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Bioethanol production from the acid hydrolysate of the carrageenophyte *Kappaphycus alvarezii* (cottonii)

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Abstract Seaweed resources can be used as raw materials to produce bioethanol, a renewable biofuel, to overcome fossil fuel depletion and environmental problems. Red seaweeds possess high amount of bioethanol-producible carbohydrates. Among 55 species tested, the carrageenophyte *Kappaphycus alvarezii* (also known as cottonii) was selected as the best resource for bioethanol production. This species is one of the most abundant and easily cultured red seaweeds. The main components of carrageenan are D-galactose-4-sulfate and 3,6-anhydro-D-galactose-2-sulfate, which are potentially fermentable D-typed carbohydrates. The seaweed powder was hydrolyzed with 0.2 M sulfuric acid and fermented with brewer's yeast. The ethanol yield from the *K. alvarezii* hydrolysate was 0.21 g g^{-1}-galactose, which corresponded to a 41% theoretical yield. It revealed a relative ethanol production of 66% comparing to that of pure galactose.

Keywords Acid hydrolysis · Bioethanol · Carrageenophyte · Cottonii · *Kappaphycus alvarezii*

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Introduction

Bioethanol has attracted attention as an alternative renewable fuel to replace our dependency on fossil fuel. Unlike fossil fuels, bioethanol is a renewable energy source produced through sugar fermentation (Chandel et al. 2007) of terrestrial plants such as corn and sugar cane. However, future limitations on terrestrial resources will require that we shift our focus from terrestrial-based resources to marine-based resources. Seaweed has been researched as a potential resource for bioethanol production (Horn et al. 2000; Wi et al. 2009; Goh and Lee 2010). The potential volume of ethanol producible from seaweed was estimated to be 23,400 L ha^{-1} y^{-1} (Adams et al. 2009). Converting seaweed to bioethanol rather than using terrestrial plant resources has some advantages, i.e., no negative impact on food security, rich in sugar content, lower lignin content than lignocellulosics, and higher mass production (Adams et al. 2009; Wi et al. 2009). The most common polysaccharides from seaweeds are galactans (agar and carrageenan) from red seaweeds and alginate from brown seaweeds (de Ruiter and Rudolph 1997). Galactans consist entirely of galactose and 3,6-anhydrogalactose. The substitution pattern of the sulfate groups and the amount of 3,6-anhydrogalactose vary in different genera, influenced by ecological conditions (Percival 1979; de Ruiter and Rudolph 1997; Jol et al. 1999). Carrageenan is mostly obtained from *Eucheuma*, *Chondrus*, and *Gigartina* (Percival 1979). There are three basic molecular types of carrageenans: kappa-carrageenan from *Kappaphycus alvarezii* (also known as cottonii), iota-carrageenan from *Eucheuma spinosum*, and lambda-carrageenan from *Gigartina pistillata* or *Chondrus crispus* (Jol et al. 1999; Pereira et al. 2009). McHugh (2003) estimated that 168,400 t y^{-1} of carrageenan could be harvested as a main polysaccharide resource from seaweed around the world. The rhodophyte *K. alvarezii* is one of the

largest tropical carrageenophytes and one of the most abundant biomass resources produced by aquaculture. It is very fast-growing, known to double its biomass in 15 days, and being cultivated between about 20° north and south of the equator, mostly in Indonesia and Philippines (Neish 2008). Production of about 12–18 dry t ha^{-1} y^{-1} is fairly typical for well-tended family farms. In this study, we focused on *K. alvarezii*, which is the most promising candidate for bioethanol production because of its high amount of biomass and D-typed galactose. The seaweed powder was hydrolyzed to monosugars by sulfuric acid treatment and then fermented to produce ethanol using brewer's yeast.

Materials and methods

Seaweed material

Dried specimens of *Kappaphycus alvarezii* were collected mostly from Karimunjawa, Indonesia, during 2007 and 2010. The seaweed samples were rinsed in distilled water to eliminate salt and debris, and dried again to a constant weight at 60°C. The dried seaweed was finely ground into a powder using a coffee grinder for 5 min. To compare tissues collected at different locations, dried specimens were obtained from different local farmers who attended the First Indonesian Seaweed Forum and Exhibition, Makassar, in October 2008; the specimens were used directly without rinsing.

