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Review

Utilization of Microalgal Biofractions for Bioethanol, Higher Alcohols, and Biodiesel Production: A Review

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Received: 27 October 2017; Accepted: 8 December 2017; Published: 12 December 2017

Abstract: Biomass is a crucial energy resource used for the generation of electricity and transportation fuels. Microalgae exhibit a high content of biocomponents which makes them a potential feedstock for the generation of ecofriendly biofuels. Biofuels derived from microalgae are suitable carbon-neutral replacements for petroleum. Fermentation is the major process for metabolic conversion of microalgal biocompounds into biofuels such as bioethanol and higher alcohols. In this review, we explored the use of all three major biocomponents of microalgal biomass including carbohydrates, proteins, and lipids for maximum biofuel generation. Application of several pretreatment methods for enhancement the bioavailability of substrates (simple sugar, amino acid, and fatty acid) was discussed. This review goes one step further to discuss how to direct these biocomponents for the generation of various biofuels (bioethanol, higher alcohol, and biodiesel) through fermentation and transesterification processes. Such an approach would result in the maximum utilization of biomasses for economically feasible biofuel production.

Keywords: microalgae; pretreatment; fermentation; bioethanol; biobutanol; biodiesel

1. Introduction

Concerns related to climatic changes, rising energy demands due to depletion of fossil fuels, and the fluctuating economics of crude oil generation are gradually emphasizing the need of other alternatives to fulfil the growing energy requirements [1,2]. International Energy Outlook 2016 predicted that the world energy-related carbon dioxide (CO_2) emissions will be increased by 10% in 2020 and 34% by the end of 2040 [3]. Emissions of CO_2 result primarily from the combustion of fossil fuels. The ignition of fossil fuels is estimated to release about six gigatons of carbon into the atmosphere (in the form of CO_2) per year [4]. As a result of human activities, CO_2 levels in the atmosphere have increased by ~25% over the past 150 years [5]. Increasing the energy demand and environmental pollution (Figure 1a) are thus two major challenges that threaten the world [6–8]. In this context, a higher supply of food (70%), fuel (50%), and water (50%) will be required by 2050, in addition to a 50% reduction in CO_2 emissions to maintain social, political, and climate security [9]. Thus, it is immensely important to develop new technologies in order to reduce the CO_2 emissions. Increases in the use of renewable and nuclear energy will reduce the share of fossil fuels to 78% [10].

The development of sustainable water-use practices and production methods for food and energy is required to prevent the use of all available land resources for agricultural purposes. Replacement

sources of energy are necessary to substitute for the shortfall in fossil fuels, and bioenergy is one such suitable source to supplement the limited fossil-fuel reserves [11]. Bioenergy is vital for the socio-economic development of a nation [12]. Depending on the type of feedstock used, bioenergy production from sugar/starch feedstocks are stated as the first-generation biofuels, whereas bioethanol generated from lignocellulosic-waste biomass is referred to a second-generation biofuel. Lignocellulosic biomass and waste materials (i.e., grasses, sawdust, livestock manure, wood chips, crop residues, and sludge) are alternative economical feedstocks, which can be hydrolyzed by enzymes to simple sugars (monosaccharides) for subsequent production of biofuel [13]. The use of reducing sugars as feedstock for the generation of biofuels has limited feasibility because of the low product yield and excessive cost of the hydrolysis process. The main shortcomings of first and second generation biofuels can be solved to a great extent by third generation biofuels (Figure 1b) [14].

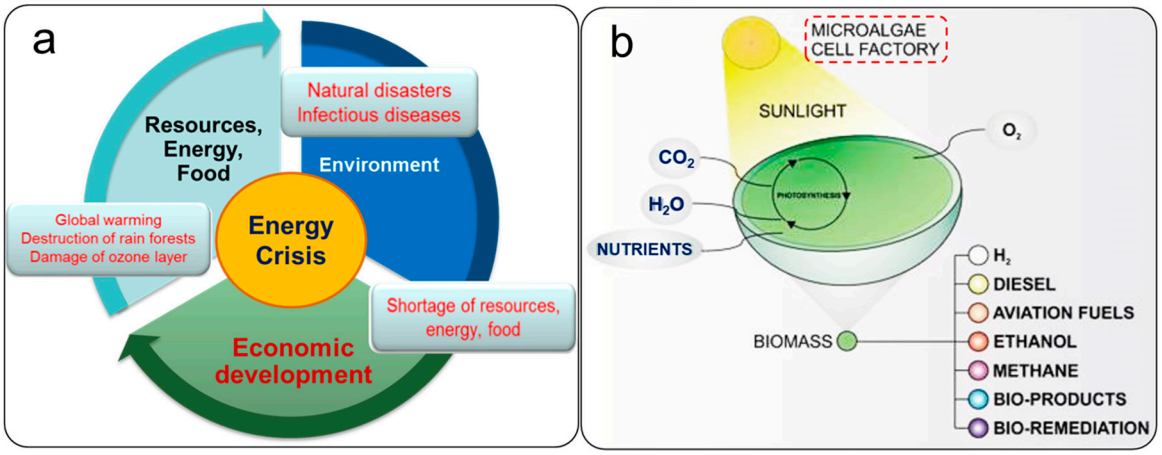

Figure 1. Resources, food demand, economic development and environmental pollution in the world as the main reasons for energy concerns (**a**). The use of microalgae cells as a factory for the production of various biofuels and value-added products (**b**), adapted from [12].

One concern associated with substantially increasing biofuels production is the limited availability or competition for suitable land. In particular, the GHG (greenhouse gases) mitigation benefits of biofuels can be negated if land with high carbon intensity is cleared for the production of biofuel feedstocks. Biofuels which could be generated without increases in arable land or reductions in tropical rainforests remain attractive.

Algal biomass-based-routes to biofuels have potential in this context [15]. Microalgae consume 1.83 tons of CO_2 to produce 1.0 ton of biomass. Moreover, studies conducted on the ability of microalgae to fix flue gas CO_2 showed an overall 90% reduction in CO_2 emissions [16]. This capacity to sequester CO_2 makes microalgae an ideal candidate for CO_2 mitigation and biofuel production. The major compounds of microalgae used for biofuel production are carbohydrates in the form of reducing sugars, proteins as various amino acids, and lipids in the form of fatty acids (Table 1). Most of previous reviews focused on conversion of a specific component of the biomass (carbohydrates, proteins, or lipids) into biofuel while considering the other portions of the biomass to be a waste, which might be the main cause for the economic infeasibility of microalgal biofuels [17–20]. In this review, we explored the use of all three major biocomponents of microalgal biomass including carbohydrates, proteins, and lipids for maximum biofuel generation. Application of different pretreatment approaches for improvement the bioavailability of substrates (simple sugar, amino acid, and fatty acid) was discussed. This review goes one step further to discuss how to direct these biocompounds for the generation of various biofuels (bioethanol, higher alcohol, and biodiesel) through fermentation and transesterification processes. Such approach would result in the maximum utilization of biomasses for economically feasible biofuel production.

