

Prospects for Bioethanol Production from Macroalgae

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Macroalgae (mainly marine macroalgae, *i.e.* seaweeds) are considered as a very promising source for bioethanol production, because they have high carbohydrate contents, superior productivity, and wide adaptability. Macroalgae are generally grouped into three major categories: red, green, and brown algae. Each category has thousands of species, and each species possesses its unique cellular structure, biochemistry, and constitutes. Converting macroalgae to bioethanol involves pretreatment, saccharification, fermentation, and distillation; and the establishment of economic pretreatment methods is always the first key step for bioethanol production. In present, dilute-acid or alkali hydrolysis is typically used to treat macroalgal biomass. Macroalgae can be depolymerized under mild conditions as they have low lignin content. The resulting polysaccharides can be converted to ethanol through enzymatic hydrolysis, followed by adding bacteria, such as *Saccharomyces cerevisiae* and recombinant *Escherichia coli* KO11. Compared with the separate hydrolysis and fermentation process, the simultaneous saccharification and fermentation process often provided higher ethanol titer and conversion efficiency. However, the research on bioethanol production from macroalgae is still in its early stage due to both technical and economic barriers, significant amount of research and development work is needed prior to the commercialization of bioethanol manufacture from macroalgae.

Keywords: Macroalgae; Bioethanol; Marine Macroalgae; Seaweeds; Pretreatment; Hydrolysis; Saccharification; Fermentation

1. Introduction

Bioethanol is a clean, safe, and bio-based energy, which is commonly regarded as one of the primary candidates to replace a fraction of liquid fossil fuels [1]. The importance of using bioethanol as a vehicle fuel is increasing domestic energy production, decreasing greenhouse gas emissions, and preventing environmental pollutions [2]. The global bioethanol production rose rapidly in recent years. Table 1 shows the production of bioethanol in different countries from year 2004 to 2014. The first generation bioethanol is mainly produced from sugars and starch-rich materials. The United States and Brazil are leaders in bioethanol production, making bioethanol from corn and sugarcane, respectively. In Europe and China, mainly cereals and sugars are used as the feedstock. As the development of fuels from biomass continues apace, the consumption of edible crops and sugars has raised food security, morality, and ethics issues [3].

Table 1 Bioethanol production in different countries from 2004 to 2014 (million liters) (modified from [3])

Country	Major feedstock	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Argentina	sugarcane	174	157	205	225	315	416	442	455	469	483	498
Australia	sugarcane	-	27	63	100	156	238	384	387	389	392	395
Brazil	sugarcane	15,208	15,807	17,932	22,446	27,674	25,804	28,960	31,392	34,299	37,396	40,625
Canada	cereal	396	406	545	839	1083	1131	1573	1703	1714	1730	1721
China	cereal/sugarcane/ cassava	3673	3438	3509	3679	3964	4109	4368	4649	4824	4962	5121
EU-27	cereal/sugar beet	2576	2940	3701	3887	5021	5762	6465	7539	9155	10,79	11,774
India	sugarcane/wheat	1178	1120	1664	2082	2085	1680	1704	2430	2482	2532	2575
Indonesia	cassava	163	177	176	196	208	240	425	441	462	485	510
Japan	cereal	-	113	113	110	110	100	130	130	130	130	130
United State	corn	12,596	15,332	20,171	28,929	35,191	40,544	46,024	49,114	51,322	54,058	57,200

In order to overcome these issues, the second generation bioethanol, refined from lignocellulosic biomass, is developed to meet economic growth and morality requirements [4, 5]. However, the cultivation of terrestrial plants requires the resources that could be used for food production. In addition, due to the structural complexity of lignocellulosic biomass, the current conversion technologies including pretreatment, saccharification, fermentation, and separation of final products are relatively costly and low-yield [6]. Among all technical barriers, the delignification is often considered as the major obstacle, which must be combated before the commercialization of lignocellulosic bioethanol can become reality [7].

