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Review

From Microalgae to Bioenergy: Recent Advances in Biochemical Conversion Processes

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Abstract: Concerns about rising energy demand, fossil fuel depletion, and global warming have increased interest in developing and utilizing alternate renewable energy sources. Among the available renewable resources, microalgae biomass, a third-generation feedstock, is promising for energy production due to its rich biochemical composition, metabolic elasticity, and ability to produce numerous bioenergy products, including biomethane, biohydrogen, and bioethanol. However, the true potential of microalgae biomass in the future bioenergy economy is yet to be realized. This review provides a comprehensive overview of various biochemical conversion processes (anaerobic digestion, direct biophotolysis, indirect biophotolysis, photo fermentation, dark fermentation, microalgae-catalyzed photo fermentation, microalgae-catalyzed dark fermentation, and traditional alcoholic fermentation by ethanologenic microorganisms) that could be adapted to transform microalgae biomass into different bioenergy products. Recent advances in biochemical conversion processes are compiled and critically analyzed, and their limitations in terms of process viability, efficacy, scalability, and economic and environmental sustainability are highlighted. Based on the current research stage and technological development, biomethane production from anaerobic digestion and bioethanol production from traditional fermentation are identified as promising methods for the future commercialization of microalgae-based bioenergy. However, significant challenges to these technologies' commercialization remain, including the high microalgae production costs and low energy recovery efficiency. Future research should focus on reducing microalgae production costs, developing an integrated biorefinery approach, and effectively utilizing artificial intelligence tools for process optimization and scale-up to solve the current challenges and accelerate the development of microalgae-based bioenergy.

Keywords: anaerobic digestion; biomethane; biohydrogen; biophotolysis; dark fermentation; photo fermentation; alcoholic fermentation; bioethanol

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1. Introduction

Energy has played a crucial role in economic and social development [1]. However, over 80% of our energy demand is fulfilled by non-renewable and less environment-friendly fossil fuels such as coal, natural gas, and oil [2]. These fuels are the primary source of carbon dioxide emissions, responsible for over 90% of global carbon emissions [3]. This dependency on fossil fuels has created two crises: fossil fuel depletion and global climate change. Despite this, energy demand continues to rise and is projected to increase by 47% in the next 30 years, particularly in developing countries [4]. It is, thus, imperative to find alternative renewable and clean energy sources to ensure a sustainable future. Bioenergy, the oldest known form of energy derived from biomass, is a promising option for meeting

growing energy demand sustainably [5]. Biomass can be replenished in a relatively shorter time when compared with fossil fuels, making bioenergy a renewable energy source [6]. Bioenergy is carbon-neutral, releasing only the carbon dioxide that biomass consumes during its growth. Compared to bioenergy, fossil fuels release new carbon dioxide into the atmosphere that was sequestered and stored under the earth's crust for millions of years [7]. Another advantage of bioenergy is its local production, reducing the need for long-distance energy transportation [8]. Local bioenergy production reduces the environmental impact of energy production and stimulates the local economy by providing energy security and economic opportunities [9].

Bioenergy is a broad term that refers to energy derived from various biomass sources, including food/non-food crops, agricultural/forest residue, and even algae [10]. Biomass can be divided into three generations based on its type. First-generation biomass includes traditional food and energy crops, such as corn, sugarcane, maize, soybean, and palm. Second-generation biomass comprises non-food crops and waste, such as woody/grassy plants and forest product residues [11]. Currently, a majority of biofuel is derived from first-generation biomasses, such as sugar (36.3 billion liters/year), maize (61.8 billion liters/year), palm oil (18.3 billion liters/year), and soybean oil (13.6 billion liters/year) [12]. However, using first-generation feedstocks for bioenergy production presents a food vs. fuel dilemma and challenges the food security [13]. Although second-generation feedstocks do not include food crops, these still compete with food production systems for resources such as arable land, freshwater, and fertilizers [14], making them unsustainable for fuel production. Third-generation feedstock, algae, has the potential to meet future bioenergy demands without compromising resources used for food production [15].

Microalgae are highly efficient photosynthetic organisms that rapidly grow throughout the year in various habitats, including aquatic (fresh or marine) and terrestrial ecosystems [16]. Microalgae exhibit relatively higher growth rates than terrestrial plants, completing an entire growth cycle in just a few days [17]. Moreover, microalgae can be cultivated on non-arable land using wastewater or seawater as a source of nutrients [18]. High biomass yield, minimal resource requirements, and a consistent biomass supply make microalgae a leading candidate for bioenergy production. Initially, the interest in using microalgae for bioenergy production was primarily due to its high lipid content (up to 60–70%), which could be converted into biodiesel using transesterification processes [19]. However, microalgae biomass can also be converted into other forms of bioenergy using biochemical and thermochemical processes. Biochemical conversion mainly includes anaerobic digestion [20], alcoholic fermentation [21], and fermentation/biophotolysis [22] processes for biomethane, bioethanol, and biohydrogen production, respectively. Thermochemical conversion includes pyrolysis [23], gasification [24], and hydrothermal processes [25] for bio-oil/biochar/syngas, syngas, and bio-oil/hydrochar/biogas production, respectively. Both biochemical and thermochemical processes are efficient pathways to recover energy from microalgae biomass since these methods utilize the whole biomass and do not depend on extracting specific macromolecules [26]. However, biochemical processes have a competitive advantage over thermochemical processes due to their ability to process wet biomass, operate at ambient processing conditions (temperature and pressure), and exhibit high selectivity toward the desired product, making them more sustainable and environment-friendly in the long run [27].

Most review articles regarding microalgal bioenergy concentrate on a single biochemical conversion technology [28–30]. However, conducting a detailed review of the available biochemical conversion technologies is crucial to selecting appropriate and sustainable methods for converting microalgae into bioenergy efficiently. This article provides a comprehensive overview of various biochemical conversion technologies, highlighting the most recent developments and critically discussing their limitations. This review article aims to provide valuable information to scientists, entrepreneurs, and governments, assisting them in identifying further research and development opportunities in microalgae-based bioenergy production.

2. Microalgae Biomass and Its Components

Algae are efficient, sunlight-driven green cell factories that convert carbon dioxide into various biomolecules, including lipids, carbohydrates, and proteins [31]. The cellular content of these biomolecules varies significantly between different algae species [32]. Algae can be classified into two broad categories: microalgae and macroalgae. Microalgae are unicellular and smaller (up to a few millimeters), whereas macroalgae are large (up to a few meters) and multicellular. There are more than 50,000 microalgae species in nature, of which 4000 species have been identified, and only a few species (<50) have been commercialized [33]. Therefore, microalgae biomass has immense untapped and unexplored potential. Table 1 displays the biochemical composition of commonly used microalgae species.

Table 1. Biochemical composition of some microalgae species (% *w*/*w*, on a dry mass basis).

Microalgae Species	Lipid	Protein	Carbohydrate	References
Botryococcus braunii	25–75	1.5	4–55	[16]
Chlorella emersonii	23–63	36	41	[19]
Chlorella protothecoides	40–60	10–28	11–15	[19]
Chlamydomonas reinhardtii	15–18	9.2	59.7	[16]
Chlorella sorokiniana	26.2	45.5	23.7	[34]
Chlorella vulgaris	41–58	51–58	12–17	[19]
Dunaliella salina	6–25	57	32	[16]
Euglena gracilis	4–20	39–61	14–18	[16]
Isochrysis galbana	11	27	34	[35]
Nannochloropsis gaditana	23.3	48.3	9.3	[20]
Nannochloropsis granulata	24–28	27–36	18–34	[35]
Neochloris oleoabundans	35–65	10–27	17–27	[19]
Porphyridium cruentum	9–14	28–39	40–57	[20]
Scenedesmus dimorphus	16–40	8–18	21–52	[19]
Scenedesmus obliquus	30–50	10–45	20–40	[19]
Tetraselmis chuii	12	31–46	12	[35]

All biomolecules in microalgae cells play crucial roles in growth, reproduction, metabolism, and other cellular functions [36]. Lipids, essential components of cell membranes, energy storage, and cell signaling molecules, can be categorized into two groups in microalgae: polar lipids and non-polar lipids [37]. Polar lipids, such as phospholipids and glycolipids, are structural lipids that help maintain cell shape and structure. Polar lipids constitute 41–92% of the total lipids in microalgae biomass. Non-polar lipids, or neutral lipids, such as sterols and free fatty acids (FFA), usually function as energy storage molecules and make up 5–51% of the total microalgal lipids [38]. Neutral lipids, stored as triacylglycerols (TAGs), are preferred for biodiesel production due to their lower degree of unsaturation and the fact that industrial-scale transesterification is designed to process acylglycerols and has limited efficacy on other lipid types [39]. This makes microalgae species with high concentrations of neutral lipids (TAGs) promising candidates for biodiesel production. Besides lipids, carbohydrates (polysaccharides or oligosaccharides) are energy-storage molecules and structural elements in all living cells [40]. Structural carbohydrates are primarily present in the cell wall, whereas storage components can accumulate inside or outside the chloroplast [19]. Some carbohydrates may also be excreted as exopolysaccharides [41]. Carbohydrates in microalgae cells consist mainly of cellulose and starch and are free of lignin [42], making them a suitable candidate for bioethanol production through fermentation or biomethane production through anaerobic digestion. In addition to carbohydrates and lipids, proteins play essential roles in living organisms. Proteins serve as the major structural components of cells and transport nutrients and other molecules

in and out of cells. They also act as enzymes or catalysts for various cellular biochemical reactions [43]. However, the high protein content in microalgae biomass may lead to a low carbon/nitrogen (C/N) ratio, limiting the biomass conversion to bioenergy [44]. To address the issue of low C/N ratio, various physiochemical approaches have been developed (Table 2) to lower protein content and enhance the lipid and carbohydrate content of microalgae biomass. Nevertheless, as shown in Table 2, high lipid or carbohydrate accumulation in microalgae does not always coincide with high biomass production, posing challenges for using microalgae as a bioenergy feedstock.

Table 2. Different approaches to enhance lipid and carbohydrate content in the microalgae biomass as well as biomass yield and their outcomes (↑: increase, ↓: decrease, and -: not available).

Approach	Outcomes			References
	Lipid	Carbohydrate	Biomass	
Nutrient stress				
Nitrogen deprivation/starvation	↑	↑	↓	[19,45]
Phosphorus deprivation/starvation	↑/↓	-/↓	↓	[19,45]
Trace metal availability	↑/↓	↑	↑/↓	[19,46]
Carbon availability	↑	-	↑	[19,47]
pH stress				
Acidic	↑/↓	↑/↓	↑/↓	[19,48]
Alkaline	↑/↓	↑/↓	↑/↓	[19,48]
Temperature stress				
High temperature	↑/↓	-	↑/↓	[49]
Low temperature	↑/↓	-	↑/↓	[49]
Light stress				
Low Light	↑/↓	↑/↓	↓	[50]
High Light	↑/↓	↑/↓	↑/↓	[50]
Saline stress	↑	↑	↑/↓	[19,51]

With the advent of metabolic engineering and its suite of genome-editing tools, some researchers are now genetically modifying microalgae cells by overexpressing or knocking out specific genes to alter metabolic pathways and improving the productivity of the compound of interest [52]. For instance, knocking out the phospholipase A_2 gene from *Chlamydomonas reinhardtii* using the CRISPR-Cas9 system resulted in a 64% increase in lipid productivity without compensating for the biomass growth rate [53]. In another study, the individual expression of three genes (glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT)) in *Neochloris oleoabundans* resulted in a 52% increase in lipid content without significantly affecting the microalgae growth [54]. Additionally, the cloning and transforming of specific genes from other species of microorganisms like *S. cerevisiae* can help improve biomolecule biosynthesis pathways in microalgae cells [55]. Due to the potential for manipulating microalgae to produce high yields of energy-rich compounds like lipids and carbohydrates, it is considered one of the most important feedstocks for bioenergy production [56]. Figure 1 presents an overview of the conversion technologies that can transform microalgae biomass into bioenergy.

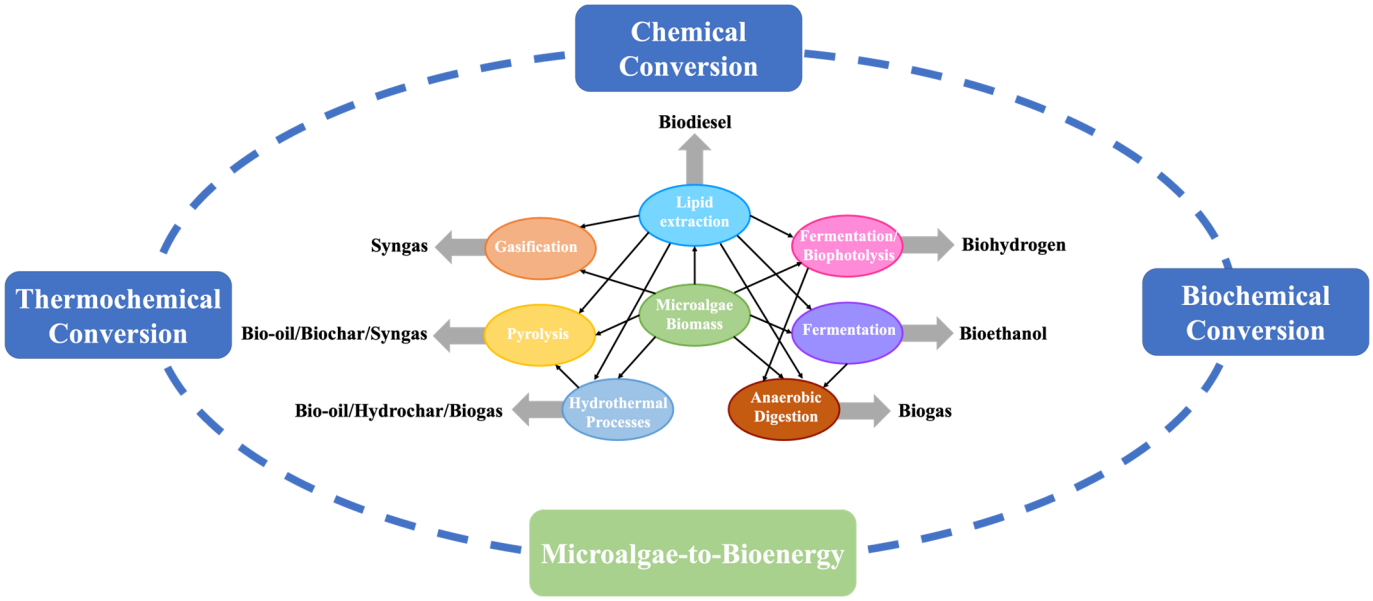

Figure 1. Different biomass conversion pathways to produce bioenergy from microalgae.