Table 1. Biochemical composition (wt. %, dry-basis) of various microalgae strains, adapted from [21–25].

Microalgae strains	Carbohydrates	Proteins	Lipids
Scenedesmus (S-2)	54.2	30.1	17.8
Scenedesmus (S-1)	50.4	7.15	35.7
Chlamydomonas mexicana	52.6	37.0	10.4
Chlorella (C-2)	49.7	14.6	30.3
Nannochloropsis (N-4)	8.92	62.8	18.1
Nannochloropsis oculata	8.00	57.0	32.0
Nannochloropsis sp.	21.0	56.0	9.00
Chlorella vulgaris	9.10	54.9	15.5
Scenedesmus sp.	31.0	50.0	8.00
Chlamydomonas reinhardtii	15.1	47.4	18.1
Pavlova (P-1)	28.0	46.9	13.9
Chlorella (C-1)	16.1	46.8	15.8
Nannochloropsis (N-3)	9.21	46.6	20.1
Chlamydomonas reinhardtii $CW15^{+}$	11.5	45.7	22.4
Porphyridium cruentum	40.0	43.0	8.00
Aurantiochytrium sp. KRS101	5.80	30.0	57.5
Nannochloropsis (N-1)	12.9	12.9	55.4
Chlorella protothecoides	29.0	11.0	53.0
Nannochloropsis (N-2)	15.9	18.2	49.3

Microalgal Biomass as a Potential Source for Biofuel Generation

Microalgae has gained considerable attention as an alternative biofuel feedstock, as recent studies have indicated that most of microalgal biomass is exceedingly rich in carbohydrates, lipids, and proteins [26]. Biofuels from microalgae have promise as carbon-neutral replacements for petroleum, and these are referred to as third generation biofuels [27]. The values of microalgae biocompounds from many previous works fall in the following range: carbohydrates (10–40%), lipids (20–80%), and proteins (10–50%) [28]. These percentages vary with the type of microalgae, cultivation method and condition. Carbohydrates derived from microalgal biomass can be easily saccharified, and the microalgal biomass requires relatively minimal pretreatment, making it a highly competitive feedstock for biofuel production compared to lignocellulosic materials [29]. Understanding the basics of carbohydrate metabolism in microalgae is necessary for developing effective approaches to increase the carbohydrate productivity [30]. Carbohydrates derived from the process of photosynthesis and CO_2 fixation. The intrinsic carbohydrate contents of selected microalgae along with the carbohydrate contents under specific cultivation and environmental conditions are listed in Table 2. Carbohydrates can be fermented to produce bioethanol. Bioethanol from microalgae can be produced by dark fermentation or yeast fermentation. Microalgal lipids are mainly composed of unsaturated fatty acids and some saturated fatty acids [31,32]. The lipid is extracted using organic solvent and is then transesterified to biodiesel in the presence of base or acid catalysts [33].

Table 2. Intrinsic and enhanced carbohydrates biomass content after use of a cultivation technique.

Microalgae	Intrinsic Carbohydrate, %	Enhanced Carbohydrate, %	Carbohydrates Productivity, mg L^{-1} d^{-1}	Biomass Concentration, g L^{-1}	Technique	Reference
Chlorella vulgaris	12–17	41	199	-	Nitrogen starvation	[34]
		55	-	~0.6	Phosphorus starvation	[35]
		38	-	~0.2	Nitrogen starvation	
		60	-	~1.0	Sulfur starvation	
		44	66–112	-	Grow on glucose	[36]
		23	18–20	-	Grow on acetate	
		29–34	26–35	-	Grow on glycerol	
Spirulina platensis	8–14	55–65	-	0.15–0.52	Nitrogen starvation	[37]
		63	170	0.94	Phosphorus starvation	
		50	290	1.6	Light intensity and nitrate supply	
Spirulina maxima	13–16	34	-	~1.3	Light intensity	[38]
		60–70	-	~1.3	Nitrogen starvation	
		50	-	~1.2	Salt stress	

Microalgae are the source of a wide range of value-added products, including nutraceuticals, pharmaceuticals, pigments, fertilizers, and feed for livestock. Microalgae contain proportions of biocompounds similar to those of human food sources [39]. Microalgal proteins are mixtures of amino acids and peptides, and they can be converted to higher alcohols [40]. Studies on transforming amino acids to biobutanol have recently been conducted (Figure 2). A few studies have suggested the utilization of biomass (containing carbohydrates, and protein fractions) left after fatty acid extraction for bioethanol, biobutanol, and biohydrogen production [41,42]. Various conversion methods are used for the generation of different forms of biofuels for example, bioethanol, biodiesel, bio-oil, and methane from microalgal biomass components (Figure 3).

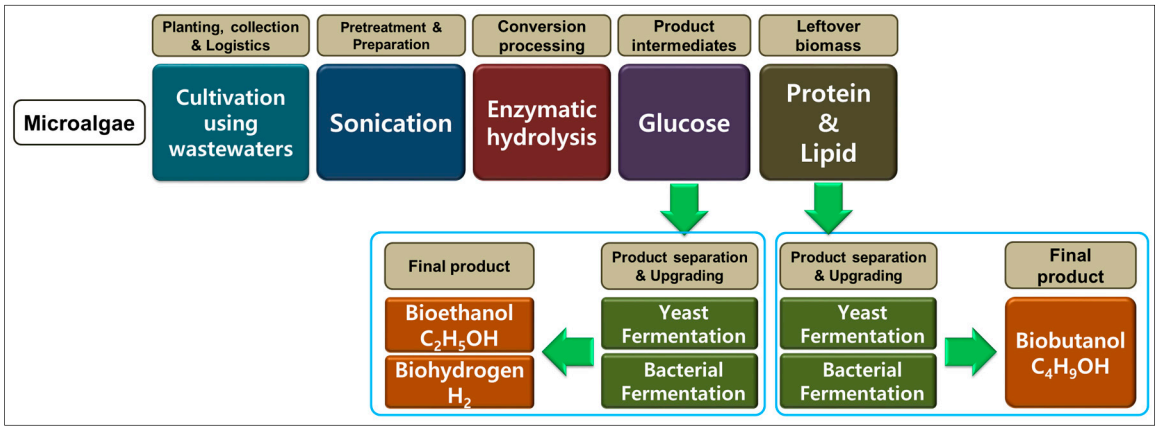

Figure 2. Microalgal technology for biofuel production through serial processes of cultivation, pretreatment, and conversion of each biocompounds.