Recently, algae are viewed as the source of third-generation biofuels [8]. Generally, algae are grouped into microalgae and macroalgae, based on their morphology and size. This paper reviews the development of bioethanol production from marine macroalgae, since the production of freshwater macroalgae is not significant [9]. The words of macroalgae, marine macroalgae, and seaweed are used interchangeably within the context of this article. The major advantages offered by marine macroalgae over terrestrial plants are: (1) no competing with conventional agricultural plants for land, and utilization of different water sources (seawater, brackish water, and wastewater), (2) high area productivity, (3) non-dependence on agricultural input (fertilizer, pesticides, etc.), (4) being hydrolyzed easily into glucose as they contain lower lignin content in the cell wall [10, 11], and (5) easier harvesting as their plant-like characteristics [12]. All of those features enable macroalgae to become a very promising biofuel feedstock for the future.

2. Macroalgae Availability and Chemical Composition

Macroalgae, namely seaweeds, are conventionally classified into three major groups based on their photosynthetic pigments: red algae (*Rhodophyta*), green algae (*Chlorophyta*), and brown algae (*Phaeophyta*) [13]. The green algae can grow in all types of water environments. While red algae grow mainly in intertropical zones, and brown algae especially grow in tempered to cold or very cold waters [14]. Macroalgae can be mass-cultivated based on current farming technologies. Up-to-date, brown and red macroalgae are cultivated more than green species. The production of brown algae alone reached 15.8 million wet tons in 2010, which were harvested from both wild habitats and coastal farms [15]. At present macroalgae are grown for food production, fertilizers, and hydrocolloid extraction in Asia (mainly in China, Korea, Philippines, and Japan)

accounting for about 72% of global annual production [16]. The macroalgae productivity ranged from 150 to 600 t/ha·y fresh weight [14], and the total worldwide production attains 19 million tonnes dry matter in 2014 [17]. The amount of the mass-cultivated macroalgae is six orders of magnitude greater than that of lignocellulosic biomass [18]. That implies that macroalgae could supply sufficient feedstocks for bioethanol production.

Macroalgae are significantly different from terrestrial plants in terms of their chemical compositions. Macroalgae have agar, carrageenan, laminarin, mannitol, mannan, ulvan, fucoidin, and alginate, which are not available in lignocellulosic biomass [13, 18]. A summary of macroalgal divisions, compositions of their cell walls, and most significant characteristics is given in Table 2.

Table 2 Three macroalgae divisions and significant characteristics (modified from [19])

	Red algae	Green algae	Brown algae
Species	6000	4500	2000
Pigments	Chlorophyll <i>a</i> (<i>d</i> in some Florideophyceae); R- and C-phycoerythrin; R- and B-phycoerythrin; allophycoerythrin; α - and β -carotene and several xanthophylls	Chlorophyll <i>a</i> , <i>b</i> ; α -, β - and γ -carotenes and several xanthophylls	Chlorophyll <i>a,c</i> ; β -carotene and fucoxanthin and several other xanthophylls
Storage product	Floridean starch (amylopectin-like)	Starch (amylose and amylopectin)	Laminaran (β -1,3-glucopyranoside); Mannitol
Cell wall	Cellulose, xylans, several sulfated polysaccharides (galactans), alginate in corallinaceae	Cellulose (β -1,4-glucopyranoside), Hydroxyproline glucosides; xylans and mannans	Cellulose, alginic acid, and sulfated mucopolysaccharides (fucoidan)
Representative	<i>Gracilaria spp.</i>	<i>Ulva fasciata</i>	<i>Laminaria spp.</i>
Carbohydrate (%wt)	76.7	43	60
Protein (%wt)	16.0	14.4	12
Lipid (%wt)	1.2	1.8	2
Ash (%wt)	6.1	16	26
Source	[20]	[10]	[21]

The pigment in red macroalgae is R-phycoerythrin, and their cell walls contain a small quantity of cellulose. Because the great majority of their components is gelatinous or amorphous sulfated galactan polymers, such as carrageenan (up to 75% dry wt.), agar (up to 52%), and funoran, red macroalgae are also called as carrageenophytes and agarophytes [22]. Another distinctive feature for red algae is accumulating floridean starch and floridoside, which are similar to starch. But green and brown algae do not have these carbohydrates [23, 24].

