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Bioethanol Production from *Hydrodictyon reticulatum* by Fed-Batch Fermentation Using *Saccharomyces cerevisiae* KCTC7017

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The aim of this study was to develop a potential process for bioethanol production from *Hydrodictyon reticulatum* (HR), a filamentous freshwater alga, using *Saccharomyces cerevisiae* (KCTC7017). From the sugar solutions prepared by the four different hydrolysis methods, bioethanol production ranged from 11.0 g/100 g dried material (acid hydrolysis) to 22.3 g/ 100 g dried material (enzymatic hydrolysis, EH). Bioethanol was fermented from a highly concentrated sugar solution obtained by a decompression-mediated (vacuum) enrichment method (VE). As the results, ethanol was more efficiently produced from HR when sugar solutions were concentrated by VE following EH (EH/VE). Using multiple feeding of the sugar solution prepared by EH/VE from HR, ethanol reached up to a concentration of 54.3 g/l, corresponding to 24.9 g/100 g dried material, which attained the economic level of product concentration (approximately 5%). The results indicate that by using HR, it is feasible to establish a bioethanol production process, which is effective for using microalgae as the raw material for ethanol production.

Keywords: Bioethanol, fermentation, freshwater algae, *Hydrodictyon reticulatum,* hydrolysis

Introduction

Owing to the depletion of fossil fuels, the rising demand for energy, and global warming caused by carbon dioxide emission, there are increasing interest and demand for bioenergy [1]. Bioenergy can be grouped into liquid fuels, such as bioethanol and biodiesel, and gas fuels like methane and hydrogen. However, if edible food crops for human and livestock are used as raw materials, it is most likely that an ethical problem will emerge due to the rise of food prices triggered by competitive demands for food [2, 3]. Therefore, it is more desirable to produce bioethanol using non-food biomass.

There are some advantages for lignocellulosic biomass such as the easiness to obtain the biomass in a large scale at a lower cost, and better option for addressing the food and

energy security and environmental concerns. However, there are many difficulties in the bioethanol production process, which requires not only high energy consumption, but also a relatively high cost of production at present. In view of the aforementioned issues, currently no commercial-scale cellulosic ethanol plants are largely in operation [4]. As an alternative way to solve this problem, there is increasing interest in bioenergy production from algae [5–8].

There are many advantages for algal biomass because algae develop and grow quickly, which leads to high biomass productivity [9]. Many algal species display high productivity for specifically effective components such as carbohydrates and lipids [10–13]. Moreover, algae can produce large amounts of metabolites in a relatively short period, such as carotenoids, lutein, chlorophyll *a* and *b*, and other pigments [14]. Algal species have soft tissues with

low lignin content, which makes the bioenergy production process simple and cheap, as a non-food raw material. They do not cause problems like food price hikes. In addition, because it is known that they utilize CO_2 more efficiently than higher plants and can utilize nutrients like nitrogen and phosphorus in wastewater [15], algae can be subsidiarily used for reduction of CO_2 in the air and purification of water. Moreover, algae grow rapidly and can be easily grown in various aquatic environments such as fresh water, saline water, or municipal wastewater [4]. Thus, algae can provide enough supplies to meet ethanol production demands compared with other feedstock.

According to other reports, macroalgae are mostly being used for bioethanol production among other algae at present [5, 16–20]. In this case, owing to the relatively high content of non-fermentable monosaccharides, macroalgae tend to have less effective bioethanol production. On the other hand, as with microalgae, unicellular algae are used as materials for bioethanol in most cases, in which it is very difficult to harvest them economically [21, 22].