3. Biochemical Conversion

The essential part of the biochemical conversion process is to use microorganisms or enzymes to convert biomass into bioenergy [27]. Biochemical conversion technologies can handle biomass with high water content (>50%) [57], making it suitable for processing wet algae biomass. Since drying microalgae biomass is an energy-intensive step [58], biochemical conversion technologies are promising for transforming microalgae into bioenergy. The classical biochemical conversion processes include anaerobic digestion for biomethane production, alcoholic fermentation for bioethanol production, and biological hydrogen production.

3.1. Anaerobic Digestion

Anaerobic digestion (AD) is a complex process in which microorganisms degrade organic biomass under anaerobic conditions and convert it into biogas, majorly comprising methane and other products, such as carbon dioxide, trace amounts of hydrogen, and ammonia [59]. Biogas produced from the AD process can be directly combusted in gas boilers to generate heat or electricity or upgraded into natural gas-quality biomethane and injected into gas grids [60]. AD process consists of four steps, hydrolysis, acidogenesis, acetogenesis, and methanogenesis, where each step is led by a unique functional group of microorganisms [61]. In the first stage of AD (hydrolysis), existing macromolecules in the biomass, such as proteins, lipids, and carbohydrates, are broken down into simpler molecules, such as amino acids, long-chain fatty acids, and simple sugars, respectively. Hydrolysis is achieved by hydrolytic bacteria, mainly belonging to the phyla *Firmicutes* and *Bacteroidetes*, secreting a mixture of hydrolytic enzymes comprising cellulase, xylanase, pectinase, amylase, lipase, and protease [62]. In the second stage of AD (acidogenesis), hydrolyzed products obtained at the end of the hydrolysis stage are converted into volatile fatty acids, such as acetates, propionate, butyrate, valerate, and isobutyrate, using facultative and obligate anaerobic bacteria species, majorly belonging to the phyla *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and *Atribacteria*. Alcohol and other inorganic compounds, such as hydrogen, carbon dioxide, ammonia, and hydrogen sulfide, are also produced during the acidogenesis stage [63]. During the third stage of AD (acetogenesis), the acidogenesis products, such as propionate, butyrate, isobutyrate, valerate, and isovalerate, are further broken down into acetate as well as hydrogen and carbon dioxide using obligate anaerobic bacteria species, majorly belonging to phyla *Firmicutes*, *Synergistota*, and *Myxococcota* [64]. In the final step of AD (methanogenesis), methanogens (a specialized group of archaea belonging to the phyla

Euryarchaeota, *Bathyarchaeota*, and *Verstaraeteachaeota*) convert acetic acid and hydrogen into methane and carbon dioxide [65]. Tuning these four metabolic stages in the AD process can influence final product yields [61]. AD is a well-established commercial technology that is currently being applied to a wide range of organic substrates (sewage waste [66], food waste [67], high-strength organic wastewater [68], and agricultural or forest residue [69]).

Research on anaerobic digestion (AD) of microalgal biomass began in 1957 when microalgae (*Chlorella* and *Scenedesmus*) were cultivated for wastewater treatment and subjected to AD for biomethane production [70]. However, the resulting biomethane yields were significantly lower due to two main factors: the rigidity of microalgae cell walls and the low carbon-to-nitrogen (C/N) ratio in microalgae biomass. Most microalgae cell walls are rigid, which limits anaerobic microorganisms' access to biodegradable microalgal organic matter [28]. The rigidity of microalgae cell walls can be attributed to components such as hemicellulose, cellulose, glycoprotein structures, and certain carbohydrates (e.g., glucose, xylose, rhamnose, and galactose) [71]. Some types of microalgae species also contain algaenan in their outer cell walls. Algaenan is a heteropolymer compound, highly resistant to acidic and basic environments [72]. Several studies have reported intact microalgae cells in the AD effluent after a hydraulic retention time of 30–180 days [73,74], highlighting the recalcitrant nature of microalgal cell walls and their resistance to bacterial degradation during the AD process. Since insufficient biodegradation leads to lower biomethane production, a pretreatment step is necessary to disrupt the microalgae cell walls. Secondly, a C/N ratio of 20–30 (especially 25) is recommended for the optimal functioning of the AD process [75]. If the ratio falls below 20, high amounts of ammonia-nitrogen are released in the anaerobic digester due to the imbalance between microbial carbon and nitrogen requirements. Ammonia buildup can inhibit methanogen growth, leading to volatile fatty acid (VFA) accumulation and process failure [76]. However, the C/N ratio in microalgae biomass is usually between 4–8 [33]. To address the low C/N ratio in microalgae, several researchers have suggested co-digesting microalgae biomass with other biomass streams with a high C/N content [77]. Various biomass pretreatment and co-digestion technologies have been developed in the past decade to improve biomethane production from microalgae biomass. These strategies are discussed in detail in the following sections.

3.1.1. Pretreatment Technologies

Biomass pretreatment technologies focus on enhancing the biodegradability of microalgae biomass by disrupting cell walls. These technologies can be categorized into three groups: physical, chemical, and biological (Table 3). Physical pretreatment technologies consist of mechanical (high-pressure homogenization, bead-milling, microwave, and ultrasonication) and thermal (steam explosion, thermal hydrolysis, and hydrothermal treatment) methods [78]. Mechanical pretreatment uses mechanical forces to disrupt cell walls through size reduction (high-pressure homogenization and milling) or physical damage induction (microwave and ultrasonication) [79]. Among the available mechanical pretreatments, ultrasonication is more commonly applied to microalgae biomass [44]. Ultrasonication pretreatment uses high-frequency sonic waves (>20 kHz) to initiate a cavitation process that propagates shock waves in the medium surrounding cells and causes cell wall disruption by high shear forces [80]. A study dealing with AD of *Scenedesmus* sp. and *Pinnularia* sp. reported 65–71% higher biomethane production when both microalgae species were ultrasonically pretreated before AD [81]. In addition to ultrasonication, microwave pretreatment is an effective cell wall disruption method. Microwaves are electromagnetic waves with shorter wavelengths ranging from one meter to one millimeter corresponding to frequencies of 0.3 to 300 GHz, respectively [82]. Microwaves damage cell walls through athermal and thermal effects [83]. In the athermal part of reactions, microwaves polarize and realign macromolecules along the electromagnetic field, altering the macromolecular structure through hydrogen bond breakage. On the other hand, the thermal effect generates heat inside the cells by absorbing microwave energy through cellular organic complexes or surrounding aqueous media, resulting in cell damage. Passos et al. [84] analyzed the effect

of ultrasound (with an energy input of 26.7 MJ/kg TS (TS: Total solids)) and microwave (with an energy input of 34.3 MJ/kg TS) pretreatment on the mixed microalgae biomass grown in high-rate algal ponds and reported an 8% and 21% increase in biomethane production, respectively, compared to untreated biomass. Apart from ultrasonication and microwave pretreatment, other mechanical pretreatment methods, such as high-pressure homogenization and bead milling, have also been widely studied for the pretreatment of microalgae biomass. Córdova et al. [34] reported a 39% increase in biomethane production when *Chlorella sorokiniana* was homogenized before AD. A 51% higher biomethane was produced when *Acutodesmus obliquus* was pretreated with glass beads [85]. Recently, Straessner et al. [86] used pulsed electric field (PEF) for the first time as a pretreatment technology to improve biomethane yield from *Auxenochlorella protothecoides*. PEF is a well-established technology commonly used for extracting molecules from microalgae biomass. It can permeabilize microalgae cell walls by applying electrical pulses. The authors reported a 10% increase in biomethane production in PEF-treated biomass compared to raw biomass. However, more PEF studies using different microalgae species are needed to prove the practicality of this approach in biomethane improvement. Nevertheless, based on the examples listed in Table 3, it can be seen that higher power input or longer exposure time during the treatment has generally favored mechanical pretreatments, leading to higher biomethane production. The high energy required by mechanical pretreatment makes it unfavorable from an energy perspective [84]. Therefore, achieving a net positive energy balance (energy output > energy input) through improved biomethane yield is crucial to the success of mechanical pretreatment applications.

Thermal pretreatment is used to solubilize cell walls by exposing the biomass to temperatures ranging from 50–240 °C [79]. When thermal pretreatment is conducted under atmospheric pressure at and below 100 °C, it is called thermal hydrolysis, whereas pretreatment at temperatures between 100 °C and 180 °C under gradual pressure (<2 MPa) is called hydrothermal pretreatment. Córdova et al. [34] studied the efficiency of thermal hydrolysis pretreatment on *Chlorella sorokiniana* biomass at different temperatures (60, 70, and 80 °C) and revealed a 6, 9, and 18% higher biomethane production, respectively. Wang et al. [87] compared the efficiency of thermal hydrolysis and hydrothermal pretreatment in improving biomethane yield from *Chlorella* sp. and obtained 114% higher biomethane from hydrothermally treated biomass, while biomass pretreated with thermal hydrolysis yielded 39–47% higher biomethane. Although hydrothermal pretreatment seems promising, a significant amount of energy is required to heat the biomass from ambient temperature to the desired pretreatment temperature (>100 °C), hindering the commercial viability of this approach. In recent studies, solar-driven hydrothermal pretreatment methods have been proposed to reduce energy expenditure [88]. Xiao et al. [89] utilized parabolic trough collectors to concentrate solar radiations for hydrothermal pretreatment of microalgae biomass and obtained 57% higher biomethane production. Furthermore, Xiao et al. [90] evaluated the thermodynamic performance of the solar-driven hydrothermal system using exergy analysis, which considers both the quantity and quality of energy. The solar-driven hydrothermal system achieved the highest exergy efficiency of 41%, whereas the exergy efficiencies of hydrothermal and control (without pretreatment) systems were 36 and 26%, respectively. Despite these promising results, solar-driven hydrothermal systems are still in the nascent development phase and require further engineering innovations to reduce capital costs and allow better integration with the existing bioenergy infrastructure. Apart from thermal hydrolysis and hydrothermal pretreatments, the steam explosion is a widely used thermal pretreatment method, especially in commercial refineries dealing with lignocellulosic biomass. In the steam explosion method, the biomass is first exposed to saturated steam (180–240 °C and 1.03–3.45 MPa) for several minutes, followed by sudden depressurization to ambient conditions [91]. However, studies have shown that steam explosion may not be a good pretreatment option for microalgae biomass. Martín Juárez et al. [92] observed no significant improvement in biomethane production when microalgae biomass was pretreated with a steam explosion (130 °C for 0.08 h and 170 °C for 0.34 h) before AD. Another

study dealing with AD of *Chlorella sorokiniana* reported a reduction in biomethane production by 28–60% when biomass was pretreated with a steam output of 75 kg/h at 4 bar for 0.08–0.25 h [34]. The steam explosion pretreatment of microalgae biomass may have produced or released inhibitory compounds that affected the activity of AD microorganisms, resulting in lower biomethane production. However, the inhibitory mechanism observed during the steam explosion pretreatment of microalgae biomass remains unknown, and further studies need to be conducted to understand the process better.