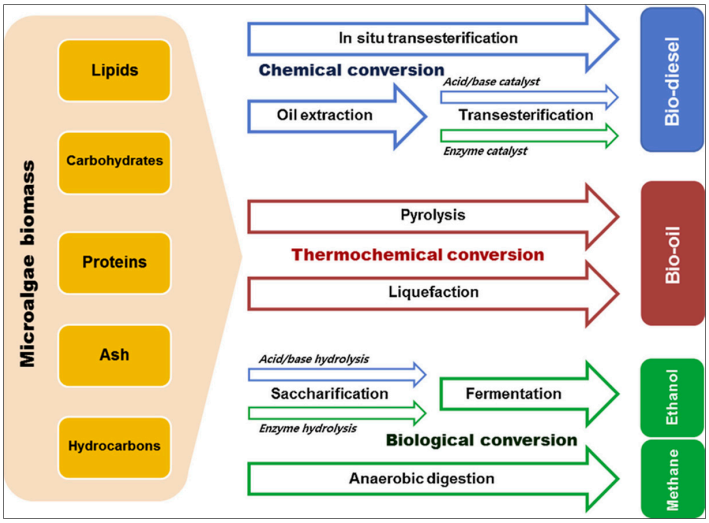

Figure 3. Several thermochemical and biochemical practices for the generation of biofuels from microalgal biomass, adapted from [43].

In the following subsections, we briefly discuss different products obtainable from microalgae. A significant amount of research and development (R and D) work is being conducted to make microalgal biofuels production a cost-effective process at a commercial scale. Some of the biggest challenges in this area are associated with finding economical and sustainable ways to cultivate, harvest, extract, and convert useful components into biofuels. The complete utilization of microalgal biomass should be considered to produce several biofuels and value-added products, and to improve the economic feasibility of microalgae.

2. Various Pretreatment Methods for the Extraction of Microalgal Biofractions

Several pretreatment methods such as physical, chemical, and biological pretreatments can be used for the hydrolysis of feedstock. Microalgae (such as *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp.) have an abundance of carbohydrates (cellulose) and proteins that can be converted to bioalcohol under specific fermentation conditions [44–46]. Biomass pretreatment enhances the hydrolysis rate of substrate as it enhances the solubility of sugar, increases surface area, improves the digestibility of substrate by weakening the cell wall, making enzymes more accessible [47]. There are four major operations required for biofuel production: pretreatment, hydrolysis, fermentation and distillation (recovery of the product) [48]. Pretreatment of biomass is usually a costly step in bioethanol production; thus, an economical pretreatment method should be applied to make bioethanol generation economically attractive process [49]. An effective pretreatment should be cost effective, energy efficient, simple to apply, and should not convert the fermentable sugars into an unusable form. Biomass can be pretreated using physical, chemical, and biological methods (Figure 4a).

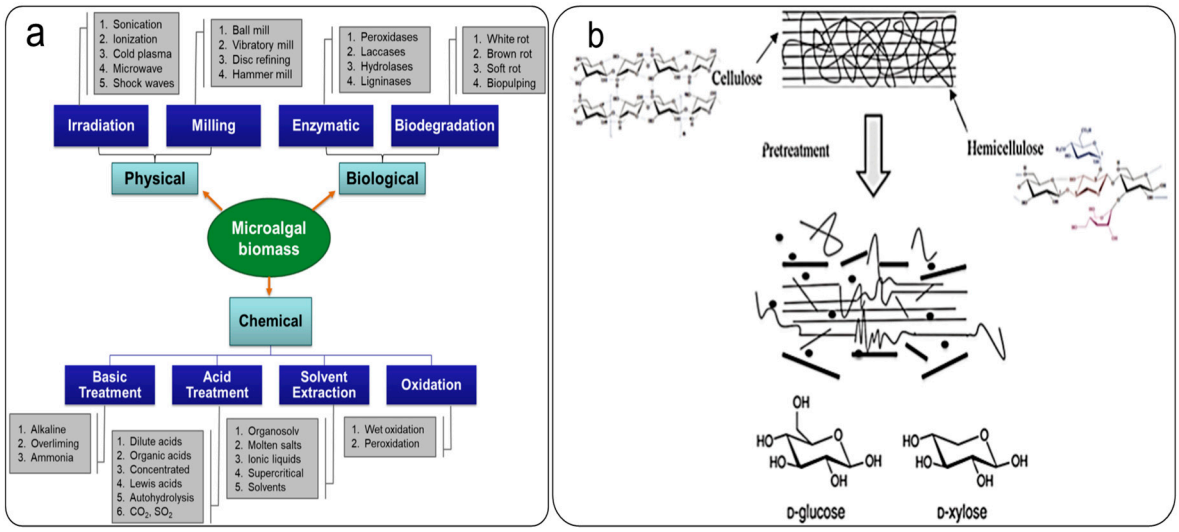

Figure 4. Physical, chemical, and biological pretreatment methods: pretreatment of biomass showing the structure of cellulose and hemicellulose (**a**) and their hydrolysis to glucose and xylose (**b**).

2.1. Physical Pretreatment

Physical pretreatment normally uses combination of mechanical and heat treatment to reduce the particle sizes of the microalgal biomass and increase the specific surface area by reducing the cellulose crystallinity. Pyrolysis is widely used as a physical pretreatment in which a high temperature is applied on the biomass for short time duration. However, the cost associated with its high energy consumption restricts its implementation at a commercial scale production [50]. Ultrasonication is a developing technology with the potential to decrease chemical loading and reaction time. It effectively modifies the surface structure of biomass which lead to enhanced saccharification [45,51]. It has been widely used for homogenization and disruption of the rigid cell wall of microalgae. Pretreatment using sonication under optimum conditions of 2.2 Kw, 40 kHz, for 15 min at 50 °C released 74 mg g^{-1} of total reducing sugars (TRS) based on the dry cell weight [25]. Other physical methods including steam explosion and autoclaving, rupture the microalgal cell wall, resulting in the release and recovery of biocomponents. The steam explosion method provides accessibility to the degradation of cellulose. Steam explosion is increasingly considered to be one of the most efficient, eco-friendly and cost-effective processes for commercial application and thus, it has been widely tested at the pilot scale for various biomasses [52]. This method consists of heat treatment (160–270 °C) to biomass under high-pressure steam (20–50 bar)

for a short duration (a few minutes); then, the reaction is stopped when the pressure conditions reach atmospheric conditions.