To solve these problems, we intended to establish a method for bioethanol production from *Hydrodictyon reticulatum* (HR). HR is a filamentous freshwater microalga living anywhere in the world and it grows very rapidly, which causes algal bloom in some places [8]. HR shows a net-like structure, in which 5–6 cells are fused together to make pentagonal or hexagonal patterns, and it lives as a colony. Therefore, it is easy to watch it grow with the naked eye and to harvest it [23]. HR is multinucleated, its cell wall is composed of cellulose and hemicelluloses, and polysaccharides and starches are accumulated as storage carbohydrates [24–26]. HR is hydrolyzed very easily and its hydrolyzed carbohydrates are mainly glucose and mannose, which are especially easy to be fermented by microorganisms [27, 28]. The previous reports suggest that HR is a useful biomass for bioethanol production at a low production cost. Therefore, we carried out various experiments to develop a new process to efficiently produce bioethanol from HR.

Materials and Methods

HR Algal Culture

HR was collected from Gab-Chun stream located in Yuseong-Gu, Daejeon-Si. Nutrients were added to a container (1.5 m^2) filled with 100 L of degased tap water and the basic composition of the culture medium was 20 mg $Ca(NO_3)_2$/l, 12.4 mg K_2HPO_4/l, 25 mg $MgSO_4$/l, 215.9 mg $NaHCO_3$/l, 2.25 mg EDTAFeNa/l, 2.25 mg $EDTANa_2$/l, 2.48 mg H_3BO_3/l, 1.39 mg $MnCl_2$/l, 1 mg $(NH_4)_6Mo_7O_{24}$/l,

and 57 mg Na_2SiO_3/l. Forty-five grams of HR, calculated as dewatered fresh weight biomass, was added and cultivated in the greenhouse for 8 days, and the same amount of nutrients as initially added was fed 3 days after the onset of cultivation. Cultivating conditions in the greenhouse were 25–30°C (day)/20–25°C (night) and a 14 h photoperiod with light intensity of 90−500 μmol/m^2/s. At the end of cultivation, HR was harvested using a steel sieve (40 mesh), passed through a spin extractor (WS-6600; Hanil, Korea), and then transferred to a drier for 2–4 days at 45°C. Dewatered fresh weight 265 g of HR (dried HR 39.2 g) was pulverized and then stored at 4°C until used. The HR was analyzed as described in the Korea Food Standard Codex. The moisture content of HR was 3.8% and contained 10.3% protein, 15.5% ash, 2.5% lipid, and 67.9% carbohydrate (w/w).

Hydrolysis Methods

Enzymatic hydrolysis (EH), acid hydrolysis (AH), and combined acid and enzymatic hydrolysis (AEH) methods were performed following a previous paper [27]. For EH of HR, two enzymes were used. Celluclast Conc BG (CC100133) was purchased from Novozymes (Denmark) in solid form with an activity of 385 FPU/g, and 1 g powder was dissolved in 5 ml of 1 M citrate buffer (pH 4.8) (64 FPU/ml; E1). Cellobiase (C6105: E2) from *Aspergillus niger* was purchased from Sigma–Aldrich Co., and its β-glucosidase activity was 311 U/ml. The EH method was performed by using 6 g of HR, 1.2 ml of E1, and 0.24 ml of E2 in 100 ml of 50 mM Na-citrate buffer at 50°C, 150 rpm for 48 h. The AH method was performed by using 1 g of HR, and 1.5 ml of 72% H_2SO_4 at 60°C for 1 h, and then 23.5 ml of distilled water was added for the hydrolysis reaction at 120°C for 1 h. For the AEH method, 40 ml of 2% HCl was added to 10 g of HR, and incubated at 121°C for 20 mins. The pH of the liquid was adjusted to 4.8–5.0 by adding 10 N NaOH, and then 47.76 ml of 0.1 M Na-citrate buffer, 2 ml of E1, and 0.4 ml of E2 were added to the liquid and incubated at 50°C, 150 rpm for 24 h in the shaking incubator. For the hydrolysis of HR by combined heating-water and enzyme hydrolysis (HEH), 10 g of HR and 40 ml of distilled water were added to the reactor and subjected to heating-water treatment for 30 min at 150°C. Then, 47.76 ml of 0.1 M Na-citrate buffer (pH 4.8), 2 ml of E1, and 0.4 ml of E2 were added to the pre-treated liquid in a sterile condition and incubated at 50°C,150 rpm for 24 h in a shaking incubator.