Chemical pretreatment involves using acidic, alkaline, oxidizing agents, or organic solvents at varying temperatures to solubilize microalgae biomass. Acidic pretreatment uses sulfuric, hydrochloric, and free nitrous acids to hydrolyze cellulosic and hemicellulosic matrices in the cell wall into simple sugars [79]. Marques et al. [93] achieved a 93% higher biomethane production by treating *Scenedesmus obliquus* biomass with 0.1% (*v*/*v*) sulfuric acid at 150 °C for an hour. Bai et al. [94] reported a 55% increase in biomethane production from *Tetraselmis striata* M8 using free nitrous acid pretreatment. In contrast to acidic pretreatments, alkaline pretreatment uses alkalis such as sodium, potassium, or calcium hydroxide to permeabilize cell walls through saponification of uronic acids and esters present in cell walls, inducing swelling and increasing specific surface areas available for microbial degradation and protein solubilization [44]. Fu et al. [95] observed a 77% increase in biomethane production when *Chlorella pyrenoidosa* was pretreated with 1.5% *w*/*v* sodium hydroxide solution at 90 °C for two hours. Biomethane yield was enhanced by 133% when a 2 M sodium hydroxide solution was used to pretreat mixed microalgae consortium at 121 °C for an hour [92]. The higher biomethane yield observed in the latter study could be attributed to the high concentration of sodium hydroxide used. However, alkalis, such as sodium hydroxide, are expensive. Recently, a cheaper alkali alternative (lime) was investigated, and 25% higher biomethane was obtained when the mixed culture of *Chlorella* sp. and *Scenedesmus* sp. was pretreated with 10% *w*/*v* lime at 72 °C for four hours [96]. Despite the promise of acidic and alkaline pretreatments in enhancing biomethane production, these methods may lead to process equipment corrosion and contaminate the biomass by introducing toxic ions and molecules during the pretreatment [44]. Besides acidic and alkaline pretreatments, some studies have used organic solvent pretreatment. For example, Caporgno et al. [97] studied the effect of N-methylmorpholine-N-oxide (NMMO) solvent to pretreat *Nannochloropsis oculata* and achieved a 42% increase in biomethane production. However, the authors used an additional step of evaporation to remove residual solvent from the biomass before anaerobic digestion, which may increase the process cost. Another type of chemical pretreatment is oxidative pretreatment, which uses oxidizing agents to react with aromatic and unsaturated compounds and break down cell walls [98]. A recent study used peroxymonosulfate oxidant to pretreat *Microcystis* sp. before AD, but it only improved the biomethane yield by 4% [99]. In contrast, Cardeña et al. [100] achieved up to a 66% improvement in biomethane yield by applying different doses of ozone pretreatment to a mixed microalgae consortium. In another study, a mixed microalgae culture was subjected to 0.5 *w*/*w*% hydrogen peroxide at 50 °C for an hour before AD, resulting in a 72% increase in the biomethane production [92]. Li et al. [101] studied the effect of zero-valent iron dosage on ultrasonically pretreated *Microcystis* sp. and achieved a 64% higher biomethane. Oxidative pretreatments can be conducted under mild temperature and pressure conditions without using concentrated acids or bases. This makes the oxidative pretreatment method a less costly and more environmentally friendly alternative to traditional chemical pretreatment methods [78]. However, further research is needed to determine the cost-effectiveness of the oxidative pretreatment method based on the chemical and energy input. Recently, a research study conducted by Wang et al. [102] demonstrated that free ammonia (FA), which can be directly obtained from anaerobic digester effluent, improved biomethane production by 17%. Although the reported results of FA pretreatment are low compared to other chemical pretreatment methods, it is a closed-loop technology that can significantly reduce the environmental impact of the pretreatment process. More investigations should be carried out to improve the performance of FA pretreatments.

Biological pretreatment uses hydrolytic enzymes, such as cellulase, hemicellulase, protease, lipase, α-amylase, and amyloglucosidase, to break down microalgae cells. The type of enzyme used in biological pretreatment depends mainly on the microalgae cell wall composition. It is advantageous over other pretreatments due to its low energy requirements, higher selectivity, mild operational conditions, and no production of inhibitory metabolites. Biological pretreatment can be divided into two types: external addition of commercial-specific enzymes and in situ production of crude enzymes by microbial activity. External addition of commercial-specific enzymes involves the addition of isolated and purified enzymes to the biomass. The commercial enzymes used for this purpose are typically selected or genetically engineered to possess high activity and specificity for the targeted compounds, making the process more efficient and cost-effective. In situ production of crude enzymes, on the other hand, involves utilizing microbial activity to produce enzymes on-site. This is typically conducted by inoculating the biomass with a single or mixed community of microorganisms, such as fungi or bacteria, that excrete hydrolytic enzymes naturally or as a result of genetic engineering. Kendir Çakmak et al. [103] studied the effect of commercial enzyme pretreatment on biomethane production from *Phorphyridium cruentum* and reported a 109 and 102% increase with single enzyme (protease) and enzyme mixture (protease and viscozyme) addition, respectively. Another study reported a 48–62% and 143–162% increase in biomethane production when *Chlorella vulgaris* biomass was pretreated with a single and mixture of enzymes, respectively [104]. Nevertheless, the high cost of commercial enzymes discourages their wide-scale application in biomethane enhancement. To reduce the high costs of enzymes, they can be either produced using low-cost substrates, such as waste [105] or immobilized to allow their recovery and reuse in several pretreatment cycles [106]. However, producing enzymes using low-cost substrates and immobilizing them for reuse remains a subject of high interest where intense research is ongoing. Another strategy to make biological pretreatment cost-effective is producing crude enzymes in situ using fungi or bacteria capable of secreting extracellular enzymes. Hom-Diaz et al. [107] pretreated mixed microalgae consortium with fungal broth and observed a 74% increase in biomethane production. Kavitha et al. [108] reported a 217–334% increase in biomethane production when a mixed microalgae consortium was pretreated using different bacterial population sets secreting protease, amylase, and cellulase enzymes. Aydin et al. [109] used 20% cow rumen fluid containing fungi, such as *Anaeromyces* sp., *Orpinomyces* sp., *Piromyces* sp., and *Neocallimastix* sp., to anaerobically digest *Haematococcus pluvialis* and observed a two-time increase in biomethane production. However, selecting appropriate microorganisms, preparing their starter culture, and fixing their inoculum ratio with microalgae biomass are the two main hurdles of this process. Another factor that needs to be considered is the possible loss of microalgae biomass due to its use as a substrate by other microorganisms. Both biological pretreatment methods have their advantages and disadvantages. The external addition of commercial-specific enzymes is more efficient and predictable, but it can also be more expensive due to the cost of the enzymes. The in-situ production of crude enzymes, on the other hand, is cheaper and more sustainable, but it is also less predictable and may require more time to achieve the desired results.

Based on the above discussion and the examples listed in Table 3, it can be concluded that each pretreatment type can effectively enhance biomethane production from microalgae to a certain extent. However, solar-driven thermal, microbial, and oxidative pretreatments seem more promising than traditional pretreatments, thus requiring further exploration. It should also be noted that the most efficient pretreatment method may not be the same for each microalgae species since cell wall structures and compositions vary with microalgae species [78]. A careful selection of the pretreatment method and its process parameters will be required to enhance biomethane production from different microalgae species.

Table 3. Effect of pretreatment technologies on biomethane production from microalgae biomass.

Microalgae Species	Pretreatment Strategy and Operating Conditions	Biomethane Yield (mL CH_4/g VS)			References
		Without Pretreatment	With Pretreatment	% Improvement in Yield	
Physical pretreatment (Mechanical)					
Scenedesmus sp.	Ultrasound pretreatment at 400 W power for 200 s	183 ± 25	313 ± 15	71	[81]
Pinnularia sp.		152 ± 21	250 ± 21	65	
Mixed microalgae and bacteria consortium dominated by green microalgae *(Stigeoclonium* sp. and *Monoraphidium* sp.) and diatoms *(Nitzschia* sp. and *Navicula* sp.	Ultrasound pretreatment at 70 W power for 0.5 h	106 ± 2	114 ± 2	8	[84]
	Microwave pretreatment at 900 W power for 180 s		128 ± 5	21	
Chlorella sorokiniana	Homogenization pretreatment at 200 W power for 0.5 h	318 ± 1	442 ± 29	39	[34]
Acutodesmus obliquus	Bead milling pretreatment using 0.35 mm glass beads at 40 g of glass beads/100 g of wet algae for 0.34 h at 8500 rpm	191	289	51	[85]
Auxenochlorella protothecoides	Pulsed electric field pretreatment at 40 kV/cm electric field and 1 µs pulse duration (3 Hz)	425	467	10	[86]
Physical pretreatment (Thermal)					
Chlorella sorokiniana	Thermal hydrolysis pretreatment at 60 °C for 0.5 h	318 ± 1	337 ± 12	6	[34]
	Thermal hydrolysis pretreatment at 70 °C for 0.5 h		347 ± 39	9	
	Thermal hydrolysis pretreatment at 80 °C for 0.5 h		375 ± 53	18	
Chlorella sp.	Thermal hydrolysis pretreatment at 70 °C for 0.5 h	155	215	39	[87]
	Thermal hydrolysis pretreatment at 90 °C for 0.5 h		228	47	
	Hydrothermal hydrolysis pretreatment at 121 °C for 0.5 h		332	114	
Chlorella pyrenoidosa	Solar-driven hydrothermal pretreatment at 723 W/m^2 irradiation (~160 °C) for 0.5 h	222	348	57	[89]
Chlorella sorokiniana	Steam explosion pretreatment at 4 bars for 0.08 h	318 ± 1	137 ± 5	−57	[34]
	Steam explosion pretreatment at 4 bars for 0.17 h		128 ± 7	−60	
	Steam explosion pretreatment at 4 bars for 0.25 h		230 ± 4	−28	

Table 3. *Cont.*

Microalgae Species	Pretreatment Strategy and Operating Conditions	Biomethane Yield (mL CH_4/g VS)			References
		Without Pretreatment	With Pretreatment	% Improvement in Yield	
Chemical pretreatment					
Scenedesmus obliquus	Acidic pretreatment with 0.1% *v*/*v* sulfuric acid at 150 °C for 1 h	131 ± 26	253 ± 51	93	[93]
Tetraselmis striata M8	Acidic pretreatment with 2.31 mg/L free nitrous acid at 5.5 pH for 48 h	161 ± 7	250 ± 2	55	[94]
Chlorella pyrenoidosa	Alkaline pretreatment with 1.5% (*w*/*v*) NaOH at 90 °C for 2 h	218	386	77	[95]
Mixed microalgae consortium	Alkaline pretreatment with 0.5 M NaOH at 121 °C for 1 h	162	173	7	[92]
	Alkaline pretreatment with 2 M NaOH at 121 °C for 1 h		377	133	
	Oxidative pretreatment with 0.5% *w*/*w* hydrogen peroxide (11.5 pH) at 50 °C for 1 h		279	72	
Mixed microalgae consortium of *Chlorella* sp. and *Scenedesmus* sp.	Alkaline pretreatment with 4% (*w*/*v*) CaO at 55 °C for 24 h	260 ± 8	255 ± 6	−2	[96]
	Alkaline pretreatment with 10% (*w*/*v*) CaO at 55 °C for 24 h		292 ± 11	12	
	Alkaline pretreatment with 4% (*w*/*v*) CaO at 72 °C for 24 h		287 ± 4	11	
	Alkaline pretreatment with 10% (*w*/*v*) CaO at 72 °C for 24 h		325 ± 12	25	
Nannochloropsis oculata	Organosolv treatment with N-methylmorpholine-N-oxide	238 ± 6	339 ± 4	42	[97]
Microcystis sp.	Oxidative pretreatment with 0.1 g peroxymonosulfate/g algae (TSS)	291 (mL CH_4/g COD)	303 (mL CH_4/g COD)	4	[99]

Table 3. *Cont.*

Microalgae Species	Pretreatment Strategy and Operating Conditions	Biomethane Yield (mL CH_4/g VS)			References
		Without Pretreatment	With Pretreatment	% Improvement in Yield	
Mixed microalgae consortium	Oxidative pretreatment with 96 mg of ozone/g algae VS at 23 °C	260	306	18	[100]
	Oxidative pretreatment with 191 mg of ozone/g algae VS at 23 °C		334	28	
	Oxidative pretreatment with 383 mg of ozone/g algae VS at 23 °C		433	66	
Ultrasonically pretreated *Microcystis* sp.	Oxidative pretreatment with 20 g of zero-valent iron/g algae (TS)	37 (mL CH_4/g COD)	61 (mL CH_4/g COD)	64	[101]
Mixed microalgae consortium	Free ammonia pretreatment with 530 mg NH_3-N/L at 22 °C for 24 h (pH 9.5)	188	219	17	[102]
Biological pretreatment					
Porphyridium cruentum	Single enzymatic pretreatment with 0.5 mL/g dry biomass commercial cellulase at 55 °C for 24 h (pH 5–5.5)	130	152	17	[103]
	Single enzymatic pretreatment with 0.5 mL/g dry biomass commercial protease at 55 °C for 24 h (pH 8–8.5)		271	109	
	Cocktail enzymatic pretreatment with 0.5 mL/g dry biomass commercial viscozyme (carbohydrase mix) at 55 °C for 24 h (pH 4–4.5)		242	86	
	Cocktail enzymatic pretreatment with 0.5 mL/g dry biomass enzyme mix (commercial protease and viscozyme) at 55 °C for 9 h (pH 8–8.5 for first 4.5 h and 4–4.5 for next 4.5 h)		263	102	

Table 3. *Cont.*

Microalgae Species	Pretreatment Strategy and Operating Conditions	Biomethane Yield (mL CH_4/g VS)			References
		Without Pretreatment	With Pretreatment	% Improvement in Yield	
Chlorella vulgaris	Single enzymatic pretreatment with 1% *w*/*v* commercial cellulase at 55 °C for 24 h	120 ± 15	183 ± 12	53	[104]
	Single enzymatic pretreatment with 1% *w*/*v* commercial protease at 55 °C for 24 h		194 ± 1	62	
	Single enzymatic pretreatment with 1% *w*/*v* commercial amylase at 55 °C for 24 h		177 ± 14	48	
	Cocktail enzymatic pretreatment with 1% *w*/*v* enzyme mix (commercial cellulase and protease) at 55 °C for 24 h		314 ± 11	162	
	Cocktail enzymatic pretreatment with 1% *w*/*v* enzyme mix (commercial cellulase and amylase) at 55 °C for 24 h		291 ± 5	143	
Mixed microalgae–bacteria consortium	Single enzyme pretreatment with 100 U/L commercial laccase at 25 °C	83 ± 1	100 ± 7	21	[107]
	Fungal pretreatment with 100 U/L laccase-rich broth from Trametes versicolor		144 ± 2	74	
Mixed microalgae consortium	Bacterial pretreatment with bacterial consortium secreting protease, amylase (*Bacillus jerish* 03 and *Bacillus jerish* 04)	0.06 (g $COD_{converted}$/g COD_{added})	0.19 (g $COD_{converted}$/g COD_{added})	217	[108]
	Bacterial pretreatment with cellulase secreting bacteria (*Bacillus* sp.)		0.21 (g $COD_{converted}$/g COD_{added})	250	
	Bacterial pretreatment with mixed bacteria population (*Bacillus jerish* 03, *Bacillus jerish* 04 and *Bacillus* sp.)		0.26 (g $COD_{converted}$/g COD_{added})	334	
Mixed microalgae consortium	Cow rumen fluid mixed with anaerobic granular sludge inoculum at 1:4 *v*/*v* ratio	300	600	100	[109]

Note: VS—volatile solids, TSS—total suspended solids, COD—chemical oxygen demand, TS—total solids, g $COD_{converted}$/g COD_{added}—biodegradable fraction of COD converted to biomethane.