Acid hydrolysis For the main ethanol production, acid hydrolysis was conducted in 250-mL flasks. Ten grams of seaweed powder were hydrolyzed with 100 mL of 0.2 M H_2SO_4 in an autoclave at 130°C for 15 min. For optimizing parameters of acid hydrolysis, it was conducted in 100-mL flasks with 50 mL of 0.2 M H_2SO_4. After hydrolysis, the residue was separated from the liquid by filtration using glass fiber. The clean yellowish liquid after removing the residue was used for analyzing sugar content and for bioethanol production after adjusting to pH 5 with NaOH.

Fermentation Commercial brewer's yeast, freeze-dried *Saccharomyces cerevisiae* (Jenico, Seoul, South Korea), was directly used for the bioethanol fermentation. The basal medium consisted of 0.02% $(NH_4)_2SO_4$ and 0.006% NaH_2PO_4, adjusted to pH 5 (Prescott and Dun 1959). Fermentation broth was consisted of hydrolysate and basal medium with a ratio of 1:2. The fermentation was conducted with 3 mL broth in 8-mL screw cap vials during 24 h for optimization and scaled up to 120 mL in 200-mL reagent bottles with cap during 72 h for main ethanol production. The medium was incubated in a shaking incubator at 30°C with gentle shaking at 120 rpm. Samples of 0.5 mL for measuring sugars and ethanol content were collected at each time during fermentation.

Analysis of biochemicals, by-products, and ethanol From dry tissue samples, total carbohydrate was determined by the phenol-sulfuric acid method (Kochert 1978), using carrageenan as the standard. Total lipids were extracted with hexane and isopropanol (3:2) as the solvent (Radin 1981) and were quantified gravimetrically. The amount of soluble protein in the tissue was estimated according to the method of Lowry et al. (1951) after heating the tissue suspension to 100°C in 1 N NaOH for 2 h to obtain complete solubilization of the protein. Bovine serum albumin was used as the standard for protein determination. Ash content was determined by heating tissue samples at 575°C for 5 h. Moisture content was measured by drying tissues at 105°C for 18 h until they reached a stable weight. After acid hydrolysis, the amount of reducing sugar in the hydrolysate was determined using dinitrosalicylic acid (Chaplin 1986). Contents of monosaccharides (galactose and glucose) and by-products (levulinic acid and 5-hydroxy-methyl-furfural) were quantified by each standard curve using high-performance liquid chromatography with an Alltech IOA 1000 organic acid column (7.8 mm ID×30 cm), which was equipped with a refractive index detector and maintained at 60°C. The mobile phase was 2.5 mM sulfuric acid at a flow rate of 0.3 mL min^{-1}. Ethanol content was quantified using gas chromatography (Agilent Model 6890 N, USA) with a 2B-WAX column (Agilent Technologies, USA). The injection volume was 2 μL with an inlet split ratio of 30:1. The initial and maximum oven temperatures were 35°C and 250°C, respectively. Ethanol yield (Y) was calculated using the following equation: $Y = [EtOH]_{max}/[Sugar]_{ini}$, where $[EtOH]_{max}$ = maximum ethanol concentration achieved during fermentation (g L^{-1}), $[Sugar]_{ini}$ = sum of the initial galactose and glucose concentrations at onset of fermentation (g L^{-1}). Percent theoretical yield (Y%) was calculated as: $Y\% = (Y/0.51) \times 100$, where 0.51=theoretical maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g g^{-1}). Relative ethanol production (%) was calculated by dividing the ethanol yield of hydrolysate by the ethanol yield of pure galactose with $[Sugar]_{ini}$ concentration, and multiplied by 100.

Activated charcoal treatment To remove fermentation inhibitors from the H_2SO_4 hydrolysate, 5% (w/v) activated charcoal was added to the hydrolysate and shaken at 30°C for 30 min with a 130 rpm shaking rate (Miyafuji et al. 2003). After removing the charcoal by filtration, the clean hydrolysate was used to determine sugar and inhibitor contents and then fermented for ethanol production. The removal rate (%) by the charcoal was calculated as a relative rate: $[(B-A)/B] \times 100$, where B is amount of each

compound before treatment, and *A* is amount of each compound after treatment.