Preparation of Concentrated Sugar Solution

Two methods were employed to obtain highly concentrated sugar solution (HCSS). First, in a decompression-mediated (vacuum) enrichment method (VE), water was removed by lowering the pressure from 1,005 mbar to 40 mbar at 45°C. The second method, membrane filtration-mediated concentration (MF), was performed at room temperature (25°C). To remove impurities including proteins other than sugars, using an ultrafiltration membrane (molecular weight cutoff: 10 kDa, pore size: 2 nm, 2 inch × 10 inch; Deerfos Membranes, Korea), the first filtration was performed at 4

bar on HR hydrolysates that were produced by the EH and AEH methods with glucose concentrations of 25 g/l and 29 g/l, respectively. The obtained solutions were subjected to the second filtration using a reverse osmosis membrane (pore size: 0.2 nm; 1.8 inch × 12 inch; glucose removal efficiency: 99.9%; Woong Jin Chemical, Korea). Surface areas for the ultrafiltration membrane and reverse osmosis membrane module were 1 m^2.

Ethanol Fermentation

Microorganisms and general fermentation. *Saccharomyces cerevisiae* (KCTC7017), *Zymomonas mobilis* (KCTC1534), and *S. cerevisiae* (KCTC7928) were used for ethanol fermentation. The seed cultures were prepared by incubating these strains at 30°C, 150 rpm for 24 h in media containing 3.0 g of yeast extract (YE)/l, 3.0 g of malt extract/l, 5.0 g of peptone/l, and 10 g of dextrose/l. The optical density of each culture was adjusted to 1.0 at 600 nm. The pre-culture, at a level of 10%, was inoculated to HR hydrolysate supplemented with YE and peptone at concentrations of 20 g/l and 10 g/l, respectively. Nitrogen gas was injected to the culture medium to remove oxygen, using a one-way air valve. Fermentation was carried out in 50 ml of medium at 30°C, 150 rpm for 48 h.

Fed-batch fermentation. A HCSS (240 g reducing sugar/l) obtained by using EH and VE (EH/VE) was used for fed-batch fermentation. The fed-batch fermentation was performed in a 5-L jar fermentor with an initial working volume of 1 L using HCSS supplemented with 20 g of YE/l and 10 g of peptone/l. The fermentation was initiated by adding pre-culture (strain KCTC7017) at a level of 5%. Nitrogen gas was purged into the reactor to keep the level of dissolved oxygen lower than 0.5 ppm. The temperature and agitation speed were maintained at 30°C and 150 rpm for 120 h. To maintain the reducing sugar (RS) concentration in the medium, 259, 326, and 410 ml of the HCSS (240 g RS/l) were fed to the culture medium at 16, 24, and 32 h after the onset of cultivation, respectively. The sample was taken out at the specified time points to analyze the D-glucose, ethanol, and RS.

Analytical Methods

The levels of RS and D-glucose in the culture broths as well as the yield were estimated and calculated using previously described methods [28]. The supernatants were diluted to 0.01−0.12 g ethanol/l for analysis. The ethanol concentration was estimated using an enzymatic kit (Megazyme International Ireland Ltd., Ireland) according to the manufacturer's instructions. Hydroxy-methylfurfural (HMF) and furfural were analyzed by HPLC (Waters 996) equipped with a diode array detector and a 250 mm × 4.6 mm Sphaerisorb C_{18} column (particle size of 5 μm) at a flow rate of 0.6 ml/min. The solvents constituting the mobile phase were 100% water (solvent A) and 100% methanol using the following gradient program: 0−10 min, linear gradient solvent system of 2%−25% B; 10−15 min, linear gradient solvent system of 25%−60% B; 15−20 min, linear gradient solvent system of 60%−100% B; 20−30 min, 100% B. [29].