3.1.2. Co-Digestion

Most microalgae species have a low C/N ratio [33], and it is often associated with destabilization and reduced biomethane production in the AD process due to ammonia release and inhibition [110]. As a result, anaerobic digesters that solely process microalgae biomass operate at low organic loading rates (<2 g VS/L.d) to avoid ammonia build-up and subsequent process failure [77]. However, operations at low organic loading rates compromise the economic feasibility of microalgal AD. Co-digestion of microalgae biomass with carbon-rich streams is an opportunity to overcome the risk of ammonia inhibition by improving the C/N ratio through the simultaneous digestion of microalgae biomass with one or more highly biodegradable carbon-rich materials. Among different carbon-rich materials, carbon-rich waste streams are considered ideal co-substrates for co-digestion because they can economically enhance biomethane production while readjusting the nutrient balance (C/N ratio) in the digester. Table 4 summarizes a few examples of microalgae co-digestion with carbon-rich waste streams. Primary sewage and waste-activated sludge are the most researched co-substrates for microalgae digestion. Wágner et al. [111] co-digested a mixed-microalgae consortium of *Chlorella sorokiniana* and *Scenedesmus* sp. with waste-activated sludge and observed a 21–69% increase in biomethane production. In another study [112], biomethane production was increased by 12% when *Scenedesmus quadricauda* was co-digested with thickened waste-activated sludge. Solé-Bundó et al. [113] varied the microalgae to the co-substrate ratio (VS basis) in the AD from 100:0 to 75:25, 50:50, and 25:75, and reported 48, 140, and 223% higher biomethane production, respectively. Microalgae biomass has been successfully digested with other types of organic substrates, including food waste [114], animal manure [115], agro-industrial waste [116,117], glycerol (a by-product of the biodiesel industry) [110], and fat, oil, and grease (FOG) waste [118] to produce 19–500% higher biomethane. Recent studies have suggested pretreating microalgae biomass before co-digestion to achieve better biomethane yields. For instance, Fu et al. [95] treated *Chlorella pyrenoidosa* with a thermo-alkaline method before co-digestion with sewage sludge and observed an 83% increase in biomethane production. Similarly, a 20% higher biomethane was produced when thermally pretreated microalgae biomass was co-digested with sewage sludge [119]. In these studies, biomass pretreatment may have improved microalgae biomass solubilization by rupturing rigid microalgae cell walls.

The co-digestion of microalgae biomass has shown promising results, but a reduction in biomethane production has also been reported when microalgae biomass was co-digested [120]. This highlights the need for a better understanding of co-substrate dosing strategies. For example, Avila et al. [121] observed a reduction of about four times in biomethane production when enzymatically pretreated microalgae biomass was co-digested with waste-activated sludge, mixed at a ratio of 7:93 (VS basis). Similarly, co-digestion of microalgae biomass with piggery wastewater (40:60 organic matter basis) produced 13% lesser biomethane [122]. Currently, the microalgae co-digestion approach primarily focuses on balancing the C/N ratio of the feedstock, but it may not be sufficient to enhance biomethane production. Other variables, such as operating conditions, digester configurations, and underlying microbial dynamics, may affect the biomethane output. Therefore, linking these factors with process monitoring, such as pH, daily biomethane production, and accumulation of intermediate metabolites, can be extremely helpful in understanding and optimizing the co-digestion process [123]. More lab and pilot-scale batch and continuous studies equipped with online process monitoring are needed to gain insights into the co-digestion process.

Table 4. Co-digestion of microalgae biomass with carbon-rich waste streams.

Microalgae Species	Co-Substrate	Microalgae/ Co-Substrate Ratio	Operating Conditions	Biomethane Yield (mL CH_4/g VS)	Improvement in Biomethane Yield with Co-Digestion (%)	References
Mixed microalgae consortium of *Chlorella sorokiniana* and *Scenedesmus* sp.	None	NA	Batch; Mesophilic (37 °C)	331 ± 76	NA	[111]
	Waste-activated sludge from the aerobic phase of the wastewater treatment plant	0.1 g of algae/1 g of sludge (TS)		400 ± 22	21	
	Waste-activated sludge from the anaerobic phase of the wastewater treatment plant	0.1 g of algae/1 g of sludge (TS)		560 ± 24	69	
Scenedesmus quadricauda	None	NA	Batch; Mesophilic (35 °C)	197	NA	[112]
	Thickened waste-activated sludge from the wastewater treatment plant	49 g of algae/51 g of sludge (VS)		222	12	
Mixed microalgae–bacteria consortium	None	NA	Semi-continuous; 30 d HRT; Mesophilic (37 °C)	90 ± 2	NA	[113]
	Thickened primary sludge from the wastewater treatment plant	75 g of algae/25 g of sludge (VS)		133 ± 6	48	
		50 g of algae/50 g of sludge (VS)		216 ± 1	140	
		25 g of algae/75 g of sludge (VS)		291 ± 9	223	
Mixed microalgae–bacteria consortium, dominated by *Dictyosphaerium* sp.	None	NA	Batch; Mesophilic (35 °C)	332	NA	[114]
	Synthetic food waste (20% rice, 15% meat, 20% beans, 25% lettuce, 10% carrot, and 10% tomato)	50 g of algae/50 g of food waste (VS)		409	23	
		25 g of algae/75 g of food waste (VS)		514	55	
Mixed microalgae–bacteria consortium dominated by *Chlorella* sp., *Scenedesmus* sp., and pennate diatoms	None	NA	Batch; Mesophilic (25–32 °C)	274	NA	[115]
	Swine wastewater	50: 50 (*v*/*v*)		326	19	

Table 4. *Cont.*

Microalgae Species	Co-Substrate	Microalgae/ Co-Substrate Ratio	Operating Conditions	Biomethane Yield (mL CH_4/g VS)	Improvement in Biomethane Yield with Co-Digestion (%)	References
Mixed microalgae–bacteria consortium	None	NA	Batch; Mesophilic (34.5–35.5)	174 ± 2	NA	[117]
	Deproteinated cheese whey	17 g algae/83 g of whey (VS)		302 ± 7	74	
	Cellulose	16 g of algae/84 g of cellulose (VS)		272 ± 12	56	
	None	NA	Semi-continuous; 30 d HRT; Mesophilic (33–35 °C)	36–81 mL CH_4/g COD	NA	
	Deproteinated cheese whey	17 g algae/83 g of whey (VS)		210–222 mL CH_4/g COD	167–500	
	Cellulose	16 g of algae/84 g of cellulose (VS)		97–122 mL CH_4/g COD	51–169	
Chlorella vulgaris	None	NA	Batch; Mesophilic (35 °C)	167	NA	[116]
	Potato processing waste (discarded parts and peels)	25 g of algae/75 g of potato waste (VS)		266	59	
Chlorella vulgaris	Potato processing waste (discarded parts)	25 g of algae/75 g of potato waste (VS)	Semi-continuous; 20 d HRT; Mesophilic (37 °C)	300 mL CH_4/g COD	NA	[110]
	Potato processing waste (discarded parts) + glycerol	25 g of algae/75 g of potato waste (VS) + 1% glycerol (*v*/*v*)		730 mL CH_4/g COD	143	
	Potato processing waste (peels)	25 g of algae/75 g of potato waste (VS)		330 mL CH_4/g COD	NA	
	Potato processing waste (peels) + glycerol	25 g of algae/75 g of potato waste (VS) + 1% glycerol (*v*/*v*)		550 mL CH_4/g COD	67	
Mixed microalgae–bacteria consortium	None	NA	Batch; Mesophilic (35 °C)	140 ± 4	NA	[118]
	Thickened primary sludge	50 g of algae/50 g of sludge (VS)		207 ± 5	48	
	Thickened primary sludge and fat, oil, and grease (FOG)	50 g of algae/50 g of sludge + 10% FOG (VS)		259 ± 13	85	
		50 g of algae/50 g of sludge + 20% FOG (VS)		293 ± 8	109	

Note: NA—not applicable, TS—total solids, VS—volatile solids, HRT—hydraulic retention time, COD—chemical oxygen demand.

3.2. Biohydrogen Production

Biohydrogen is considered the fuel of the future due to its higher heating value (142 kJ/g) and ability to produce energy without emitting carbon dioxide [124]. It can be used in fuel cells to generate electricity and as a fuel for automobiles, providing a carbon-neutral solution to current energy crises [125]. To tap into the potential of biohydrogen as a future fuel, microalgae have emerged as a promising source for its production. Microalgae can produce biohydrogen through two different pathways: bio-photolysis and fermentation. During biophotolysis, microalgae cells act as a biocatalyst to directly produce biohydrogen, whereas in fermentation, microalgae biomass serves as a feedstock for biohydrogen-producing microorganisms [126]. The following sections will discuss these two biohydrogen production strategies in detail.

3.2.1. Biophotolysis

Biophotolysis can be classified into direct and indirect types and some of their examples have been listed in Table 5 [127]. During direct bio-photolysis, microalgae generate biohydrogen through light energy-driven water splitting ($2H_2O$ + light energy $\rightarrow$ $2H_2$ + O_2) [128]. In brief, microalgae use photosystems II (PSII) to harvest light energy and split water into protons and electrons. These electrons flow linearly from water through two photosystems (PSII to PSI) to the hydrogen-producing enzyme, hydrogenase, under special conditions, via an electronic carrier (Ferredoxin). The hydrogenase enzyme is activated when the microalgae culture is exposed to anaerobic conditions. Finally, the hydrogenase enzyme catalyzes the reaction between protons and electrons to produce biohydrogen [129]. Gaffron and Rubin [130] first reported direct biophotolysis in 1942 while studying *Scenedesmus obliquus*. This phenomenon was later observed in other green microalgae species, such as *Chlamydomonas reinhardtii*, *Chlorella fusca*, *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Chlorococcum littorale*, *Monoraphidium* sp., *Platymonas subcordiformis*, and *Tetraspora* sp. [131]. The conversion of readily available substrates, solar energy, and water into biohydrogen makes direct biophotolysis promising. However, its practical application has been limited for the last 70 years due to the lack of efficient techniques to overcome the oxygen sensitivity of hydrogenase.

Even in small amounts (<2% *v*/*v*), oxygen generated as a by-product of PSII activity suppresses all hydrogenase-catalyzed reactions, allowing only transient biohydrogen production (lasting for a few minutes) from the direct biophotolysis [132]. To avoid oxygen inhibition of hydrogenase in *Chlamydomonas reinhardtii*, Reeves and Greenbaum [133] continuously purged inert gas in the system, extending the biohydrogen production for up to 160 h. Hamed et al. [134] reported a 2–10-fold increase in biohydrogen production from four microalgae species by purging the system with argon gas. Maswanna et al. [135,136] reported 6.5 times higher biohydrogen production under argon gas purging. However, continuous inert gas purging is an expensive and operationally impractical strategy for large-scale biohydrogen production systems. Pow and Krasna [137] trialed an alternative approach to remove the photosynthetically generated oxygen from the photobioreactor system. The authors used oxygen absorbers, such as Fieser's reagent, 20% potassium hydroxide solution, sodium dithionite, and diuron. Although Fieser's reagent, 20% potassium hydroxide solution, and diuron resulted in little to no significant biohydrogen production, sustained biohydrogen production for up to 6 h was reported when sodium dithionite was directly added to the microalgae culture. Paramesh and Chandrasekhar [138] tested the efficiency of three oxygen-scavenging agents (sodium sulfite, sodium metabisulfite, and sodium dithionate), and found that sodium sulfite was the most efficient agent in extending biohydrogen production. However, the direct addition of exogenous reductants to the algae culture may compromise the cell viability and biohydrogen production yield in the long term [139].