The major photosynthetic product of green macroalgae is starch, and the cell walls of their outer and inner layers are predominantly cellulose and pectin, respectively. *Ulva spp.* and *Enteromorpha spp.* have 38-52% (dry wt.) of water-soluble ulvan and insoluble cellulose in the cell walls. Ulvan, the unique carbohydrates of green algae, is composed mainly of D-xylose, D-glucuronic acid, L-rhamnose, and sulfate [18].

Brown macroalgal cell walls are composed of cellulose, alginic acid, and other polysaccharides [19]. The accumulation product of this group are the carbohydrates of laminarin and mannitol [20]. Laminarin (*i.e.*, β -1,3-glucans) is a unique polysaccharide present in brown seaweeds [21]. Alginate accounts for up to 40% dry wt. as a principal material of the cell wall [14], and is composed of three different uronic acids: guluronic

acid blocks, mannuronic acid blocks, and alternative blocks of mannuronic and guluronic units.

Macroalgae biomass is easier to be converted into simple sugars than land-plant biomass due to lack of lignin. Besides cellulose and hemicellulose, many algal species accumulate high content of starch as their food material. Carbohydrate contents of macroalgae vary widely by species and cultivar, representing 30-70%, 25-40%, and 30-50% of dry wt. for red, green, and brown algae, respectively [4, 18, 25]. Macroalgae species with high carbohydrate contents include: *Sargassum*, *Gracilaria*, *Euglena gracilis*, *Prymnesium parvum*, *Gelidium amansii* [26], and *Laminaria* [27]. Further species selection is still needed to develop strains with higher carbohydrate contents for use as the promising candidates for bioethanol production.

3. Bioethanol Conversion Processes from Macroalgae

In general, the steps for bioethanol production from biomass include pretreatment, enzymatic hydrolysis, fermentation, and distillation. Almost all kinds of macroalgae can be converted to bioethanol by decomposing their polysaccharides into simple sugars, followed by fermentation with suitable bacteria. However, the development of macroalgal conversion technology is still at an early stage, and the researches were conducted mainly on lab-scale.

Figure 1 shows the flow diagram of bioethanol conversion processes from macroalgae. Unlike the terrestrial biomass, macroalgae contain contaminants from the growth environment and unique chemicals, thus there are some differences in the bioethanol technological processes from other feedstock. The fresh algal biomass collected from the cultivation site need to be processed prior to bioethanol conversion steps [28]. The biomass can be washed with tap water to remove adhering salt, sand, epiphytes, and then sun-dried. Dry seaweed is more easily transported and stored. The granular dried seaweeds can be cooked with hot water and alkali or acid to extract the polysaccharides, or be directly extracted by using supercritical fluids. The extract may be purified and separated through filtration or centrifugation. Since macroalgae have various carbohydrates such as starch, cellulose, carrageenan, laminarin, mannitol, and agar, the saccharification of them is different from that of lignocellulosic biomass [13]. The hydrolysis of macroalgae commonly conducted by using dilute sulfuric acid and enzymes. And then bacteria, such as *Saccharomyces cerevisiae* (NCIM 3455 and ATCC 24858) and recombinant *Escherichia coli* KO11 (ATCC 55124), were added to the algae hydrolysates for ethanol fermentation. There are two methods for fermentation: one is the separate hydrolysis and fermentation process (SHF), and the other is the simultaneous saccharification and fermentation process (SSF). Bioethanol distillation in the lab is often carried out by using vacuum evaporation or small-scale distillation columns.