Results

Carbohydrate was the main component in various seaweed species and reached a maximum of approximately 64% (*w*/*w*) as a dry weight basis in our preliminary experiments. *K. alvarezii*, a major carrageenan producer, possessed the highest amount of carbohydrate among the 54 seaweed species tested (data not shown). We conducted sulfuric acid hydrolysis using *K. alvarezii* to maximize productivity of galactose content and ethanol fermentation from the hydrolysate. The process was optimized by applying different H_2SO_4 concentrations (0 to 1 M), hydrolysis times (0 to 30 min), amounts of seaweed materials (0 to 133 g L^{-1}), and inoculum amounts of *S. cerevisiae* (0 to 6.7 g L^{-1}). Among different H_2SO_4 concentrations, the highest production of reducing sugar, galactose, and ethanol was obtained with 0.2 M H_2SO_4 under the standard conditions of hydrolysis at 130°C for 15 min and fermentation with 3.3 g L^{-1} of *S. cerevisiae* for 24 h (Fig. 1a). It yielded 30.5 g L^{-1} reducing sugar, 25.6 g L^{-1} galactose, and 1.31 g L^{-1} ethanol from the acid hydrolysis. At lower and higher concentrations than 0.2 M H_2SO_4, the reducing sugar, galactose, and ethanol concentrations decreased. The hydrolysis time with 0.2 M H_2SO_4 was optimal at 15 min under standard conditions of hydrolysis at 130°C (Fig. 1b). Increasing the hydrolysis time to longer than 15 min resulted in decreased production of reducing sugars, galactose, and ethanol, suggesting that higher H_2SO_4 concentrations and longer reaction times might degrade the sugar compounds and produce more by-product inhibitors such as 5-hydroxy-methyl-furfural and levulinic acid. The optimal amount of *K. alvarezii* powder was 100 g L^{-1} under standard conditions of hydrolysis at 130°C for 15 min with 0.2 M H_2SO_4 (Fig. 1c). Figure 1d shows the effect of different *S. cerevisiae* inoculum amounts on ethanol production. The highest ethanol production was obtained with an inoculum amount of 3.3 g L^{-1}. Thus, the optimum conditions were concluded at 130°C hydrolysis for 15 min with 0.2 M H_2SO_4, 100 g L^{-1} seaweed and 3.3 g L^{-1} yeast, same as the standard conditions.

We measured the time course of main ethanol fermentation from the 120 mL *K. alvarezii* hydrolysate (Fig. 2). The *K. alvarezii* hydrolysate containing 50.0 g L^{-1} reducing sugar, 20.4 g L^{-1} galactose, and 0.8 g L^{-1} glucose was fermented in

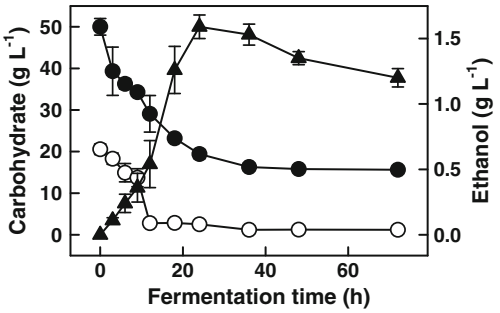

Fig. 2 Time course of sugar consumption and ethanol production during fermentation of a *K. alvarezii* hydrolysate. Amounts of reducing sugar (*black circle*), galactose (*white circle*), and ethanol production (*black triangle*) were measured. Fermentation was conducted in broth of hydrolysate and basal medium (1:2). Values represent the mean±SD ($n \geq 3$)