To combat the oxygen inhibition of hydrogenase and sustain biohydrogen production in microalgae during biophotolysis, a two-stage process (indirect biophotolysis) has been developed, separating hydrogen production activities from oxygen evolution [140]. In the

first stage, cells undergo photosynthesis under aerobic conditions to fix carbon dioxide into biomass and release oxygen ($6H_2O + 6CO_2$ + light energy $\rightarrow C_6H_{12}O_6 + 6O_2$). In the second stage, cells catabolize stored organic compounds under anaerobic conditions to produce hydrogen ($C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$ and $2CH_3COOH + 4H_2O$ + light energy $\rightarrow 8H_2 + 4CO_2$). To induce anaerobiosis in the second stage, nutrient (sulfur, nitrogen, and phosphorus) deprivation has been extensively studied, where cells are first cultivated under nutrient-replete conditions (first stage) and then subjected to nutrient-deprived conditions in the second stage [141]. Daniel et al. [142], Maswanna et al. [135], and Pongpadung et al. [143] deprived the *Coccomyxa chodatii*, *Tetraspora* sp., and *Chlorella sorokiniana* cultures of sulfur and reported a 4-, 1.2-, and 69-fold increase in biohydrogen production, respectively. Sulfur is an essential component of amino acids, cysteine, and methionine, which play a vital role in protein synthesis during the PSII repair cycle [125]. When microalgae cells are deprived of sulfur, protein biosynthesis is inhibited, partially deactivating PSII and resulting in lower water-splitting activity. This slows down the oxygen evolution rate from PSII compared to the cell's respirational oxygen consumption rate, and the cultures become anaerobic, enhancing the hydrogenase activity. Hence, the technique of inducing anaerobiosis through sulfur deprivation is an attractive option to sustain biohydrogen production from microalgae [126]. Besides sulfur, phosphorus deprivation also inhibits oxygen-evolving activity from PSII since phosphorus is an essential component of nucleic acids, including DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) [144]. Maswanna et al. [135] reported a 1.1-fold increase in biohydrogen production when *Tetraspora* culture was deprived of sulfur and phosphorus compared to only sulfur deprivation. Moreover, phosphorus deprivation is a promising approach for enhancing biohydrogen production, especially from marine algae, as the sulfur deprivation strategy cannot be applied to seawater-based media due to its high sulfate concentrations (2649 mg/L) [145]. In addition to phosphorus and sulfur deprivation, nitrogen deprivation has been investigated to enhance biohydrogen production from microalgae. Pongpadung et al. [143], Hamed et al. [146], and Li et al. [147] deprived the *Chlorella sorokiniana*, *Parachlorella kessleri*, and *Chlorella pyrenoidosa* cultures of nitrogen and reported a 17, 1.2, and 8943-fold increase in biohydrogen production, respectively. Although sulfur, nitrogen, and phosphorus deprivation were the focus of many studies, magnesium and potassium deprivation have also exhibited promising results. Volgusheva et al. [148] compared the effect of sulfur and magnesium deprivation on *Chlamydomonas reinhardtii* cells and observed that magnesium-deprived cells produced 60% more biohydrogen. *Tetraspora* sp. generated 2.6 times higher biohydrogen under potassium deprivation conditions [149]. In addition to single-nutrient deprivation, some research studies used double- or multiple-nutrient stress to enhance biohydrogen production. Pongpadung et al. [143] exposed nitrogen-limited *Chlorella sorokiniana* culture to phosphorus and sulfur deprivation and reported 6.4-, 8.2-, and 10.4-times higher biohydrogen production when cultures were deprived of only sulfur, only phosphorus, and both sulfur and phosphorus, respectively.

Even though nutrient-deprived conditions seem promising in enhancing biohydrogen production from microalgae during two-stage growth, their overall impact can be limited by the amount of intracellular organic carbon present inside the autotrophically grown microalgae biomass [150]. During indirect biophotolysis, intracellular organic carbon compounds degrade to supply the electrons and protons required for biohydrogen production, linking the biohydrogen yield directly with the amount of intracellular organic compounds present in the biomass [125]. Photosynthetically fixed carbon may not be sufficient to produce desired levels of biohydrogen [150]. One approach to increasing the intracellular organic carbon content and the resulting electron supply is to utilize exogenic organic substrates [151]. Microalgae can be cultivated using hetero- and mixotrophic modes in addition to phototrophic cultivation. During phototrophic cultivation, cells harvest light energy and assimilate carbon dioxide, whereas in the absence of light (heterotrophic mode), cells utilize organic carbon as the sole energy source. In the mixotrophic method, both photosynthetic and heterotrophic metabolisms occur concurrently, and cells simultaneously

assimilate both carbon dioxide and organic carbon sources in the presence of light. It has been reported that the mixotrophic mode of microalgae cultivation can yield higher biomass than the other two modes [152]. Liu et al. [150] demonstrated that the biohydrogen yield from sulfur-deprived *Chlorella pyrenoidosa* culture almost doubled from 65.6 to 121.1 mL/L with the addition of 0.7 g/L glucose, showing a positive correlation between biohydrogen production and glucose consumption by microalgae. Another study reported 2.7 times higher biohydrogen production from *Chlorella vulgaris* when the initial glucose concentration was increased from 5 to 10 g/L [153]. Biohydrogen production in *Chlorella* sp. was increased from 0 to 128 and 150 µmol/mg Chl (Chl: Chlorophyll) when glucose and acetate were supplied, respectively [154]. However, supplementing commercial carbon sources for biohydrogen production may be an economic burden for the overall process. Hence, it is essential to use alternate organic carbon sources that are cheaper and abundant. Sengmee et al. [155] supplemented *Chlorella* sp. culture with 16 g/L of crude glycerol (a byproduct of the biodiesel industry) and achieved 2.6 times higher biohydrogen production. Dudek et al. [156] used 50% diluted aerobically pretreated dairy wastewater to cultivate *Tetraselmis subcordiformis* and produced 1.3 times higher biohydrogen. These studies highlight the possibility of substituting commercial organic compounds with waste resources to enhance microalgae-based biohydrogen production.

Another challenge with the nutrient deprivation approach is that nutrient-deprived cells may suffer from acute oxidative stress, resulting in diminished biohydrogen production over time [157]. However, it is possible to establish an anaerobic environment without nutrient deprivation by adding acetate, using specific illumination protocols, and co-culturing microalgae with bacteria. Recently, Hwang et al. [158] used an acetate-rich fermenter effluent (at an acetate/Cl^- ratio of 150) as a natural PSII oxygen regulator in nutrient-replete *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* cultures to demonstrate a continuous biohydrogen production process for 15 days. In another study, biohydrogen production was sustained for up to three days in *Chlamydomonas reinhardtii* culture without any nutrient deprivation when the culture was illuminated by a train of short pulses (1 s), followed by long dark periods (9 s) instead of continuous illumination [159]. Co-culturing microalgae with bacteria is an alternate approach to induce anaerobiosis through the bacterial partner's respiration [160]. Ben et al. [161] cultured *Chlamydomonas reinhardtii* with different isolates of *Pseudomonas* sp. and found 12 times higher biohydrogen production compared to the pure algal culture. Shetty et al. [162] achieved a three-fold increase in biohydrogen production when 5% enriched microbial inoculum was added to the *Chlorella vulgaris* culture utilizing pretreated brewery effluent as a cultivation media. Although co-culturing microalgae with bacteria can benefit biohydrogen production, some bacteria may compete with microalgae for nutrients, negatively impacting microalgal growth [31]. This phenomenon was observed in the study conducted by Fakhimi and Tavakoli [163] in which *Chlamydomonas reinhardtii* was co-cultivated with *Escherichia coli, Pseudomonas stutzeri*, *Pseudomonas putida*, and an unknown bacterial consortium, resulting in up to 24, 46, 32, and 56%, higher biohydrogen production, respectively. However, in their study, *P. stutzeri* and *P. putida* negatively influenced the microalgae growth at all tested initial cell concentrations (optical density: 0.01–0.5), whereas *E. coli* reduced microalgae growth at higher concentrations (optical density >0.3). This highlights the importance of selecting appropriate bacterial cultures and cultivation conditions to benefit from algal-bacteria symbiosis in biohydrogen production. Biohydrogen production without nutrient deprivation is an emerging area of research in biophotolysis, and it should be further explored for direct and efficient biohydrogen production from microalgae.

Table 5. Effect of different strategies on biohydrogen production through microalgal biophotolysis.

Microalgae Species	Biohydrogen Production Strategy and Experimental Conditions	Biohydrogen Production		Biohydrogen Production Duration	References
		Without Pretreatment	With Pretreatment		
Oxygen removal by inert gas purging					
Synechocystis sp. PCC 6803	Direct photolysis with and without anaerobic conditions	0.12–0.7 mmol/mg Chla.h	1–4 mmol/mg Chla.h	168 h	[134]
Parachlorella kessleri EMCCN 3073		0.04–0.2 mmol/mg Chla.h	0.4–1 mmol/mg Chla.h		
Nostoc spongiaeforme		0.1–0.8 mmol/mg Chla.h	0.2–2.1 mmol/mg Chla.h		
Nostoc sp.		0.07–0.2 mmol/mg Chla.h	0.2–0.9 mmol/mg Chla.h		
Immobilized *Tetraspora* sp. CU2551 in sodium alginate beads	Two-stage growth (sulfur deprivation in the second stage) with and without anaerobic conditions	182 nmol/mg DW.h	1183 nmol/mg DW.h	108 h (Anaerobic); 1034 h (Aerobic)	[135,136]
Oxygen removal by scavenging agents					
Scenedesmus obliquus 393	Direct photolysis with and without sodium dithionite addition	≈0	570 µL	6 h	[137]
Chlorococcum minutum	Direct photolysis with and without sodium sulfite addition	NA	300 µmol	24 h	[138]
	Direct photolysis with and without sodium metabisulfite addition		300 µmol		
	Direct photolysis with and without sodium dithionite addition		135 µmol		
Nutrient deprivation					
Chlamydomonas reinhardtii (CC425)	Two-stage growth with and without sulfur deprivation in the second stage under anaerobic conditions	NA	61 ± 7 mL/L (17 ± 4 µmol/L.h)	204 h	[141]
Chlamydomonas moewusii (SAG24.91)		NA	21 ± 3 mL/L (5 ± 0.4 µmol/L.h)		
Coccomyxa chodatii SAG 216–2	Direct photolysis with and without sulfur deprivation and malate supplementation	<50 mL/L	200 mL/L	120 h	[142]

Table 5. *Cont.*

Microalgae Species	Biohydrogen Production Strategy and Experimental Conditions	Biohydrogen Production		Biohydrogen Production Duration	References
		Without Pretreatment	With Pretreatment		
Immobilized *Tetraspora* sp. CU2551 in sodium alginate beads	Two-stage growth with and without sulfur deprivation in the second stage under aerobic conditions	0.46 mL per 25 mL of medium	0.55 mL per 25 mL of medium	48 h	[135]
	Two-stage growth with and without sulfur and phosphorus deprivation in the second stage under aerobic conditions		0.61 mL per 25 mL of medium		
Chlorella sp. IOAC707S	Two-stage growth with and without phosphorus deprivation in the second stage under aerobic conditions	NA	Up to 40 mL/L	650 h	[144]
Chlorella sorokiniana KU204	Two-stage growth with and without sulfur deprivation in the second stage under anaerobic conditions	0.7 mL/L	48 mL/L	84 h	[143]
	Two-stage growth with and without sulfur and nitrogen deprivation in the second stage under anaerobic conditions		98 mL/L		
	Two-stage growth with and without nitrogen deprivation in the second stage under anaerobic conditions		12 mL/L		
	Two-stage growth with and without phosphorus and nitrogen deprivation in the second stage under anaerobic conditions		77 mL/L		
	Two-stage growth with and without sulfur, nitrogen, and phosphorus deprivation in the second stage under anaerobic conditions		125 mL/L		
Parachlorella kessleri EMCCN 3073	Two-stage growth with and without nitrogen deprivation in the second stage under anaerobic conditions	250 µL/L	300 µL/L	9 d	[146]

Table 5. *Cont.*

Microalgae Species	Biohydrogen Production Strategy and Experimental Conditions	Biohydrogen Production		Biohydrogen Production Duration	References
		Without Pretreatment	With Pretreatment		
Chlorella pyrenoidosa IOAC707S	Two-stage growth with and without nitrogen deprivation in the second stage under anaerobic conditions	0.003 mL/L	26.83 mL/L	92 h	[147]
Chlamydomonas Reinhardtii wild type strain 137^{+}	Two-stage growth with and without sulfur deprivation in the second stage under aerobic conditions	NA	1.3 mmol/10^6 cells	100 h	[148]
	Three-stage growth with and without magnesium deprivation in the third stage under aerobic conditions	NA	2.1 mmol/10^6 cells	230 h	
Tetraspora sp. CU2551	Two-stage growth with and without potassium deprivation in the second stage under anaerobic conditions	3.6 ± 0.1 µmol/mg DW	9.2 ± 0.1 µmol/mg DW	32 h	[149]
Addition of exogenic substrates					
Immobilized *Chlorella vulgaris* in sodium alginate beads	Two-stage growth (under purple light) with and without sulfur deprivation and exogenous organic carbon addition (10 g/L of glucose) in the second stage under anaerobic conditions	NA	60 mL/L (39 mL/L.d)	50 h	[151]
Immobilized *Scenedesmus obliquus* in sodium alginate beads		NA	128 mL/L (205 mL/L.d)	70 h	
Chlorella pyrenoidosa	Two-stage growth with and without sulfur deprivation and exogenous organic carbon addition (0.7 g/L of glucose) in the second stage under anaerobic conditions	65.5 mL/L	121.1 mL/L	120 h	[150]
Chlorella vulgaris	Direct biophotolysis with and without exogenous organic carbon addition (5–10 g/L of glucose) under anaerobic conditions	0	0.75–2 mL/h	174 h	[153]