2.2. Chemical Pretreatment

The chemicals that are usually used for the pretreatment procedures are either acids or alkaline. These are industrial chemicals with minimal toxicity in their applied concentrations. The chemical pretreatment of microalgal biomass improved effective extraction of intracellular lipids, carbohydrates and proteins. Acid pretreatments are preferable over alkali treatments, as they provide higher convertibility of cellulosic materials into reducing sugars [53]. During the acid pretreatment, several parameters such as acid concentration, treatment time, temperature, and amount of substrate loading influence the process efficiency. Microalgal polysaccharides are encapsulated in the cell wall [54], which must be released from the cell well and hydrolyzed into simple sugars so that microorganisms can ferment it to bioethanol.

Maximizing the bioavailability of fermentable biomass components is a key challenge in biomass pretreatment due to the loss of sugars during conventional pretreatment approaches [55]. Pretreatment of fruit peels and wastes (FPWs) with dilute acetic acid helped maximize sugar recovery (99.9%) under the optimized conditions (0.2 M acetic acid, 100 °C, 1 h) at 10% substrate loading [56]. Nguyen et al. [57] documented that pretreatment of *Chlamydomonas reinhardtii* biomass with 3% sulfuric acid for 15–20 min at 110 °C released up to 58 wt. % glucose and yielded ~29 wt. % (g ethanol g^{-1} microalgae) ethanol. This demonstrates the possibility of applying acid pretreatment to microalgal biomass.

Alkali has also been used for pretreatment of macroalgae. Harun et al. [58], applied alkali treatment (120 °C, 30 min, 0.75% NaOH) to recover the carbohydrate portion from *Chlorococum infusionum* with a maximum glucose yield of 0.35 g sugar g^{-1} algal biomass. To commercialize the process of bioethanol production from microalgal biomass, in-depth investigations on pretreatment processes, mostly focusing on the optimization of the process parameters are essential.

2.3. Biological Pretreatment

Biological pretreatment utilizes microbes and enzymes to disintegrate the biomass and release the simple sugars for the subsequent fermentation (Figure 4b). This approach is the most environmentally friendly method since it avoids using chemical regents, consumes less energy, does not require specific vessels that are resistant to rust and pressure, and forms few inhibitors [48]. However, biological pretreatment is associated with a slow hydrolysis rate, which prolongs this processing step. The algal cell wall contains cellulose and other nutrients that support the growth of hydrolytic microorganisms for biofuel production. Hydrolytic aerobic bacteria have been used for degrading the algal cell wall [59]. Moreover, anaerobic bacteria could be used for pretreatment of microalgal cells for anaerobic digestion and biofuel production.

Microbial enzymes for the pretreatment convert the complex compounds in the algal cells into simple soluble sugars. Enzymatic hydrolysis is an efficient process for the hydrolysis of the microalgal biofractions [60,61]. This strategy has advantages over chemical hydrolysis due to its high selectivity and generation of low toxic hydrolysates as compared to acid/alkali hydrolysis. Commercial enzymes under anaerobic digestion were investigated for the pretreatment of *Rhizoclonium* biomass to improve biomethane production [62]. Because of the complexity of the algal cell wall, enzyme cocktail is recommended for pretreatment instead of using a single enzyme. Various enzymes have been applied for the hydrolysis of microalgae biomass for increasing the total reducing sugar production (Table 3).

Carbohydrases are used for the disintegration of cell wall and retrieval of proteins at neutral pH [63]. Carbohydrases attack the carbohydrates in the cell wall, leading to destruction of cell wall and which subsequently increases the release of intracellular protein pool. Enzymatic hydrolysis treatment under the optimum conditions of 50 °C, pH 5 and enzyme [E]: to substrate [S] ratio of [1]:[5] enhanced the TRS yield by four-fold (2814.9 mg g^{-1}) [25]. Combined pretreatment approaches such as integrated sonication with enzymatic treatment improved the soluble proteins content up to

27.1 mg g^{-1} biomass [21]. Once the microalgal biomass is pretreated by different methods, it can be used for lipid extraction (for biodiesel), releasing simple sugars (for bioethanol) through fermentation or biomethane production through anaerobic digestion.

Table 3. Comparison of the results documented in earlier studies for total reducing sugar (TRS) release from microalgae using enzymatic treatment and subsequent bioethanol production.

Enzyme	pH	Temp., °C	Substrate	Total Reducing Sugar Yield	Ethanol Concentration	Reference
Cellulase (≥1 U mg^{-1})	5	50	*Chlamydomonas mexicana*	445.5 mg (g biomass)$^{-1}$	10.5 g L^{-1}	[64]
(Celluclast 1.5L, Novoprime B957), + Amyloglucosidase (300 L),	5.5	55	*Dunaliella tertiolecta* LB999	42.0% (w w^{-1})	14%	[65]
α-amylase 0.2 (%, v w^{-1})	4.5	55	*Chlamydomonas reinhardtii* UTEX 90	43.6% (w w^{-1})	11.73 g L^{-1}	[66]
NaOH (0.75 w v^{-1})	-	120	*Chlorococcum infusionum*	350.13 mg (g biomass)$^{-1}$	26.13%	[67]
Sulfuric acid (3% v v^{-1})	-	160	*Chlorococcum humicola*	43.6% (w w^{-1})	6.47 g L^{-1}	[50]

Bio-Pretreatment: A New Approach to Economic Pretreatment for the Extraction of Microalgal Bioblocks

Biopretreatment is a new terminology that reflects the extraction of proteins during the fermentation of sugars. Fermentation of carbohydrate fraction from microalgal biomass for generation of bioethanol led to the concurrent release of 56% protein fraction under the combined treatment containing of sonication and hydrolysis [25]. Proteins extracted from this process are a feasible feedstock for the production of higher alcohols. Such approach will make the overall process cost effective by enhancing the biofuels yield from algal biomass.

3. Fermentation of Carbohydrates from Microalgae for Production of Bioethanol

Advanced biotechnology is vital for developing and deploying solutions for biomass conversion. Fermentation is the major process for the metabolic conversion of microalgal biocompounds into biofuel.