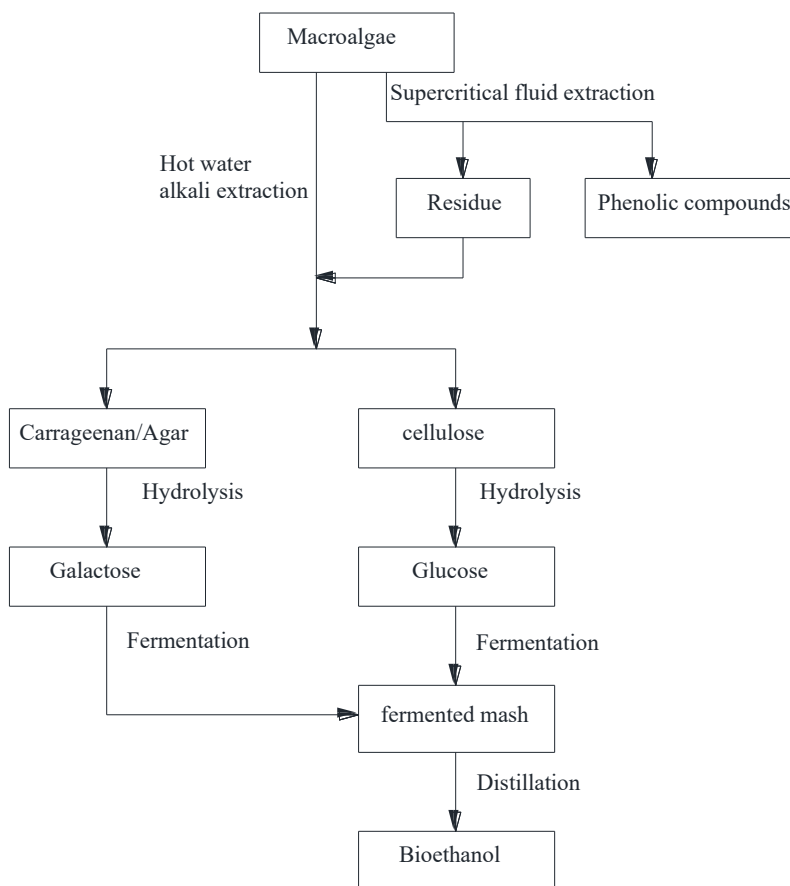


Figure 1. Block flow diagram of bioethanol conversion processes for macroalgae

4. Overview of Pretreatment Technologies for Macroalgae

The establishment of economic pretreatment methods is always the first key step for bioethanol production. The carbohydrate compositions of marine macroalgae highly depend on their species, which largely differ from those of terrestrial plants, so new efficient pretreatment methods are required to make the sugar monomers available for fermentation. Nowadays, although some physical, chemical, and biological pretreatments of macroalgae have been studied to increase the saccharification efficiency, those research activities looking for economically efficient technological solutions are in the early phase.

Different examples of bioethanol production and pretreatment technologies for macroalgae have been described in Table 3. Dilute-acid and alkali hydrolysis are typical physicochemical methods to treat raw macroalgal biomass [29, 30].

Table 3 Comparison of bioethanol yield from various seaweed feedstocks (Modified from [11]).