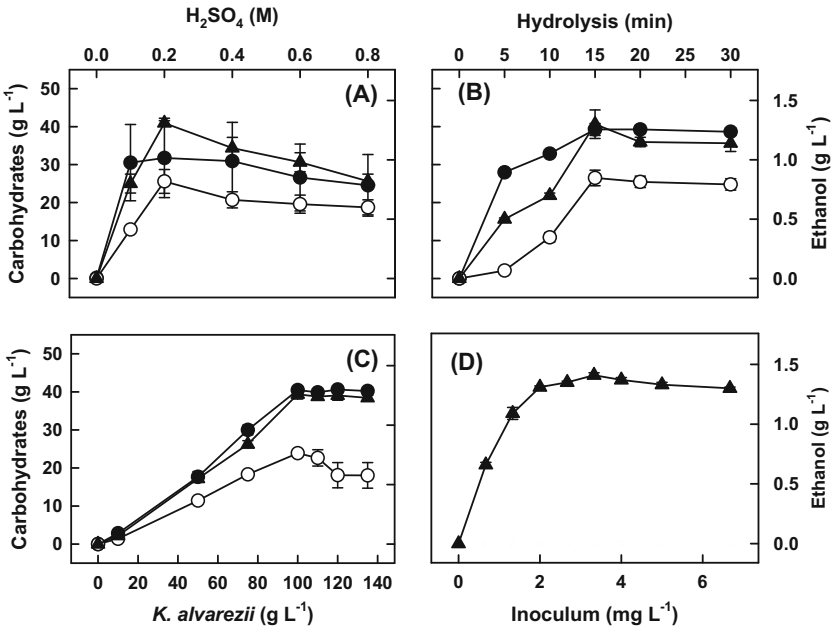

Fig. 1 Determining various parameters to optimize ethanol production conditions of a *K. alvarezii* hydrolysate. **a** effect of H_2SO_4 concentration on seaweed hydrolysis. **b** hydrolysis time with 0.2 M H_2SO_4. **c** amount of seaweed used for H_2SO_4 hydrolysis. **d** amount of yeast inoculum used for fermentation. Amounts of reducing sugar (*black circle*) and galactose (*white circle*) released after H_2SO_4 hydrolysis were measured, and fermentation was conducted in broth of hydrolysate and basal medium (1:2) to determine ethanol production (*black up-pointing triangle*). Values represent the mean±SD ($n \geq 3$)

Table 1 Comparison of fermentation inhibitors, sugars, and ethanol yield from the H_2SO_4 hydrolysate before and after activated charcoal treatment

	Before treatment	After treatment	Removal rate (%)
5-Hydroxy-methyl-furfural (g L^{-1})	4.67±0.96	1.14±0.02	75.6
Levulinic acid (g L^{-1})	1.07±0.02	0.60±0.04	43.9
Galactose (g L^{-1})	22.39±0.70	12.73±0.75	43.1
Glucose (g L^{-1})	0.78±0.02	0.45±0.09	42.3
Ethanol yield (g L^{-1})	1.30±0.35	1.70±0.10	N/A

The charcoal (5%, *w/v*) treatment was conducted at 30°C for 30 min on a shaker. Values represent the mean±SD ($n \geq 3$)

broth, and produced 1.5 g L^{-1} of ethanol in 24 h. The broth consisted of hydrolysate and basal medium with a ratio of 1:2. The ethanol production rate in the early phase of the culture was relatively slow but rapidly increased after 12 h and reached a maximum after 24 h of fermentation. The galactose was exhausted after 24 h. Sugar consumption was consistent with the ethanol production time, and ethanol productivity was 0.063 g L^{-1} h^{-1}, which was lower than that of a reference (0.095 g L^{-1} h^{-1}) containing pure galactose of the same concentration. This result indicated that some inhibitors or toxic compounds against fermentation might have been present and decreased ethanol production. The ethanol yield during fermentation of the *K. alvarezii* hydrolysate was 0.21 g g^{-1} of the sum of galactose and glucose, which corresponded to a 41% theoretical yield. This was a relatively lower result compared to the ethanol yield of 0.32 g g^{-1} of pure galactose, which corresponds to 63% of the theoretical yield in the reference. Thus, it shows a relative ethanol production of 66% comparing to the ethanol yield by pure galactose, namely 34% was inhibited in fermentation by hydrolysate by-products.