3.1. Metabolic Pathways

The most commonly used microbial species for commercial production of bioethanol is *Saccharomyces cerevisiae*, which has proved to be robust and well suited to the fermentation of cellulosic, hemicellulosic and lignocellulosic hydrolysates (Figure 5a). *Saccharomyces cerevisiae* is particularly safe and convenient. *S. cerevisiae* is nonpathogenic, classified as "generally regarded as safe" (GRAS), and has been used to produce consumables like ethanol. Thus, the fermentation and processing technology for large-scale production is well established in *S. cerevisiae* [68]. Renewable feedstocks (such as microalgae) are promising for the use with yeast for ethanol and higher alcohol production, as it is composed of cellulose and other carbohydrates. Yeast converts most of the glucose they uptake into pyruvate thorough the glycolytic pathway, where glucose is oxidized to 2 pyruvate molecules (which release CO_2), then, 2 acetaldehyde molecules are reduced to 2 ethanol molecules (Figure 5b). A *S. cerevisiae* strain with high glucose consumption rate was developed via multi-copy integration using metabolic engineering. Among 350 screened metabolically engineered strains, YPH499/dPdA3-34 showed the highest glucose consumption rate and 1.3-fold higher cell growth rate than the wild (control) strain [69].

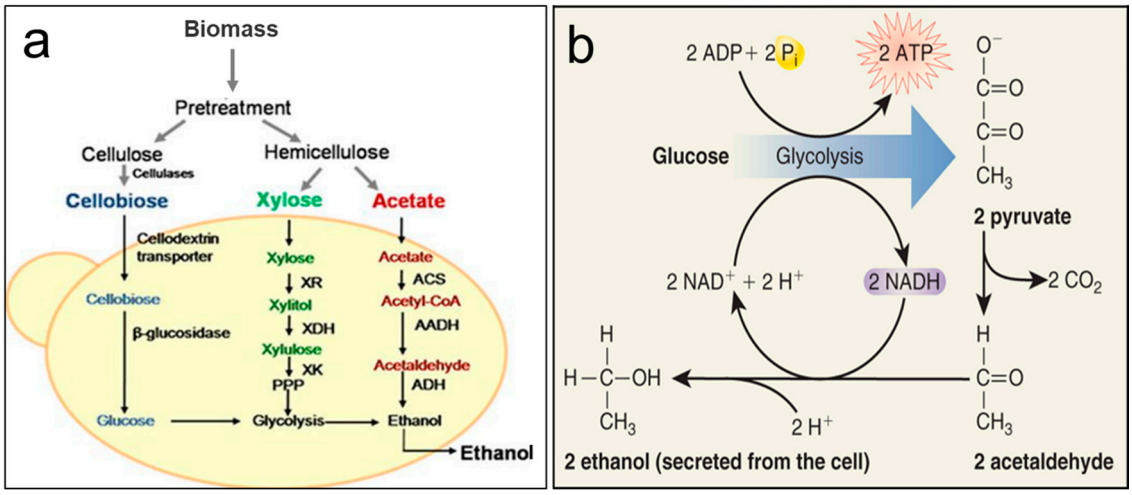

Figure 5. Schematic overview of bioethanol production through utilization of cellobiose, xylose and acetic acid from biomass by yeast (**a**) and the metabolic pathways (glycolysis and fermentation) (**b**), adapted from [70].

3.2. Saccharification and Fermentation Strategies

Microalgae-rich carbohydrates have high reducing sugars, thus their saccharification is much easier, making algae a sustainable feedstock for bioethanol production. The pretreatment is followed by hydrolysis and fermentation. Both these steps can be conducted either separately or simultaneously. Enzymatic treatment catalyze by amylases, cellulases, and glucoamylases to hydrolyze microalgal biomass to obtain simple sugars, and the fermentation is performed by yeast or bacteria [71]. Some microalgae can participate in self-fermentation (SF) processes to produce bioethanol. For example, *Chlorococcum littorale* managed to produce 450 mmol (ethanol) g^{-1} at 30 °C via dark fermentation [29].

Most explored cellulase systems were isolated from fungi such as *Fusarium solani, Trichoderma viride* and *T. reesei* [72]. Pretreatment of *Chlorococum humicola* (macroalgae) by enzymes (from *T. reesei*) resulted in 64.2% (W W^{-1}) saccharification yield [73]. The efficiency of enzymes depends both on the appropriate proportional ratio of the various components and on the presence of all cellulose components. Several process combinations are possible if enzymatic hydrolysis is applied: separate (or sequential) hydrolysis and fermentation (SHF), simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP) and simultaneous saccharification and fermentation (SSF) (Figure 6a). During SHF, enzymatic hydrolysis and fermentation are carried out separately in two different vessels in a subsequent process at optimum process conditions. One problem can be a negative aspect of SHF is accumulation of glucose and cellobiose in the hydrolysis step inhibit the activity of the cellulases.

In SSF, the hydrolysis process carried out by the enzymes and the subsequent fermentative process are performed in the same reactor; therefore, glucose released due to cellulases activity is directly converted to ethanol by fermentative microorganism. Thus, SSF provides a benefit over SHF, as continuous utilization of glucose in the reactor decreases the end-product inhibition of enzymes [64]. Moreover, the presence of ethanol in the medium prevents the contamination by unnecessary microorganisms. Such benefits resulted in an enhanced saccharification rate and higher total ethanol yield compared to SHF. The SSCF is carried out by combining the cellulose hydrolysis step with co-fermentation of pentose and hexose sugars in a single reactor (Figure 6b). This make the process simpler and reduces the number of reactors involved and problem of product inhibition associated with enzymes.

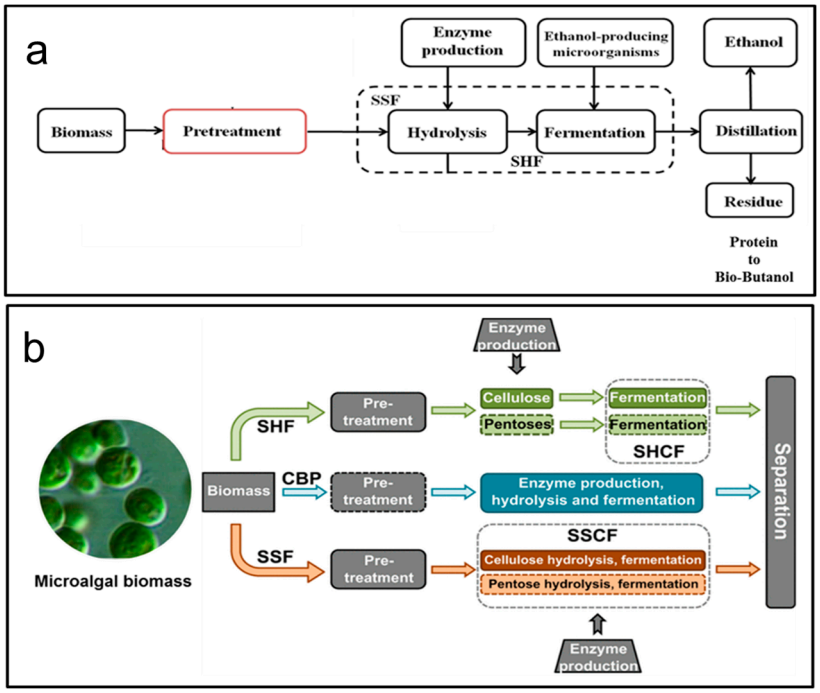

Figure 6. Process flow of separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) (**a**) and simultaneous saccharification and co-fermentation (SSCF) (**b**).