Table 5. *Cont.*

Microalgae Species	Biohydrogen Production Strategy and Experimental Conditions	Biohydrogen Production		Biohydrogen Production Duration	References
		Without Pretreatment	With Pretreatment		
Chlorella sp. KLSc59	Two-stage growth with and without exogenous organic carbon addition (glucose) in the second stage under anaerobic conditions	0	128 µmol/mg Chl	42 h	[154]
	Two-stage growth with and without exogenous organic carbon addition (acetate) in the second stage under anaerobic conditions		150 µmol/mg Chl		
Chlorella sp.	Two-stage growth with and without exogenous organic carbon addition (16 g/L of crude glycerol) in the second stage under anaerobic conditions	4 mL/L	10.3 mL/L	24 h	[155]
Tetraselmis subcordiformis	Two-stage growth with and without exogenous organic carbon addition (50% diluted aerobically pretreated dairy wastewater) in the second stage under anaerobic conditions	54 ± 2 mL/g DW	69 ± 4 mL/g DW	120 h	[156]
Chlamydomonas reinhardtii UTEX 2243	Supplementing acetate-rich fermenter effluent to achieve acetate/Cl^- ratio of 150 without nutrient deprivation under aerobic conditions	NA	95 µmol/L	15 d	[158]
Chlorella sorokiniana UTEX 2714			80 µmol/L		
Light intensity manipulation					
Chlamydomonas reinhardtii	Pulse illumination (strong light pulse for 1 s, followed by dark period for 9 s) vs. continuous illumination under anaerobic conditions	0	3 mmoL/L	48 h	[159]
Chlorella sp. KLSc59	Illumination with and without light intensity of 7 µmol photons/m^2.s	5 µmol/mg Chl	63 µmol/mg Chl	60 h	[154]
	Illumination with and without light intensity of 14 µmol photons/m^2.s		130 µmol/mg Chl		
	Illumination with and without light intensity of 28 µmol photons/m^2.s		206 µmol/mg Chl		

Table 5. *Cont.*

Microalgae Species	Biohydrogen Production Strategy and Experimental Conditions	Biohydrogen Production		Biohydrogen Production Duration	References
		Without Pretreatment	With Pretreatment		
Algae–bacteria co-culture					
Chlamydomonas reinhardtii FACHB-265	Co-cultured with and without *Pseudomonas* sp. strain-D in sulfur-deprived media under aerobic conditions	10 mL/L	120 mL/L	15 d	[161]
Chlorella vulgaris MACC360	Co-cultured with and without 5% enriched microbial consortium in pretreated brewery effluent media under aerobic conditions	52 mL/L·d	154 mL/L·d	3 d	[162]
Chlamydomonas reinhardtii strain 704	Co-cultured with and without *Escherichia coli* K-12 MG1655 with acetic acid under aerobic and low light intensity conditions	NA	24% higher	5 d	[163]
	Co-cultured with and without *Pseudomonas stutzeri* A1501 with acetic acid under aerobic and low light intensity conditions		46% higher		
	Co-cultured with and without *Pseudomonas putida* 12,264 with acetic acid under aerobic and low light 56 intensity conditions		32% higher		
	Co-cultured with and without unknown bacterial consortium with acetic acid under aerobic and low light intensity conditions		56% higher		
Immobilization					
Tetraspora sp. CU2551	Two-stage growth with and without immobilized cells in the second stage under aerobic conditions	0.0025 µmol/mg DW.h	0.1 µmol/mg DW.h	NA	[135]

Note: NA—not applicable, DW—dry weight, Chla—chlorophyll a, Chl—chlorophyll. Strategies used in each treatment group are highlighted.

Currently, biohydrogen yields from biophotolysis are low, ranging from 0.015 to 1.084 mmol/L.h (Table 5), which is not competitive with that of the fermentation approach (0.35 to 10.26 mmol/L.h) [22]. As a result, biophotolysis remains far from commercialization. However, understanding the underlying molecular mechanisms of biohydrogen production and genetically reengineering those metabolic pathways to achieve optimal biohydrogen productivity can accelerate its commercial implementation. The primary focus of genetic engineering is the development of oxygen-resistant hydrogenase, but this remains a significant challenge for the scientific community. Recent genetic engineering studies (Table 6) have shown that current approaches focus on redirecting electron pathways and promoting hydrogenase oxygen sensitivity. In addition to lower biohydrogen yields, capital and operational costs are additional issues for scaling up the biophotolysis process because separate photobioreactors are needed for biomass generation and biohydrogen production. This includes the energy-intensive process of centrifugation, which is conducted to exchange cultivation media and switch between oxygenic photosynthesis and biohydrogen production processes. Operational costs can be reduced by simplifying the media exchange process through microalgae immobilization. Some published studies have also reported that the immobilization of microalgae cells enhances their biohydrogen yield due to the self-shading-induced reduction in photosynthetic activity and subsequent oxygen evolution [135,151]. However, immobilization technology is still under development and warrants further research.

Table 6. Genetic engineering approaches to enhance biohydrogen production from microalgal biophotolysis.

Microalgae Species	Genetic Engineering Approach	Outcome	References
Chlamydomonas reinhardtii hpm91 mutant	Mutant lacking PGR5 (Proton Gradient Regulation 5)	Produced 7287 mL/10 $L_{reactor}$ biohydrogen in 26 days under sulfur deprivation	[164]
Chlamydomonas reinhardtii C3 mutant	Altered the ratio between PSI and PSII from 0.85 to 0.33	Produced biohydrogen with a rate of 3 mL/L.d for 42 days	[165]
Chlorella sp. DT mutant	Modified amino acid residues A105I, V265W, G113I, or V273I around the hydrogenase gas tunnel to prevent oxygen from accessing the enzyme active site via site-directed mutagenesis	Produced 7 times more biohydrogen than the wild type in the presence of 5% oxygen	[166]
Chlamydomonas reinhardtii FACHB-265	Randomly mutated by atmospheric and room temperature plasma (ARTP)	Produced 2.7–3.1 times higher biohydrogen than the wild type	[167]
Chlamydomonas reinhardtii	Used artificial miRNA (amiRNA) to regulate the function of D1-encoded gene, *psbA*	Produced 60% more biohydrogen content than the wild type	[168]

3.2.2. Fermentation

Fermentation can be categorized into photo and dark fermentation (Table 7). The photo fermentation (PF) process employs photosynthetic bacteria, mainly purple non-sulfur (PNS) bacteria, to convert organic compounds (acetate, butyrate, lactate) into biohydrogen and carbon dioxide using a light energy [140]. The ratio of biohydrogen to carbon dioxide produced during the PF process can vary depending on the type of substrate used [169]. PNS bacteria use a nitrogenase enzyme for simultaneous biohydrogen production and nitrogen fixation, making nitrogen-limited conditions a requirement for the

evolution of biohydrogen. During this process, organic acids are first degraded using light energy to generate electrons and adenosine triphosphate (ATP) that drive nitrogenase to evolve biohydrogen and fix molecular nitrogen into ammonium ions [170]. Examples of the commonly used purple non-sulfur bacteria species for biohydrogen production are *Rhodopseudomonas palustris*, *Rhodobacter capsulats*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, and *Rubrivivax gelatinosus* [169]. However, PNS bacteria involved in the PF process can only consume small organic molecules, usually organic acids, requiring microalgae biomass to undergo hydrolysis before PF. Therefore, the PF process is most likely preceded by dark fermentation [171,172].

The dark fermentation (DF) process is similar to the AD process described in Section 3.1, in which organic substrates undergo hydrolysis, acidogenesis, and acetogenesis stages. However, the methanogenic stage is suppressed during DF. In the AD process, biohydrogen is typically produced as an intermediate metabolite during the acidogenesis and acetogenesis stages, and it is then converted into biomethane by hydrogen-utilizing methanotrophs. Unlike AD, during DF, special care is taken to stop the fermentation process at the acidogenesis–acetogenesis stage by inactivating methanotrophs from the initial inoculum [173]. Similar to AD, the major hurdle in DF is the poor biodegradability of microalgal biomass due to its recalcitrant cell wall [124]. Therefore, selecting a suitable pretreatment process before fermentation is vital to efficiently break down the cell wall and convert the complex carbohydrates into simpler monosaccharides. Pretreatment methods that could be applied for microalgal cell wall disruption and carbohydrate hydrolysis are discussed in Section 3.1.1, and their effect on biohydrogen production has been summarized in Table 7. DF exhibits a low biohydrogen production efficiency, but it can be drastically increased by pairing it with PF. In addition to biohydrogen, DF produces hydrogenic effluent containing a wide range of organic acids that PF can use for additional biohydrogen production, as reported in published studies [171,172]. However, the requirement to build customized anaerobic photobioreactors with large surface areas exposed to sunlight is a bottleneck in the combined DF–PF process, as it increases the cost and complexity of the system [174].

Table 7. Photo fermentation and dark fermentation of microalgae biomass for biohydrogen production.

Microalgae Species	Pretreatment	Fermentation Microorganisms	Operating Conditions	Biohydrogen Yield (mL H_2/g VS)	References
Photofermentation					
Chlorella sp.	Acid–hydrothermal treatment followed by dark fermentation	*Rhodobacter sphaeroides* TISTR 1952	Initial pH: 7 Light: 5000 lux Inoculum size: 20% *v*/*v* Temperature: 37 °C	125	[171]
Arthrospira platensis	Acid–hydrothermal treatment followed by dark fermentation and NaCl-modified zeolite treatment	*Rhodopseudomonas palustris*	Initial pH: 7 Light: 6000 lux Inoculum size: NA Temperature: 30 °C	333	[172]
Dark fermentation					
Chlorella sp.	1.5% *v*/*v* HCl, 180 °C, 0.25 h	Heat-treated (105 °C for 3 h) anaerobic granules (dominated by *Clostridium* sp.) collected from brewery wastewater treatment plant	Initial pH: 6 F/I: 1 (VS/VS) Temperature: 37 °C	47	[171]
Arthrospira platensis	1% *v*/*v* H_2SO_4, 135 °C, 0.25 h	Heat treated (100 °C, 0.5 h) anaerobic digestion sludge dominated by *Clostridium* sp.	Initial pH: 6 F/I: NA Temperature: 35 °C	96	[172]
Chlorella sp.	4% *v*/*v* H_2SO_4, 2.5 h 0.75% *v*/*v* H_2SO_4, 160 °C, 0.5 h	Heat-treated (105 °C for 3 h) anaerobic granules (dominated by *Clostridium* sp.) collected from brewery wastewater treatment plant	Initial pH: 6 F/I: 3 (VS/VS) Temperature: 35 °C	26 54	[173]
Chlorella sp.	No pretreatment	Heat treated (100 °C, 0.25 h) anaerobic sludge obtained from sewage treatment plant	Initial pH: 6 F/I: NA Temperature: 37 °C	8	[175]
Chlorella sp.	No pretreatment	Mixed anaerobic bacterial consortia	Initial pH: 7 F/I: NA Temperature: 35 °C	22	[176]

Table 7. *Cont.*

Microalgae Species	Pretreatment	Fermentation Microorganisms	Operating Conditions	Biohydrogen Yield (mL H_2/g VS)	References
Deoiled *Scenedesmus obliquus* UTEX 393	No pretreatment	Acidogenic mixed consortia (dominated by *Clostridium* sp.) developed from heat-treated (100 °C for 0.34 h) cow dung	Initial pH: 6.7 Inoculum size: 10% *v*/*v* Temperature: 37 °C	10	[177]
	Grinding			15	
	Homogenization			20	
	Autoclave			30	
	Sonication			36	
	1 N NaOH, 121 °C, 0.5 h			40	
	1 N KOH, 121 °C, 0.5 h			38	
	0.5 N H_2SO_4, 121 °C, 0.5 h			89	
	10% *w*/*v* magnetic solid acid, 121 °C, 0.5 h			53	
Scenedesmus obtusiusculus AT-UAM	No pretreatment	Granular sludge obtained from a full-scale up-flow anaerobic sludge blanket reactor fed with tequila vinasses	Initial pH: 7.5 F/I: 12 (VS/VS) Temperature: 37 °C	29	[178]
	3% HCl, 100 °C, 1.7 h			48	
Algal bloom dominated by *Microcystis* sp.	No pretreatment	Heat-treated (100 °C, 0.5 h) anaerobic digestion sludge dominated by *Clostridium* sp.	Initial pH: 6 F/I: 0.5 (VS/VS) Temperature: 35 °C	0.3	[179]
	2% *v*/*v* H_2SO_4, 135 °C, 0.25 h (steam treatment)			19	
	2% *v*/*v* H_2SO_4, 135 °C, 0.25 h (hydrothermal treatment)			25	
Wastewater-born microalgal biomass	No pretreatment	Heat-treated (90 °C, 0.5 h) anaerobic digestion sludge from sewage treatment plant	Initial pH: 9.5 F/I: 1 (TS) Temperature: 35 °C	18	[180]
	240–530 mg NH_3-N/L, 1 day, pH 9.5 (free ammonia pretreatment)			20–22	

Note: NA—not available, F/I—food/inoculum.

3.3. Alcoholic Fermentation

Bioethanol is the most extensively used renewable fuel for transportation and can be produced from microalgae using three pathways: dark fermentation (DF), photo fermentation (PF), and traditional fermentation (TF) [181]. Although DF and PF are similar process terminologies used to depict alcoholic fermentation and biohydrogen production pathways, the mechanisms involved in these pathways are different. Some examples of DF, PF, and TF for bioethanol production are listed in Tables 8 and 9. In the DF process, microalgae species, such as *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlorococcum littorale*, *Chlorogonium elongatum*, and *Chlorella fusca*, ferment intracellular polysaccharides into bioethanol under dark and anaerobic conditions [182–184]. Pyruvate, an intermediate compound, is generated through hydrolysis and glycolysis of intracellular polysaccharides (starch). Subsequently, pyruvate is converted into various end products, including acetate, ethanol, formate, glycerol, lactate, biohydrogen, and carbon dioxide, depending on the type of microalgae species and surrounding environmental conditions [185]. Studies on microalgae DF for bioethanol production reported low bioethanol yields of less than 2% *w*/*w*, as shown in Table 8. This could be attributed to the complex network of metabolic pathways involved in microalgal DF and difficulties associated with understanding and selectively manipulating those metabolic pathways to enhance bioethanol production. Therefore, the practical application of the DF pathway for bioethanol production has not received much attention.