Activated charcoal was used to remove the fermentation inhibitors from the acid hydrolysate (Table 1). During H_2SO_4 hydrolysis of the *K. alvarezii*, fermentation inhibitor by-products, such as levulinic acid (1.07 g L^{-1}) and 5-hydroxy-methyl-furfural (4.67 g L^{-1}), were detected in the hydrolysate. The 5-hydroxy-methyl-furfural was mostly removed (75.6%) by charcoal, whereas 43% of the levulinic acid and sugars also were removed by charcoal. The charcoal treatment was not specific to the inhibitors, even though the ethanol yield increased only slightly.

We compared the biochemical composition (carbohydrate, protein, lipid, and ash) and moisture content of seaweed samples collected at different locations in Indonesia (Table 2). A relatively high amount of total carbohydrate was detected in tissues collected at Papua and Lombok locations, whereas the lowest amount was observed in samples from the Madura region. High amounts of ash and moisture were detected in tissues from Madura and Kupang. Samples with more moisture content showed increased amounts of ash. Relatively high amounts of reducing sugars, galactose, and glucose were detected in tissue hydrolysates from most regions, except samples from Kupang and Madura. Generally, more 5-hydroxy-methyl-furfural and levulinic acid by-products are produced from tissue samples with greater sugar content. Relatively lower amounts of total carbohydrate from tissues and lower concentrations of reducing sugars, galactose, and

Table 2 Biochemical composition and ethanol yield from hydrolysate of different *K. alvarezii* tissues collected from various areas in Indonesia

Collection area	Relative% in dry tissue (*w/w*)					Amount (g L^{-1}) in hydrolysate				Ethanol yield (g L^{-1}) from hydrolysate
	Carbohydrate	Protein	Lipid	Ash	Moisture	Reducing sugar	Galactose	5-Hydroxy-methyl-furfural	Levulinic acid	
Bali	64.4±3.2	3.1±0.1	0.7±0.0	14.3±0.7	9.2±0.4	39.6±2.0	24.3±1.0	4.1±0.5	0.9±0.1	1.4±0.2
Bone-A	72.4±17.8	3.2±0.1	0.7±0.0	13.4±0.1	9.8±0.4	43.6±2.1	25.7±1.8	5.2±0.4	0.9±0.0	1.7±0.2
Bone-B	55.4±8.0	2.3±0.1	0.9±0.0	15.9±0.9	10.9±0.4	40.4±3.7	22.7±1.1	4.7±0.2	0.7±0.1	1.1±0.1
Karimunjawa	67.8±10.4	3.6±0.9	0.6±0.0	18.4±0.5	16.7±0.7	38.9±2.2	18.4±1.3	3.9±0.4	0.9±0.1	1.1±0.1
Kupang	64.1±12.9	5.4±0.1	0.7±0.0	19.5±0.1	17.1±0.2	28.6±3.7	15.7±0.5	2.9±0.2	0.6±0.0	1.1±0.1
Lampung-A	69.8±7.3	3.6±0.2	0.6±0.0	12.8±0.4	11.3±0.5	39.2±2.0	22.8±1.1	4.7±0.4	1.0±0.2	1.4±0.2
Lampung-B	73.2±6.6	3.7±0.2	0.7±0.0	12.6±0.1	14.3±0.6	40.7±2.0	22.7±0.9	5.1±0.4	1.1±0.2	1.3±0.2
Lombok	76.2±1.5	2.4±0.4	0.8±0.0	18.1±0.6	14.7±0.2	40.2±2.4	24.6±1.4	2.9±0.2	1.3±0.1	1.7±0.2
Madura	35.6±9.5	3.2±0.5	0.4±0.1	19.4±0.3	21.7±0.5	29.1±2.1	15.2±0.8	3.3±0.2	0.8±0.1	1.1±0.1
Papua	78.3±11.5	3.4±0.8	0.6±0.1	17.7±1.2	13.3±0.4	40.4±3.7	24.7±0.9	4.7±0.4	1.2±0.1	1.9±0.2
Takalar	73.9±3.1	4.5±1.0	0.9±0.0	16.6±0.6	15.6±0.4	44.8±2.1	25.3±1.4	4.8±0.2	1.3±0.2	1.6±0.2

All values were calculated against the dry weight of tissues after removing moisture content. Lampung-A and -B represent green and white types, respectively, from the Lampung area. Bone-A and -B represent brown and white types, respectively, from the Bone area. Tissue powders (10%) were hydrolyzed in 0.2 M H_2SO_4. Values represent the mean±SD ($n \geq 3$)

glucose from the acid hydolysates of specimens from Kupang and Maura may be related to higher moisture content compared to the other specimens.