3.3. Long-Term and Continuous Production of Bioethanol through Repeated Batch Fermentation

Repeated or continuous batch fermentation is a sophisticated process for improving bioethanol production, and this process has numerous benefits such as reduced operating cost, its simplicity, and increased productivity [74]. The major purpose of repeated batch process is to enhance the productivity by achieving a high cell density in the bioreactor. However, it is unbearable to accumulate cells when starchy substrates are used in the repeated batch process without eliminating suspended solids. As demonstrated by several studies, 'cell immobilization' is arguably the most effective way to minimize the inhibition caused by high concentrations of substrate and product, and to enhance the ethanol production with low costs investments. Immobilization systems are attractive and promising as the bioethanol yields are higher than that observed for free cells [75]. The enhanced stability of the immobilized yeast cells along with high ethanol production has been observed during a five-cycle repeated batch fermentation [64].

The procedure of immobilization in alginate beads is simple to carry out and inexpensive, thus there is a high potential for its use in industrial applications [76]. Calcium alginate beads are one of the most frequently used supports for the immobilization. They offer several advantages as a support, such as easy availability, good biocompatibility, low cost and ease of preparation. However, there are some drawbacks associated with their applications including loss of integrity, matrix degradation, severe mass transfer limitations, large pore sizes and low mechanical strength (which results in cells being released from the support) [76]. Most of the cell immobilization methods are either adsorption or entrapment. They usually have problems associated with the matrix degradation and mass transfer limitations related to oxygen, nutrient and metabolites are the highlighted problems of gel entrapment methods. On the other hand, an inexpensive and simple procedure (natural adsorption) was used for cell immobilization [75].

Some of the novel strategies can be applied to increase the ability to reusability of alginate beads. Regeneration of yeast cells in a nutrient medium after each cycle is one approach that can help to regenerate the yeast cells in terms of their cellular integrity and catalytic efficiency [64]. In addition, amendment of such micro-elements/nutrients and vitamins in fermentation media may improve

and maintain the cellular activity of the yeast cells for several repeated batches. The recovery of the immobilized beads and/or end products from the fermentation broth during regeneration process is required because, the accumulation of a high concentration of bioethanol in the suspension has harmful effects on immobilized beads and yeast cells [77]. Such practices might prevent degeneration of the beads and improve their efficiency. This may be the focus of future studies on enhancing bioethanol generation in continuous bioenergy production.

4. Conversions of Proteins into Higher Alcohol

Proteins derived from biomass are not well-established feedstocks for the synthesis of fuels because of the difficulties with deaminating protein hydrolysates. Deamination is a critical step toward the formation of keto acids (the precursors for higher alcohol generation) [78]. Considering the huge volumes of biomass that could be processed in biofuel refineries, the amount of protein waste product is likely to be substantial [79–81]. Recent technologies such as hydrothermal liquefaction (HTL) involve the conversion of whole microalgal bio-compounds (lipids, carbohydrates, and protein) into different biofuels. Biochemical and HTL conversion of algal biomass to carbohydrate and protein fractions were reported for ethanol and isobutanol [82].

The conversion of the carbohydrate fraction into bioethanol is metabolically achieved via glycolysis and fermentation, which leads to the production of bioethanol along with the accumulation of bioavailable protein by-products. These protein by-products can be used for the production of higher alcohols (C3–C5) through biological regulation of fermentative microbes [83]. Several yeast strains tend to consume the available substrates (such as amino acids) when the reducing sugars are exhausted during carbohydrate fermentation [84]. Such selective yeast species can be used to convert the protein residues left after carbohydrate fermentation into higher alcohols (Figure 7).

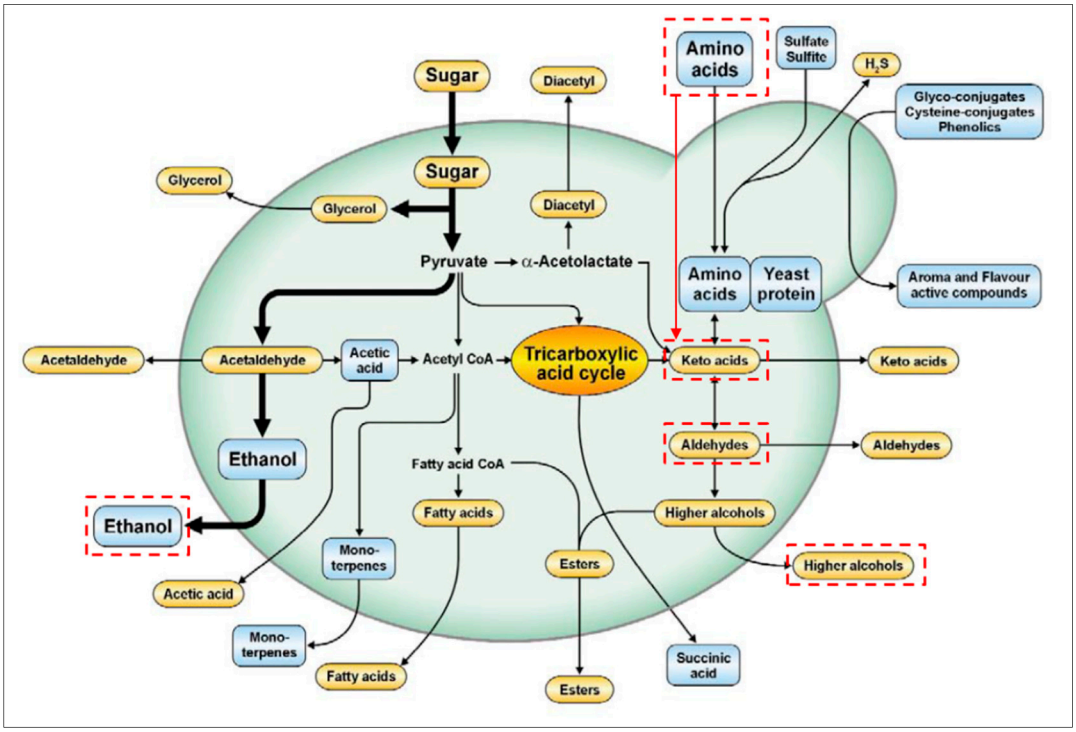

Figure 7. A schematic representation of the derivation and synthesis of flavor-active compounds from sugar, amino acids and sulfur metabolism by wine yeast, adapted from [85].