Compared to the DF pathway, the PF pathway results in a more specific and efficient bioethanol production [186]. The PF pathway comprises two steps: photosynthesis and fermentation. During the first step of photosynthesis, inorganic carbon (carbon dioxide) is fixed into organic carbon (phosphoglycerate) through the Calvin cycle and later converted to pyruvate. In the second step, pyruvate is fermented into ethanol with the help of two key enzymes, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhII*). *pdc* catalyzes the conversion of pyruvate into acetaldehyde and carbon dioxide through a nonoxidative decarboxylation reaction, whereas *adhII* oxidizes the resulting acetaldehyde into ethanol [187]. However, these enzymes are naturally missing or expressed in insufficient quantities in microalgae. Therefore, genetic engineering is used to heterologously express *pdc* and *adhII* genes in microalgae, preferably cyanobacteria, to enable direct bioethanol production [188]. Cyanobacteria have relatively well-characterized genetic backgrounds, demonstrate a high tolerance to foreign gene introduction, and exhibit amenability to genetic modifications [189]. Foreign DNA (Deoxyribonucleic acid) can be introduced into cyanobacteria under controlled conditions through shuttle vectors or by directly integrating it into the chromosome via targeted homologous recombination. A research study by Deng and Coleman [190] was the first to report the cyanobacteria *Synechococcus elongatus* PCC7942 strain as the platform of bioethanol production. The authors constructed a new *S. elongatus* strain using a shuttle vector pCB4, cloned from the coding sequences of *pdc* and *adhII* genes obtained from the bacterium *Zymomonas mobilis*. Following four weeks of culture, the transformed *S. elongatus* strain produced a bioethanol titer of 0.23 g/L. Later, Dexter and Fu [191] used the same two genes from *Z. mobilis* and integrated them into the chromosome of *Synechocystis* sp. PCC 6803 using a double homologous recombination system and produced a bioethanol titer of 0.46 g/L in six days of cultivation. Since then, several genetic engineering efforts have been made to improve the bioethanol yield from cyanobacteria while maintaining cell growth (Table 8). However, PF pathways produce much lower bioethanol yields (<6 g/L), making the ethanol separation process (distillation) too costly for large-scale applications. Poor bioethanol yields could be attributed to co-factor imbalance [192], low ethanol tolerance levels [193], competition for carbon usage between biomass synthesis and target product formation [194], and inefficient carbon fixation mechanisms [195,196]. However, there is still room for optimizing the bioethanol yield from cyanobacteria through alternate gene expression approaches.

Table 8. Dark fermentation and photo fermentation for microalgae-based bioethanol production.

Dark Fermentation					
Microalgae Species	**Operating Conditions**	**Starch Content (% of Dry Cell Weight)**	**% Starch Decomposed**	**Bioethanol Yield (% of Dry Cell Weight)**	**References**
Chlamydomonas reinhardtii UTEX 2247	Incubation under dark and anaerobic conditions at 25 °C for 46 h Slurry concentration: 15% *w*/*w*	45	NA	1	[182]
Chlamydomonas sp. YA-SH-1	Incubation under dark and anaerobic conditions at 30–35 °C for 44 h Slurry concentration: 15–25% *w*/*w*	30	NA	1.3	[183]
Chlorococcum littorale	Incubation under dark and anaerobic conditions at 30 °C for 24 h Slurry concentration: 1.4% *w*/*w*	15	46	1.6	[184]
Photo Fermentation					
Cyanobacteria	**Genes expressed (source of genes) and their expression mechanism**	**Promotor used**	**Gene deletion (Effect)**	**Bioethanol titer (g/L) and days of cultivation (d)**	**Reference**
Synechococcus elongatus PCC7942	*pdc* (*Zymomonas mobilis*), Shuttle vector; *adhII* (*Zymomonas mobilis*), Shuttle vector	*rbcLS*	NA	0.23 in 28 d	[190]
Synechocystis sp. PCC 6803	*pdc* (*Zymomonas mobilis*), Homologous recombination; *adhII* (*Zymomonas mobilis*), Homologous recombination	*psbA2*	NA	0.46 in 6 d	[191]
Synechocystis sp. PCC6803	*pdc* (*Zymomonas mobilis*), Homologous recombination; *adh*, *slr1192* (Endogenous overexpression), Homologous recombination	*Prbc*	*phaA* and *phaB* (Disrupting PHB biosynthesis pathway)	5.5 in 26 d	[197]
Synechocystis sp. PCC6803	*pdc* (*Zymomonas mobilis*), Homologous recombination; *adhII* (*Zymomonas mobilis*), Homologous recombination	*nblA*	*glgC* (Disrupting glycogen biosynthesis pathway) and *phaC* + *phaE* (Disrupting PHB biosynthesis pathway)	3 in 3 d	[194]

Table 8. *Cont.*

Synechocystis sp. PCC6803	*zwf* (Endogenous overexpression to enhance NADPH production) Homologous recombination; *pdc* (*Zymomonas mobilis*), Homologous recombination; yqhD, NADPH-dependent adh (*Escherichia coli*), Homologous recombination	*Pcpc560*	NA	0.59 in 14 d	[192]
Synechocystis sp. PCC6803	*pdc* (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector	*PnrsB*	NA	0.45 in 7 d	[196]
	pdc (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector; *rbcSC*, *slr0009-slr0011-slr0012-FLAG* with RuBisCO-encoding genes (Endogenous), Shuttle vector	*PnrsB* (for *pdc* and *adh*) and *psbA2* (for *rbcSC*, *70glpX*, *tktA*, and *fbaA*)		0.7 in 7 d	
	pdc (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector; *70glpX* with FBP/SBPase-encoding genes (Synechococcus PCC 7002), Shuttle vector			0.75 in 7 d	
	pdc (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector; *tktA*, *sll1070* with TK-encoding genes (Endogenous), Shuttle vector			0.6 in 7 d	
	pdc (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector; *fbaA*, *sll0018* with FBA-encoding genes (Endogenous), Shuttle vector			0.75 in 7 d	

Table 8. *Cont.*

Synechocystis sp. PCC6803	*pdc* (*Zymomonas mobilis*), Shuttle vector; *adh*, *slr1192* (Endogenous), Shuttle vector; *fbaA*, *sll0018* with FBA-encoding genes (Endogenous), Shuttle vector; *tktA*, *sll1070* with TK-encoding genes (Endogenous), Shuttle vector	*PnrsB* (for *pdc* and *adh*) and *psbA2* (for *tktA* and *fbaA*)	NA	1.2 in 20 d	[195]
Synechocystis sp. PCC6803 (Fe_2O_3-treated culture)	*pdc* (*Saccharomyces cerevisiae*), Shuttle vector; *adh* (Endogenous), Shuttle vector	*psbA1*	NA	4.9 in 25 d	[198]
Synechocystis sp. PCC6803 (MgO-treated culture)				5.1 in 25 d	

Note: NA—not applicable, NADPH—nicotinamide adenine dinucleotide phosphate, *pdc*—pyruvate decarboxylase, *adh*—alcohol dehydrogenase, *phaA*—polyhydroxyalkanoate-specific β-ketothiolase, *phaB*—polyhydroxyalkanoate-specific acetoacetyl-CoA reductase, PHB—polyhydroxybutyrate, *glgC*—glucose-1-phosphate adenylyltransferase, *phaC*—polyhydroxyalkanoate synthase, *phaE*—polyhydroxyalkanoate polymerase subunit, *zwf*—glucose 6-phosphate dehydrogenase, RuBisCO (*rbcSC*)—ribulose-1,5-bisphosphate carboxylase/oxygenase, FBA (*fbaA*)—Fructose-1,6-bisphosphate aldolase, FBP/SBPase (*70glpX*)—fructose-1,6-/sedoheptulose-1,7-bisphosphatase, TK (*tktA*)—transketolase.

Traditional fermentation has been the most widely studied method of bioethanol production, as it typically yields higher bioethanol quantities (21–88% *w*/*w* (% of dry cell weight) and 5–43 g/L (based on the working volume)) compared to DF (<2% *w*/*w*) and PF (<6 g/L) (Tables 8 and 9). In the traditional fermentation process, the carbohydrate content of microalgae biomass is used as a feedstock by ethanologenic microorganisms, such as yeast (*Saccharomyces cerevisiae*) and bacteria (*Zymomonas mobilis*). However, *S. cerevisiae* is more commonly used for bioethanol fermentation due to its tolerance towards low pH and high ethanol concentrations [199]. As described earlier in Section 3.1, microalgae biomass pretreatment is crucial before fermentation for easy access to intracellular microalgal compounds. Various biomass pretreatment methods are discussed in detail in Section 3.1.1, and the effects on bioethanol production have been summarized in Table 9. Shokrkar et al. [200] compared the effect of acidic and enzymatic pretreatments on the bioethanol production performance of a mixed microalgae culture and reported a 1.3 times higher bioethanol production when microalgae culture was pretreated with enzymes. De Farias Silva et al. [201] observed no significant variations between the acidic and enzymatic pretreatments for *Chlorella vulgaris* and *Scenedesmus obliquus* biomass. Another study demonstrated an alternate approach for bioethanol production without any acidic or enzymatic pretreatments [202]. The authors combined the extraction and fermentation process in which a lysozyme and calcium chloride mixture was used to extract glycogen from *Arthrospira platensis*. Extracted glycogen was simultaneously degraded to glucose with the help of a recombinant *Saccharomyces cerevisiae* culture, which produced alpha-amylase and glucoamylase. However, such an approach can only work for cyanobacteria, which lack robust cell wall structure. The examples listed in Table 9 confirm that the effectiveness of microalgae pretreatment varies depending on the species.

The traditional fermentation process can be divided into two groups: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) [203]. During SHF, hydrolysis and fermentation processes are conducted separately in different reactors, whereas, during SSF, hydrolysis and fermentation processes proceed simultaneously in the same reactor. The main advantage of the SHF process is the possibility to separately optimize the operating conditions of the hydrolysis and fermentation processes. El-Mekkawi et al. [21] used the response surface method (RSM) during SHF to optimize the process variables, such as algal biomass, yeast loading, and fermentation time, achieving a higher bioethanol concentration of 19 g/L. Other advantages of the SHF process are the potential use of cheaper chemicals, shorter residence time, and easy operation. However, its high capital cost is moving the research direction toward SSF. Kim et al. [204] compared the effect of SHF and SSF on bioethanol production from *Phorphydium cruentum* and observed that SSF produced slightly better bioethanol yields (74–80%) over SHF (70–78%). Similarly, Megawati et al. [205] observed a slightly better bioethanol production result with SSF (48.5%) compared to SHF (46%). Although most of the bioethanol production studies have used a single strain of yeast to study the fermentation process, some studies have applied a co-fermentation approach in which two or more different strains of yeast are used with a capacity to simultaneously degrade pentose and hexose sugar [201]. However, using a combination of different yeast strains would still require careful and thorough investigation.

Table 9. Pretreatment of microalgae biomass for bioethanol production through traditional fermentation.