Results and Discussion

Effects of Substrate Concentrations and Microorganisms on Ethanol Production

According to previous reports, *S. cerevisiae*, *Escherichia coli*, and *Z. mobilis* are mainly used for ethanol fermentation from algal biomass [4, 7, 21, 30, 31]. Our study investigated and compared the ethanol yield among three microorganisms widely used in industry; namely, KCTC7017 (*S. cerevisiae*), KCTC1534 (*Z. mobilis*), and KCTC 7928 (*S. cerevisiae*). The concentrations of initial RS and ethanol are presented in Fig. 1. When HR hydrolysate obtained by EH was concentrated by using a rotary evaporator and supplied at various concentrations through dilution with distilled water, there was little difference in ethanol concentration among the different microorganisms at 25 g RS/l and 75 g RS/l. The strain KCTC7017 showed higher ethanol yield than the others; it produced 10.5, 21.1, and 31.3 g/l of ethanol at 25, 50, and 75 g/l of RS, respectively, corresponding to approximately 86% of RS consumption and 95% of ethanol yield (data not shown). The results indicated that strain KCTC7017 reached higher RS conversion than the other strains especially when treated at high substrate concentrations. Therefore, only KCTC7017 was used for fermentation in subsequent experiments.

Effects of Different Hydrolysis Methods on Ethanol Production

To produce ethanol from biomass on the industrial scale, the sugar solutions should be prepared at low cost, with
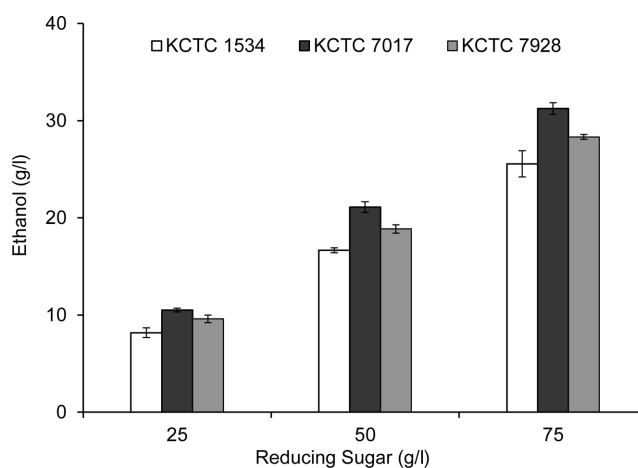

Fig. 1. Effects of different microorganisms (*Z. mobilis* KTC1534, and *S. cerevisiae* KCTC7017 and KCTC7928) on ethanol fermentation from different concentrations of *H. reticulatum* hydrolysate.
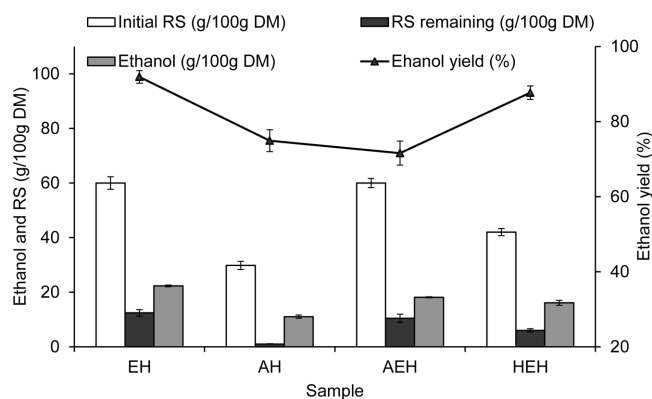
Fig. 2. Effects of different hydrolysis methods on ethanol production.
EH: enzymatic hydrolysis; AH: acid hydrolysis; AEH: combined acid and enzymatic hydrolysis; HEH: combined heating-water and enzymatic hydrolysis. Ethanol yield (%) = ethanol/(consumed RS × 0.51) × 100.