Discussion

We selected *K. alvarezii* as one of the most promising candidates for bioethanol production from seaweed resources, based on carbohydrate content and its capacity to produce ethanol. The carrageenan producer possessed the highest amount of carbohydrate among the 54 seaweed species tested in our preliminary experiments. Chapman and Chapman (1980) reported that *K. alvarezii* contains more than 50% carbohydrate on a carrageenan dry-weight basis. Carrageenan is a polymer of α-1,3 linked carrabiose (β-D-galactose-4-sulfate-β-1,4-3,6-anhydro-D-galactose-2-sulfate) substituted with sulfate groups, whereas agar consists of β-D-galactose-β-1,4-3,6-anhydro-L-galactose substituted with methyl ethers and a pyruvate group. Carrageenan has an advantage in that it is composed of D-galactose as a basic unit, compared to agar, which contains both D- and L-galactose. L-type sugars are generally non-usable during fermentation and may become reaction inhibitors; thus, one more step is needed to convert L-type sugars to D-type sugars (Yun et al. 2011). From the polysaccharides present in seaweed tissues, it is necessary to obtain fermentable sugars by hydrolysis to produce bioethanol. Acid hydrolysis is an inexpensive process than enzymatic hydrolysis from an economic point of view. The disadvantage of acid hydrolysis is that it is a complex reaction, which results in the production of by-product compounds that can inhibit ethanol-producing yeast and bacteria (Larsson et al. 1999; Klinke et al. 2004). The concentrations of 5-hydroxy-methyl-furfural and levulinic acid in the 0.2 M H_2SO_4 hydrolysate were approximately 4.67 and 1.07 g L^{-1}, respectively (Table 1). According to Mussatto and Roberto (2004), four factors can minimize the toxification of by-products in fermentation: minimizing the formation of inhibitors during hydrolysis, removal of inhibitors before fermentation, development of microbial species able to resist inhibitors, and conversion of toxic compounds into products that result in no inhibition. In this study, we could not demonstrate the perfect processes to minimize and remove inhibitors because of the co-occurrence of fermentable sugars and by-products during hydrolysis and the non-specific absorption of inhibitors and sugars by the common adsorbent charcoal. Even though carbon adsorption has numerous applications in removing pollutants from air or water streams (Ahmedna et al. 2000), the activated charcoal is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for adsorption or chemical reaction. To increase specificity for binding an inhibitor, a specific ligand must be developed using affinity chromatography to remove inhibitors.

A sufficient and inexpensive biomass is necessary to produce ethanol economically, and seaweed culture is able to fulfill this demand. Approximately 7.5–8 million tons of wet seaweed is produced annually worldwide, either by natural wild seaweed collection or by culture (McHugh 2003). Seaweed has the ability to grow at a fast rate and yield high biomass because it requires less energy to produce supporting tissues than land plants, and nutrients are taken up over their entire surface of the plant (John et al. 2011). Among seaweed species, the carrageenophyte *K. alvarezii* is now produced in mass scale by aquaculture simply using asexual tissue propagation in tropical areas of Indonesia and Philippines. Indonesia produced at least 1.5 million tons of dry cottonii from 1.1 million ha of farming area in 2009 (Pambudi et al. 2010). From Table 2, we found that cottonii samples with more moisture content generally showed more amounts of ash and lower amounts of total carbohydrate and sugars. It is assumed that the remained moisture of seawater in tissues, not completely dried, contributed to ash amount and encouraged microorganisms in decaying carbohydrate and sugars during dry, transportation and storage of the tissues. Thus, cottonii tissues are suggested to be dried quickly and completely as much as possible. Carrageenan contains the basic unit of D-typed galactose, which is easily fermentable by yeast and bacteria. For these reasons, the carrageenophyte *K. alvarezii* might be one of the best candidate bio-resources for bioenergy production when the ethanol yield is increased after removing fermentation inhibitors.

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