Microalgae Species	Pretreatment	Fermentation Microorganisms	Fermentation Operating Conditions	Bioethanol Concentration (g/L) and Yield (% of Dry Cell Weight)	References
Separate hydrolysis and fermentation					
Carotenoid-free *Chromochloris zofingiensis* SAG 211-14	Autoclave (120 °C for 0.34 h) followed by two-stage enzymatic pretreatment with α-Amylase (90 °C, 2 h, 4.5 pH) and glucoamylase (60 °C, 22 h, 6.5 pH)	*Saccharomyces cerevisiae* CCUG 53310	Initial pH: 4.8 Inoculum size: NA Temperature: 37 °C Time:	25 ± 2%	[206]
	Autoclave (120 °C for 0.34 h) followed by three-stage enzymatic pretreatment with Cellic Ctec2 and Cellic Htec2 (45 °C, 48 h, 5 pH), α-Amylase (90 °C, 2 h, 5 pH) and glucoamylase (60 °C, 22 h, 5 pH)			62 ± 2%	
Carotenoid-free *Haematococcus pluvialis* SAG 192.80	Autoclave (120 °C for 0.34 h) followed by two-stage enzymatic pretreatment with α-Amylase (90 °C, 2 h, 4.5 pH) and glucoamylase (60 °C, 22 h, 6.5 pH)	*Saccharomyces cerevisiae* CCUG 53310	Initial pH: 4.8 Inoculum size: NA Temperature: 37 °C Time:	35 ± 0.3%	[207]
	Autoclave (120 °C for 0.34 h) followed by three-stage enzymatic pretreatment with Cellic Ctec2 and Cellic Htec2 (45 °C, 49 h, 5 pH), α-Amylase (90 °C, 2 h, 5 pH) and glucoamylase (60 °C, 22 h, 5 pH)			88.1 ± 0.5%	
Chlorella vulgaris	1 N HCl, 90 °C, 1 h	*Saccharomyces cerevisiae*	Initial pH: 5 Inoculum size: 3% *v*/*v* Temperature: 30 °C Time:	46%	[205]
Chlorella vulgaris FSP-E	2% H_2SO_4, 121 °C, 0.34 h	*Saccharomyces cerevisiae* FAY-1	Initial pH: NA Inoculum size: NA Temperature: 30 °C	21% (43 g/L)	[208]
Mixed microalgae consortium	0.5 M H_2SO_4 and 2.5% (*w*/*v*) $MgSO_4$ at 121 °C, 0.67 h	*Saccharomyces cerevisiae* ATCC 7921	Initial pH: 6.5 Inoculum size: 3% *v*/*v* Temperature: 30 °C	5 g/L	[200]
	Three-stage enzymatic pretreatment with β-glucosidase/cellulase (65 °C, 3 h), α-amylase (95 °C, 3 h) and amyloglucosidase (55 °C, 3 h)			6.4 g/L	

Table 9. *Cont.*

Microalgae Species	Pretreatment	Fermentation Microorganisms	Fermentation Operating Conditions	Bioethanol Concentration (g/L) and Yield (% of Dry Cell Weight)	References
Arthrospira platensis NIES-39	1 g/L lysozyme and 100 mM $CaCl_2$	*Saccharomyces cerevisiae* strain BY4741 AASS/GASS	Initial pH: 5.2–5.4 Inoculum size: 5% *v*/*v* Temperature: 38–40 °C	32%	[202]
Wastewater-grown microalgae biomass dominated by *Microcystis*	0.5 N H_2SO_4, 120 °C, 4 h	Immobilized *Saccharomyces cereviciae* ATCC 4126	Initial pH: 4.5 Inoculum size: 15% *v*/*v* Temperature: 30 °C	19 g/L	[21]
Porphyridium cruentum KMMCC-1061	One-stage enzymatic hydrolysis with pectinase and cellulase (37 °C, 7 h, 4.8 pH)	*Saccharomyces cerevisiae* KCTC 7906	Initial pH: 4.5 Inoculum size: 0.1% *w*/*v* Temperature: 37 °C	70–78% (based on initial glucose content)	[204]
Simultaneous Saccharification and Fermentation					
Chlorella vulgaris	Two-stage enzymatic pretreatment with α-Amylase (90 °C, 6 pH) and glucoamylase (80 °C, 5 h, 6 pH)	*Saccharomyces cerevisiae*	Initial pH: 5 Inoculum size: 3% *v*/*v* Temperature: 30 °C	49%	[205]
Porphyridium cruentum KMMCC-1061	One-stage enzymatic hydrolysis with pectinase and cellulase (37 °C, 10 h, 4.8 pH)	*Saccharomyces cerevisiae* KCTC 7906	Initial pH: 4.5 Inoculum size: 0.1% *w*/*v* Temperature: 37 °C	74–80% (based on initial glucose content)	[204]
Co-fermentation					
Dried and milled *Chlorella vulgaris* biomass powder Neoalgae® (Micro seaweed products B-52501749).	3% H_2SO_4, 120 °C, 0.5 h	75% *Saccharomyces cerevisiae* Cameo S.p.A. and 25% *Pichia stipitis* ATCC 58, 785	Initial pH: 5–6 Inoculum size: 7.5 g/L Temperature: 30 °C	49 ± 5%	[201]
	One-stage enzymatic hydrolysis with Viscozyme® L, AMG 300 L, and Pectinex Ultra SP-L (50 °C, 4 h, 5 pH)			49 ± 0.5%	
Scenedesmus obliquus SAG 276.7	3% H_2SO_4, 120 °C, 0.5 h			87 ± 6%	
	Ultrasonication followed by One-stage enzymatic hydrolysis with Viscozyme® L, AMG 300 L, and Pectinex Ultra SP-L (50 °C, 8 h, 5 pH)			41 ± 1.5%	

Note: NA—Not available.

4. Challenges and Future Prospects

With rising energy demand and the gradual fossil fuel depletion, it is necessary to develop renewable and sustainable alternative energy sources, including bioenergy. Among the different available biomass resources, microalgae biomass possesses comparative advantages due to its high growth rate and ability to grow using flue gas and waste as a nutrient source. Despite its advantages, microalgae-based bioenergy is not yet commercialized. One of the main challenges is the high production cost of microalgae biomass, making it less promising than other renewable and non-renewable energy feedstocks. However, research efforts are underway to reduce microalgae biomass production costs by using waste cultivation media, redesigning photobioreactors, and developing cost-effective harvesting techniques [209].

The second challenge is the low energy recovery efficiency. Single biochemical conversion process often exhibits low energy recovery efficiencies, even under optimal processing conditions. This could be attributed to the complex biochemical composition of microalgae biomass (as shown in Table 1), limiting the energy recovered by a single biomass conversion process. To fully exploit the microalgae biomass and extract a maximum amount of bioenergy, a biorefinery framework can be applied to produce multiple bioenergy products simultaneously [210]. Table 10 lists examples of studies that have combined two or more biomass conversion processes to generate multiple bioenergy products. Such approaches help recover the maximum possible bioenergy from microalgae biomass while simplifying the downstream handling of those bioenergy products. For instance, studies have combined biohydrogen and biomethane production to increase energy recovery [171,177]. Such sequential production of biohydrogen and biomethane makes it feasible to produce biohythane (a mixture of 90–75% biomethane and 10–25% biohydrogen) on site, which can be stored and transported using the existing natural gas infrastructure, avoiding the limitation of building dedicated storage and transport infrastructures for pure hydrogen energy [211]. However, such integrated bioenergy production processes are still in their early stage of development. Although integrated processes can maximize bioenergy production from microalgal biomass, their complexity and excessive energy requirements to execute certain steps (such as biomass pretreatment and photo fermentation) will limit their commercial applicability. Further investigations on enhancing energy efficiency and simplifying the procedures, such as the development of novel energy-saving biomass pretreatment methods, high-performance photobioreactors for photo fermentation utilizing solar energy, biogas upgrading through carbon dioxide fixation during microalgae cultivation using anaerobic digestate, etc., remain to be conducted in future studies to make this integrated process more energetically and economically feasible for industrial applications.

In addition to bioenergy, microalgae biomass can also be used to produce high-value-added bioproducts, such as pigments (astaxanthin, phycocyanin, lutein, and β-carotene), polyunsaturated fatty acids (Docosahexaenoic acid and Eicosapentaenoic acid), and protein supplements [212]. Co-production of high-value-added products with bioenergy can significantly reduce the overall cost of microalgae-based biorefineries. As shown in Table 10, Mirzaei et al. [206] and Hosseini et al. [207] successfully demonstrated the co-production of astaxanthin with bioenergy (biomethane and bioethanol) using *Chromochloris zofingiensis* and *Haematococcus pluvialis*, respectively. However, techno-economical and life cycle analyses must be conducted to determine the best possible scenarios for microalgae biomass valorization in a biorefinery, concurrently producing bioenergy and high-value-added bioproducts. It is necessary to continue building new relevant solutions based on the experiences from recent advancements and challenges encountered to fully exploit the microalgae biomass and balance the sustainability aspect of microalgal biotechnology with economic gains.

Table 10. Integrated biorefinery approach for combined production of bioenergy and bioproducts from microalgae biomass.

Microalgae Species	Process	Product	Energy/Product Yield or Recovery	References
Chlorella sp.	Dark fermentation	Biohydrogen	0.5 kJ/g VS	[171]
	Dark fermentation and photo fermentation	Biohydrogen	1.9 kJ/g VS	
	Dark fermentation and anaerobic digestion	Biohydrogen and Biomethane	6 kJ/g VS	
Chlorella sp.	Dark fermentation	Biohydrogen	0.4%/g VS	[175]
	Dark fermentation and anaerobic digestion	Biohydrogen and Biomethane	57%/g VS	
Algal bloom dominated by *Microcystis* sp.	Dark fermentation	Biohydrogen	0.4%/g VS	[179]
	Dark fermentation and anaerobic digestion	Biohydrogen and Biomethane	39–44%/g VS	
Scenedesmus obliquus UTEX 393	Lipid extraction/transesterification	Biodiesel	13%/g VS	[177]
	Lipid extraction/transesterification and dark fermentation	Biodiesel and Biohydrogen	19%/g VS	
	Lipid extraction/transesterification, dark fermentation, and anaerobic digestion	Biodiesel, Biohydrogen, and Biomethane	30%/g VS	
Arthrospira platensis	Dark fermentation	Biohydrogen	0.5–1 kJ/g VS	[172]
	Dark fermentation and photo fermentation	Biohydrogen	2.4–4.6 kJ/g VS	
	Dark fermentation, photo fermentation, and anaerobic digestion	Biohydrogen and biomethane	9.9–10.5 kJ/g VS	
Chromochloris zofingiensis SAG 211-14	Anaerobic digestion	Biomethane	287 L/kg TS (10,343 kJ)	[206]
	Carotenoid extraction and anaerobic digestion	Carotenoids (Mainly astaxanthin)	10 g/kg TS	
		Biomethane	198 L/kg TS (7153 kJ)	
	Carotenoid extraction, yeast-based fermentation, and anaerobic digestion	Carotenoids (Mainly astaxanthin)	10 g/kg TS	
		Bioethanol	143 g/kg TS (3832 kJ)	
		Biomethane	123 L/kg TS (4428 kJ)	
Haematococcus pluvialis SAG 192.80	Carotenoid extraction and anaerobic digestion	Astaxanthin	39 g/kg TS	[207]
		Biomethane	192 L/kg TS (6939 kJ)	
	Carotenoid extraction, yeast-based fermentation, and anaerobic digestion	Astaxanthin	39 g/kg TS	
		Bioethanol	170 g/kg TS (4666 kJ)	
		Biomethane	67 L/kg TS (2430 kJ)	

Note: VS—volatile solids, TS—total solids.

Even though bioenergy production from microalgae biomass has increasingly exhibited promising results at the laboratory and pilot scale, studies focusing on process optimization and the industrial scale-up are scarce. Optimizing process parameters for biochemical processes is complex because it involves many permutations and combinations of operating conditions [213]. Moreover, the composition of feedstock can influence the bioenergy yield. Conventionally, trial and error or one variable at a time (OVAT) analyses are conducted to decipher the correlation between output (bioenergy yield) and input (governing factors) variables. However, these analyses involve time, cost, and labor-intensive laboratory studies. To overcome the shortcomings of conventional strategies, theory-driven (hypothesis-driven) models are developed by deriving empirical judgments from multiple experiments. However, theory-driven models often fail to accurately predict the outcomes for the bioenergy systems due to their complex and non-linear nature [214]. With the emergence of artificial intelligence (AI) tools, it is now possible to identify and use the patterns in available datasets to predict the outcome for a new input without conducting detailed laboratory studies [215]. Machine learning models (data-driven models), such as artificial neural networks, random forests, support vector machines, multilinear regression, and decision trees, have been successfully applied in microalgae biomass conversions technologies such as pyrolysis [23,216], gasification [217], hydrothermal liquefaction [218] and biological hydrogen production [219] for the prediction and optimization of the bioenergy yield. Despite their success, machine learning-assisted predictions in the bioenergy field are still in the initial stages of development. More developments in machine learning studies are required to expand the overall understanding of microalgae biomass conversion processes and obtain new insights to improve the bioenergy yield. More research should be conducted to improve the interpretability and predictability of machine learning models by developing high-quality datasets to test and apply novel machine learning algorithms, promoting the application of state-of-the-art algorithms such as multi-view and deep learning, and integrating theory-driven models.

In addition to technological breakthroughs, bioenergy cannot replace fossil fuels without significant policy changes. Government policies such as taxation for greenhouse gas emissions, subsidies for bioenergy production, and incentives for bioenergy utilization may help to relieve the cost pressure to some extent. It is still too early to predict which of the many developments summarized in this article will succeed on a large scale. However, when we contemplate the myriad of possibilities from microalgae biomass exploitation and the likelihood of continued "crises" arising from non-renewable energy usage, there is little doubt that this field will be vital in shaping the future of new and clean energy technologies. The current literature review on microalgae-based bioenergy production indicates that long-term research and development plans are required to translate laboratory studies into sustainable real-scale facilities.

5. Conclusions

This article reviewed various biochemical conversion technologies, such as anaerobic digestion, biohydrogen production (direct biophotolysis, indirect biophotolysis, photo fermentation, and dark fermentation), and alcoholic fermentation (microalgae-catalyzed photo fermentation, microalgae-catalyzed dark fermentation, and traditional fermentation by ethanologenic microorganisms) for biomethane, biohydrogen, and bioethanol production, respectively. Compared to other biochemical conversion processes, anaerobic digestion and traditional alcoholic fermentation are simple, easy to operate, and more technically advanced technologies that can pave the way for commercializing microalgae-based bioenergy. Nevertheless, the high cost of microalgae biomass production and low energy recovery efficiencies are the major bottlenecks in anaerobic digestion and traditional alcoholic fermentation technologies. To reduce biomass production costs and improve energy recovery, future research should focus on cultivating microalgae using waste resources, designing efficient photobioreactor systems, and developing cost-effective biomass harvesting and pretreatment technologies. Artificial intelligence tools can be used to accelerate process

optimization and scale-up. In addition, a biorefinery approach must be explored to fully exploit the microalgae biomass and produce high-value-added products with bioenergy. Techno-economical and life cycle analyses must be conducted to determine the best scenarios for microalgae valorization in an integrated biorefinery. Along with continuous research and development efforts, changes in government policies are also needed to incentivize bioenergy production and consumption.

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