high sugar content with high fermentability, and the level of fermentation inhibitors kept at low concentration. In an effort to find the most efficient hydrolysis method for ethanol production, the ethanol yield was investigated using sugar solutions generated by the four different hydrolysis methods, which were well established previously and optimized for HR biomass [27]. By using HR hydrolysate obtained from EH, AH, AEH, and HEH, ethanol fermentation reached 22.3, 11.0, 18.1, and 16.1 g ethanol/100 g DM, corresponding to ethanol yields of 91.9%, 74.9%, 71.6%, and 87.7%, respectively (Fig. 2). The initial RS concentrations of EH and AEH hydrolysates were approximately 60 g/100 g DM. In comparison, the initial RS concentration of the AH and HEH hydrolysates were 29.8 and 42 g/100 g DM, respectively. Considering the initial RS concentration and ethanol yield, the EH method was chosen as the best saccharification method for HR-derived ethanol production. In several previous papers, enzymatic hydrolysis methods were reported: *L. japonica* and *Sargassum fulvellum* were hydrolyzed by using Celluclast

Table 1. Effects of different hydrolysis methods on hydroxymethylfurfural (HMF) and furfural concentrations.

Saccharification	HMF (g/l)	Furfural (g/l)
EH	0.012 ± 0.00	0.013 ± 0.003
AH	0.272 ± 0.02	0.057 ± 0.005
AEH	0.362 ± 0.03	0.065 ± 0.004
HEH	0.016 ± 0.00	0.029 ± 0.006

EH: enzymatic hydrolysis; AH: acid hydrolysis; AEH: combined acid and enzymatic hydrolysis; HEH: combined heating-water and enzymatic hydrolysis.

1.5L, Viscozyme L, Novoprime 959, Novoprime 969, or AMG 300L (Novozymes A/S, Denmark) at a level of 0.01 g/g dried biomass [21]; *Ulva pertusa* Kjellman, *Alaria crassifolia* Kjellman, and *Gelidium elegans* Kuetzing were hydrolyzed by Meicelase at a level of 0.017 g enzyme/g biomass [13]; For *Chlamydomonas reinhardtii*, a mixture of α-amylase (Termamyl 120L, 0.0001%) and amyloglucosidase (AMG 300L, 0.2%) were used [2]. In our study, cellulase and β-glucosidase were used for enzymatic hydrolysis of HR at the level of 0.04 g of Celluclast Conc BG (CC100133) and 0.04 ml/g biomass. Thus, the EH efficiency of HR in this study was similar those of the several macroalgae and microalgae previously reported [2, 13, 21].

Since the lower ethanol yields in the AH and AEH methods were suspected to be caused by generation of fermentation inhibitors, the concentrations of HMF and furfural were measured. HMF and furfural are known to be derived from hexose and pentose, respectively, during acid hydrolysis and they are typical fermentation inhibitors [32]. The data in Table 1 indicate that more than 0.27 g HMF/l and 0.05 g furfural/l were detected in the AH and AEH hydrolysates. Specifically, HMF was produced more than furfural, which is thought to be related to the fact that hexoses (glucose and mannose) are more abundant in HR. Although acid hydrolysis has been studied extensively due to an advantage of a low production cost in general, our data indicate that it is not suitable for production of sugar solution and ethanol fermentation from HR, especially when the concentrated method and fed-batch mode are applied for ethanol fermentation [33]. In contrast, the concentrations of the two inhibitors in EH and HEH hydrolysates were very low. Thus, EH is more suitable than AH for production of sugar solution and ethanol fermentation from HR.