Higher alcohols (e.g., fusel oil) are alcohols that have more than two carbons and thus have a higher molecular weight and a higher boiling point. A higher alcohol is related to the amino acid from

which it is formed; for examples: leucine to 3-methylbutanol, isoleucine to 2-methylbutanol, valine to 2-methylpropanol, threonine to propanol and phenylalanine to 2-phenylethanol. Production of a variety of higher alcohols from algal proteins, including isopropanol, phenyl alcohol, isobutanol, amyl alcohol, butanediol, tert-butyl alcohol, and sesquiterpene could enhance the economic sustainability of algal biofuel production [82]. The amount of higher alcohols produced from fermentation is dependent on the genus, species and strain of yeast and the specific nutrient status (amino acids and nitrogen). Amino acids are absorbed by yeast through a number of transporters set in the cell membrane. Ehrlich pathway is the associated with the assimilation of amino acids [86], which are then transaminated using aminotransferases located in mitochondria and cytosol [87]. The resulting α-keto acid is decarboxylated to produce an aldehyde and then reduced to the corresponding higher alcohol (Figure 8a). Three exogenous transamination and deamination cycles provides an irreversible metabolic force that make sure the completion of deamination reactions (Figure 8b). Carboxylic acid derivatives may be produced instead of alcohols which strictly dependent upon the redox state of the cells.

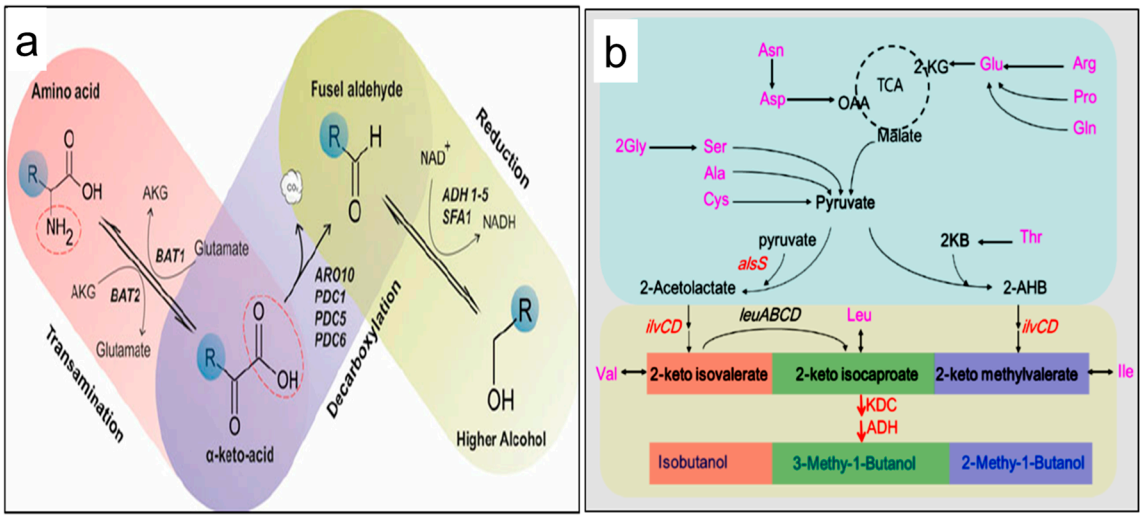

Figure 8. The Ehrlich pathway. Catabolism of branched-chain amino acids (leucine, valine and isoleucine), aromatic amino acids (phenylalanine, tyrosine and tryptophan), and sulfur-containing amino acid (methionine) leads to the formation of fusel acids and fusel alcohols (**a**). The metabolic networks for biofuel production from amino acids (**b**), adapted from [44,85].

In previous studies [78,88,89], metabolic engineering was applied to develop *Escherichia coli* that is able to deaminate protein hydrolysates, facilitating the conversion of proteins to C4, C5 alcohols at 56% of the theoretical yield [44]. *Bacillus subtilis*, *E. coli*, *Saccharomyces cerevisiae* and microalgae was used as protein sources, which yielded up to 4,035 mg L^{-1} of alcohols from biomass containing ~22 g L^{-1} of amino acids [44]. These results showed the viability of using proteins for biorefineries, for which high-protein microalgae could be used as a feedstock with the possibility of maximizing algal growth and total CO_2 fixation.

Utilizing microalgal biomass for fuel production from carbohydrate and lipid-rich biomass and from proteins (amino acids) is a novel concept for maximizing the energy recovery. An unpublished recent study by our team showed that a wild type of fermentative microbe (*Saccharomyces cerevisiae* S288C) could be used for the conversion of leftover amino acids into higher alcohols. The primary carbohydrate fermentation enhanced the release of embedded proteins to the aqueous phase (soluble), which improved the hydrolysis rate of those proteins into amino acids. Distillation made the deamination of hydrolyzed amino acids bioaccessible for utilization by yeast and conversion into higher alcohols. Such cost-effective approaches are needed to minimize waste and produce highly

sustainable biofuel. Changes in the fusel alcohol production could be associated with variations in the intracellular pools of the substrates and intermediate compounds involved in their synthesis [90].

5. Conversion of Microalgal Lipid Fraction into Biodiesel and Glycerol

Some microalgal species can yield up to 60% of their weight in the form of lipids, which can be converted to biodiesel (Table 1). Microalgae can accumulate substantial amounts of lipids under various nutrient-deficient conditions, which makes them one of the most promising feedstocks for biofuel generation. Strategies involving nutrient-deficiency such as nitrogen, phosphorus and iron were applied to activate lipid accumulation in microalgae [91]. Microalgae produce a diverse range of lipids which are a suitable feedstock for biofuel production and value added products which can be used in pharmaceutical, cosmetics and food industry [92]. Microalgae are capable of producing biodiesel 200 times more efficiently than traditional crops [93]. Lipid biosynthesis performed through a complex pathway where synthesis initiates with the formation of acetyl Coenzyme A (CoA) through acetyl CoA carboxylation by the acetyl CoA carboxylase (ACCase) gene [94]. Several microalgal species are well-adapted to environmental stress conditions which affect the cellular activities, including lipid metabolism [95]. Various sources and concentrations of carbon affect the overall composition of microalgal lipids. Alterations in carbon dioxide levels have prompted the accumulation of unsaturated and saturated fatty acids (FAs), respectively [96]. Besides carbon dioxide, several mixotrophic microalgae use carbon from other organic sources for the production of macromolecules under heterotrophic conditions.