Algae	feedstock	Pretreatment	Sugar released (g/g biomass)	Ethanol yield (g/g sugar)	Ethanol (g/L)	Reference
<i>E. cottonii</i>	Residue after agar extraction	Solid acid + enzyme	0.814	-	14.1	[31]
<i>Kappaphycus alvarezii</i>	Whole thallus	Acid	-	0.369	6.8	[32]
<i>Kappaphycus alvarezii</i>	Whole biomass plus carrageenan granule	Acid	0.306	0.39	20.6	[30]
<i>Gelidium amansii</i>	Whole thallus	Acid+ enzyme	0.566	-	-	[23]
<i>Gelidium amansii</i>	Whole thallus	Dilute acid	0.422	0.38	27.6	[33]
<i>Gelidium amansii</i>	Whole thallus	autoclave+ enzyme	0.227	-	25.7	[34]
<i>Gracilaria Salicornia</i>	Two stage hydrolysis of fresh biomass	Acid+ enzyme	0.166	0.079	-	[35]
<i>Gracilaria spp.</i>	Whole thallus	Sequential acid + Enzyme	0.592	0.48	4.93	[20]
<i>Gracilaria verrucosa</i>	Pulp after agar extraction	Enzyme	0.87 g/g cellulose	0.43	-	[11]
<i>Laminaria japonica</i>	Whole thallus	Acid+ enzyme	0.376	0.41	23–29	[23]
<i>Sargassum sagamianum</i>	Whole thallus	Thermal liquefaction	-	0.386	1-2	[36]
<i>Sargassum fulvellum</i>	Whole thallus	Acid+ enzyme	0.096	-	-	[23]
<i>Saccharina japonica</i>	Whole thallus	Enzyme	0.614	0.41	37.8	[37]
<i>Saccharina japonica</i>	Whole thallus	Thermal acid	0.456	0.169	7.7	[38]
<i>Ulva lactuca</i>	Whole thallus	Acid+ enzyme	0.194	-	-	[23]
<i>Ulva fasciata</i>	Whole thallus	Hot buffer+ enzyme	0.205	0.45	-	[10]
<i>Ulva fasciata</i>	Whole thallus	Enzymatic	0.112	0.47	-	[39]
<i>Zostera marina</i>	Supercritical CO ₂ extraction residue	Sulfuric acid + Enzyme	0.582	-	6.55	[40]

5 Ethanol Production from Marine Macroalgae

5.1 Red Macroalgae

Gelidium amansii, one of the most abundantly available red seaweed species, are known for high carbohydrate content. *G. amansii* predominantly consists of fibrin (cellulose) and agar (galactan) whose basic monomers are glucose and galactose, respectively [41]. The main products from dilute-acid hydrolysis of *G. amansii* are D-galactose, 3,6-anhydro-L-galactose (3,6-AHG), and D-glucose [42]. The galactose and glucose are classified as fermentable simple sugars, and the 3,6-AHG is non-fermentable. Since the physical morphology of agar is softer than that of cellulose, the hydrolyzed products of galactose and 3,6-AHG are released firstly under mild hydrolysis conditions. However, the 3,6-AHG, is also known as so acid-labile that it is very apt to be decomposed into 5-(Hydroxymethyl)furfural and, subsequently, into organic acids such as formic acid and levulinic acid, which act as inhibitors in the fermentation process [43].

It is well known that the fermentable sugar yields and the amount of inhibitors primarily depends on the three major factors: acid concentration, reaction temperature, and reaction time (or residence time for continuous process)[44]. A facile continuous method for dilute-acid hydrolysis of *Gelidium amansii* was developed to compare with the batch operation. The continuous acid pretreatment was done at a flow rate of 40 L/h, 15% (w/w) seaweed slurry containing 2% (w/w) of sulfuric acid at 150°C, and auto-generated pressure range of 3.0-3.5 bar. The product mixtures were continuously collected and the unreacted solid residual fibers were subsequently separated, followed by neutralization of hydrolysates by adding limestone (CaCO₃). The hydrolysate of *G. amansii* was then fermented by *Brettanomyces custersii* KCTC 18154P. Results showed the hydrolysate obtained from the continuous process attained a high sugar concentration with low quantity of inhibitors, thereby leading to the higher ethanol yield (the final

ethanol titer of 27.6 g/L after 39 h), than that of the batch reactor (11.8 g/L after 56 h) [33].

In order to produce a high quality hydrolysate with minimal inhibitors, sequential acid and enzyme hydrolysis of *Gracilaria spp.* was studied. The dilute-sulfuric acid hydrolysis process was carried out by using 2% (w/v) of dried *Gracilaria spp.*, and optimized at 121°C via varying acid concentrations (0.025-0.25 mol/L) and residence time (up to 60 min). The hydrolysates were adjusted to various pHs (pH 2–8) at the end of the acid treatment. After pH adjustment, the enzymatic hydrolysis was performed with various amounts of cellulase (0.01- 8% w/v) at 50°C for 6 h. The *Gracilaria* hydrolysate was fermented in batch and repeated batch modes by using immobilized *S. cerevisiae* Wu-2 cells. The process maximally released 11.85 g/L of glucose and galactose, yielding 4.72 g/L of ethanol at the rate of 0.48 g/g sugar-consumed with a 94% conversion efficiency [20].

