualization, supervision. RCK and RP: writing-review and editing, visualization, supervision.

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Data and Materials Availability All data used have been included in the manuscript.

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Declarations

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

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