Fatty acids with a chain length of C14–C18 carbons are preferable for biodiesel production. The compositions of fatty acids (saturated and unsaturated fatty acids) vary with different species and strains [97]. Saturated fatty acids especially C14, C16, and C18, and unsaturated fatty acids including C16:1, C16:2, C18:1, and C18:2 are vital for producing high quality biodiesel. This is due to the other unsaturated fatty acids with three or four double bonds which have reduced stability in storage [98]. The microalgal conversion pathways typically consist of a series of steps that include the concentration of harvested biomass from the cultures and lipid extraction. After this, the options are numerous for converting the residual (oil extracted) biomass to additional bio-products, which include converting the spent algal biomass to biogas using anaerobic digestion (AD) [15] or using as biofertilizer. Biodiesel produced from microalgal biomass does not produce sulfur oxide and reduces the soot particulate by >75% compared to the existing petroleum. Such environmental benefits make biodiesel a suitable alternative for conventional fuels. In the present scenario, biodiesel has been produced through transesterification process using vegetable oil. However, the need of such catalysts along with high energy requirements are the major drawbacks of such chemical processes [99].

The transesterification uses alcohol to displace the ester group and is widely used to significantly reduce the viscosity of the triglycerides. Several factors such as temperature, incubation time, molar ratio of oil to alcohol, purity of reactants, types of alcohol and catalyst could influence the transesterification reaction [95]. The Fischer-Tropsch liquid fuel which was produced from syngas showed similar properties to the biodiesel. The properties of biodiesel, blend with conventional diesel and diesel like fuels are presented in Table 4. The physico-chemical properties of microalgal biodiesel are similar to that of oil crop based biodiesel and petroleum diesel [93]. The microalgal biodiesel is characterized of having high cetane number, low sulfur and oxygen [100]. Because of high boiling point and stability, the biodiesel could avoid vaporization in the combustion chamber. Its low specific gravity enables it to get upgraded to low value and high-density refinery stream.

Table 4. Properties of different diesel-like fuels obtained through several techniques.

Property	Diesel Fuel	Biodiesel	Blend [a]
Cetane number	53	70–90	57.8
Sulfur content (mg L^{-1})	<10	<1	4.7
Distillation (°C)	180–360	265–320	249–341
Lower heating value (MJ kg^{-1})	35.7	44	36.5
Cloud point (°C)	−5	−20	−4.1
Stability	Baseline	Baseline	Baseline
Specific gravity (kg m^{-3})	835	780	827

[a] Effluent coming from the hydrodesulfurization (HDS) unit when co-feeding vacuum gas oil (VGO) and palm oil (10 wt. %).

6. Fermentation of by-Product Glycerol into Butanol

The first attempt of glycerol fermentation was described way back in 1983, where glycerol produced from halophilic algae was fermented by *Clostridium pasteurianum* [101]. The major products retrieved from this process were ethanol, n-butanol, 1,3-propanediol and acetic acid. Heyndrickx et al. [102] re-demonstrated similar experiments in continuous cultures to extract the same products, and concluded that hydrogen is evolved corresponding to the acetyl CoA formed. *Clostridium pasteurianum* CH_4 has been utilized to produce butanol from glycerol. The addition of butyrate (6 g L^{-1}) as a precursor increased the butanol yield from 0.24 to 0.34 mol butanol (mol glycerol)$^{-1}$. It was believed that butyrate addition triggered the metabolic pathway toward butanol production. One more reason for this improved performance was the in situ butanol removal using vacuum membrane distillation (VMD) which avoided the inhibition caused due to a high butanol concentration in the fermentation broth. Similar observations of enhanced butanol titer and butanol yield due to addition of butyrate and in situ butanol removal [99]. The VMD-based practices decreases the negative impact caused by the inhibitory effects of butanol. In addition, it improves the the butanol yield, making downstream processing more cost-effective and easier. *Clostridium pasteurianum*, produces acetic acid, butyric acid and 1,3-propanediol; moreover, it is also capable to convert glycerol not only to butanol but also to ethanol. The factors that lead these anaerobes to use one or the other metabolic routes are not sufficiently understood. The glycerol fermentation by *C. pasteurianum* is influenced by the culture conditions [103]. Overall, the fermentations were described by a certain variability in product formation under apparently equal or slightly diverse conditions.

7. Conclusions and Future Perspectives

The current microalgal derived-biofuel schemes have limited efficiency and economic viability, and these are influenced by the selection of microalgal strains, pretreatment and conversion pathways. In this review, we covered appropriate solutions to overcome the current limitations. The biochemical content of different microalgal strains that tend to be converted into specific biofuel was thoroughly discussed. The biochemical properties of microalgal biocompounds affect their processing for bioenergy production and are strongly involved in enhancing the characteristics of the processed fuel. Various approaches for the conversion of all microalgal biocompounds into biofuel have been reported for the first time. Conversion of carbohydrate and protein waste through fermentation is a feasible option. Establishment serial processes for maximum utilization of microalgal biocomponents will enhance the production of valuable chemicals such as sustainable fuels and reduce waste. To make microalgae biofuel a practical reality, future research should focus on genetic improvements, biorefineries, higher alcohols from microalgae and large-scale production of higher alcohol and other biofuels. The economic feasibility of microalgal based biofuels should be assessed.

Acknowledgments: This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2017R1A2B2004143) and a Korea Energy Technology Evaluation and Planning (KETEP) grant funded by the South Korean government (MSIP) (No. KETEP-20163010092250).

Author Contributions: M.M.E.-D. & B.-H.J. designed the content of the manuscript; M.M.E.-D. wrote sections #1, 3, and 4; E.-S.S. wrote sections #5 and 6; M.B.K. wrote sections #2 and 7; S.H.A.H., S.-E.O., S.K., and B.-H.J. revised the manuscript; M.M.E.-D., E.-S.S., and M.B.K. improved the presentation of Figures and Tables; B.-H.J. conceived and majorly coordinated the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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