For converting red macroalga *Gelidium amansii* (GA), GA was autoclaved at 121°C for 60 min to reduce the galactan content. After the autoclave treatment, 177 g glucose and 50 g galactose were produced from 1 kg GA. Enzymatic hydrolysis was conducted with a cocktail of cellulase (Celluclast® 1.5 L) and β -glucosidase (Novozyme 188). SHF (2% substrate loading, w/v) produced a maximum ethanol concentration of 3.33 g/L and an ethanol conversion yield of 74.7% after 6 h. In contrast, SSF achieved an ethanol concentration of 3.78 g/L and an ethanol conversion yield of 84.9% after 12 h. With an increased biomass concentration, the ethanol concentration of 25.7 g/L was attained from 15% (w/v) treated biomass after 24 h SSF processing [34]. The results indicated that autoclaving can increase the sugar yields and ethanol conversion yield of GA, and also showed that SSF is superior to SHF for ethanol production.

Carrageenan is the major polysaccharide constituent of red algae, which consists of repeating of β (1-3)-D-galactose and α (1-4)-3,6-anhydro-D-galactose [41]. Purified carrageenan is generally used for forming thick solution or gel [22]. During manufacturing carrageenan, seaweeds were treated with alkali solution (1-10% NaOH or KOH) at 80°C for 0.5-5h, resulting in 60-70% solid residues (SWBC) with high carbohydrates content. One study used this stream of seaweed wastes as the bioethanol feedstock [45]. Researchers treated seaweed wastes with peracetic acid (PAA) followed by different types of ionic liquids (ILs): 1-ethyl-3-methylimidazole acetate ([Emim][OAc]), 1-hexylpyridinium chloride ([Hpy][Cl]), and 1-ethyl-3-methylimidazolium diethylphosphate ([Emim][DEP]). For a 48-hour saccharification, the cellulose conversions of untreated and pretreated seaweed wastes with PAA followed by [Emim][OAc], [Hpy][Cl], and [Emim][DEP] were 77, 62, 91, and 84%, respectively. The untreated SWBC had a high cellulose conversion, which may be caused by the alkali pretreatment or low lignin and hemicellulose contents of this seaweed. Meanwhile, PAA-IL pretreatments did produce more amorphous cellulose structures, which are beneficial to cellulose conversion [46].

5.2 Green Macroalgae

The most common green macroalga, *Ulva prolifera* (UP), contains about 62% carbohydrates, 27% protein, 0.3% lipid, and 11% ash of dry matter [47]. However, the carbohydrates of *U. prolifera* are chiefly in the form of complex hydrocolloid ulvan, which shows very high viscosity by undergoing a random coil to double helix transition while cooling [48]. The high viscosity of ulvan is one reason that hindered the high production of bioethanol from this species. The depolymerase produced by *Catenovulum*

spp. LP, showed high efficiency and high specificity to UP for monomer sugar production. During the enzymatic hydrolysis, the viscosity of 1.2% UP solution obviously declined from initially 1127 to 7.2 mPa·s within 95 min. Reducing sugar yield attained 50.2% in 6 h at the optimal conditions of pH 6.0 and 35°C [49]. Compared to the commercial enzymes, this depolymerase might bring promising prospects for bioethanol production from *U. prolifera* biomass.

Chaetomorpha linum, one of green macroalgae species, has rigid epidermal cell walls consisting of highly crystalline cellulose [35]. The cellulose content (35-40%) of *C. linum* is higher than that of other algae, and similar to that of land-based biomass. For breaking down the cellulosic structure of *C. linum*, following five pretreatment methods have been employed: steam explosion (STEX), hydrothermal pretreatment (HTT), plasma-assisted pretreatment (PAP), wet oxidation (WO), and ball milling (BM) [50]. HTT and WO were performed with 4% *C. linum* at 200°C; *C. linum* (35%) was treated by STEX at 200-210°C for 5 min; the PAP treatment was performed with raw material (50%) for 20-60 min with 1% ozone gas flow rate of 0.01 L/s; and the BM experiment was carried out for 18 h at 180 rpm. WO, HTT, PAP, BM, and STEX resulted in glucan concentrations of 74, 60, 46, 38, 36 g/100 g dry matter, respectively. Using a SSF process with the commercial cellulase enzymes (Celluclast 1.5 L and Novozyme 188) and *S. cerevisiae* ATCC 96581 for ethanol fermentation, WO and BM showed the highest ethanol yield of 77.2% of the theoretical ethanol yield. However during WO, about 50% of the biomass (especially C5 sugars) was lost. The results suggested that physical pretreatment method like BM is already effective enough to break down the cellulosic structure of *C. linum*.

5.3 Brown Macroalgae

Conversion of *Sargassum spp.*, a brown seaweed species, was conducted by using dilute acid hydrolysis and SHF [51]. In terms of glucose and other reducing sugar yields, the optimal pretreatment condition was found to be (3.4-4.6%w/v H₂SO₄, 115°C and 1.50 h). The residue after pretreatment was hydrolyzed with cellulase (*Trichoderma reesei* ATCC 26921) and β -glucosidase, and then fermented by *S. cerevisiae* for 48 h. The ethanol conversion rate achieved 89%, which was obviously higher than the theoretical yield of 51% based on glucose as substrate. Since all glucose was consumed during fermentation, other sugar sources might be present in the hydrolysate.

Zostera marina is a source of natural antioxidants in the food and pharmaceutical industries. After washing, drying, grinding, and sieving, antioxidants (phenolic compounds) were extracted by using supercritical CO₂ from this brown alga. The contents of lignin, hemicellulose, and α -cellulose in the residues were 22.4%, 16.89%, and 27.39%, respectively. Because supercritical fluid extraction already loosen the lignin structure [40], the raffinate phase would either be directly used for SSF or hydrolyzed by enzyme/dilute acid. Under optimized conditions, a reducing sugar yield of 58.24 g/100 g dry-feed was reached by consecutive enzymatic and acid hydrolysis with a commercial cellulase (Cellic CTec2).

6 Prospect on the Utilization of Macroalgae for Biofuels Production

As an abundant and carbon-neutral renewable resource, macroalgae represent an unrealized feedstock that might expand existing bioethanol industries. Currently,

macroalgae are gaining more attention because of their plant-like characteristics, fast growth rate, superior productivity, lower energy inputs, and no land requirements. In terms of availability, the annual production of brown algae was 9.72 million tons (dry weight) in 2004, representing the largest seaweed source; and red algae produced 3.99 million tons of dry biomass at the second place [52]. Another advantage of macroalgae is high content of carbohydrates (cellulose and hemicellulose) and the paucity of lignin resistant [53]. Although, the notion of macroalgae-based bioethanol production is environmentally better than the fossil fuels, but still suffer from low cost effectiveness and technological barriers [14]. The industrial-scale technologies for seaweed conversion still require significant basic research and development.

Since the price of a final product is directly related to the cost of feedstock, the price of seaweed is an important factor in the economics of a bioethanol process. The estimated macroalgal bioethanol production cost is ca. \$0.50/kg (dw) (\$0.16 from corn) [54]. Algae production cost is connected with the available technologies for cultivation, harvesting, and processing. Although macroalgae can be cultivated both naturally and artificially, approximately 90% of total feedstock were currently harvested from cultivated sources [21]. The production cost will decrease with the increase of macroalgae yield per unit area. To date, there are limited numbers of economic assessments on seaweed-based bioethanol technologies, as the research just started. Although it is impossible to make full-scale and periodically life cycle assessment right now, the processing technologies for bioethanol production from macroalgae should be estimated not only from the viewpoints of technical feasibility and economic efficiency, but also from the environmentally friendly point and the recycling of byproducts.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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