Effects of Preparation Methods of Concentrated Sugar Solution on Ethanol Production

When HR hydrolysate with low sugar concentration was used for ethanol fermentation, the conversion of RS to bioethanol reached to over 90%, but the final ethanol concentration was low. Therefore, we sought a way to produce higher ethanol by using HCSS at the beginning of fermentation. The HCSSs were prepared with the combinations of EH/VE and EH/MF. The ethanol concentration, ethanol yield, and RS consumption produced from each concentrated hydrolysate were measured at initial RS concentrations of 80, 100, and 120 g/l (Table 2). As the results, ethanol yield from hydrolysate obtained by the EH/VE method was higher than that of EH/MF sugar solution; the ethanol

Table 2. Effect of different hydrolysis methods and sugar concentrations on ethanol yield and reducing sugar consumption rate.
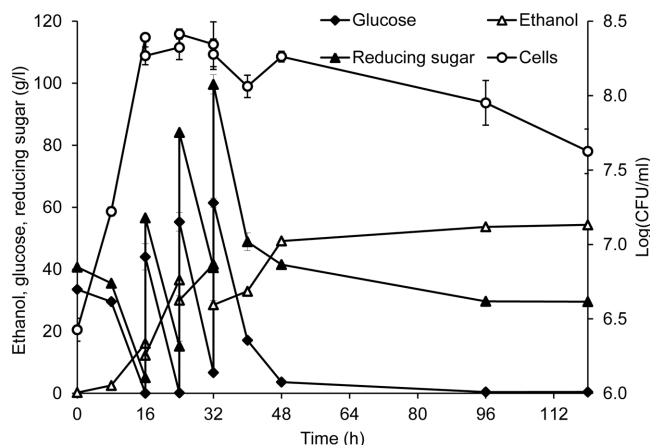
	Initial RS (g/l)	80	100	120
EH/VE	RS consumption (%)	78.5	78.8	14.9
	Consumed RS (g/l)	62.8	78.8	17.9
	Ethanol (g/l)	28.8	35.0	8.9
	Ethanol yield (%)	89.9	87.1	98.0
EH/MF	RS consumption (%)	86.3	85.1	85.2
	Consumed RS (g/l)	69.0	85.1	102.2
	Ethanol (g/l)	28.9	31.3	35.0
	Ethanol yield (%)	82.1	72.1	67.1

RS: reducing sugar; EH/VE: enzymatic hydrolysis, then vacuum enrichment; EH/MF: enzymatic hydrolysis, then membrane filtration-mediated concentration; RS consumption = 100 × consumed RS/initial RS; ethanol yield = 100 × ethanol/ (consumed RS × 0.51).

Fig. 3. Production of ethanol with multiple feeding of sugar solution at 16, 24, and 32 h after onset of fed-batch fermentation using *H. reticulatum*.

yield of the former ranged from 87.1% to 98.0%, whereas that of the latter ranged from 67.1% to 82.1%. At a concentration of 120 g RS/l, the ethanol yield reached its highest level, but the final ethanol was very low (8.9 g/l). Similar results were reported by King and Hossain [34] and Lin *et al.* [35] after 144 h. However, the reason is not clear. The data also indicate the same trend for ethanol concentration at the initial RS concentration of 80 and 100 g/l. However, it showed a contrast in the RS consumption at every initial RS concentration.

These results suggested that ethanol production can be affected by several factors, including the viscosity of the hydrolysate, generation of fermentation inhibitors during the hydrolysis process, accumulation of fermentation inhibitors during the concentration process, activities of unknown fermentation facilitators, and more likely the combinatorial and/or synergy effects of these multiple factors. Therefore, further investigations are still needed for a better interpretation of these results [36–38].

In this study, VE was better than MF for the production of HCSS from HR for ethanol production using the strain KCTC7017. However, for future applications to the industrial production process, the most effective method should be selected after overall consideration for production cost. According to a previous report [39], total energy consumption for VE is twice as much as MF. However, considering the investment costs and the unit cost of production, there is almost no difference between VE and MF. In this study, we continued with additional experiments using the HCSS prepared by EH/VE, which guaranteed high ethanol yield.

Production of Ethanol through Fed-Batch Mode

The hydrolysis rate of HR was more than 80% with EH when HR was applied at less than 150 g/l, but it decreased dramatically at high substrate concentrations [40]. Therefore, when hydrolysate was prepared from 150 g HR/l, RS in the hydrolysate reached up to approximately 68 g/l. If it was directly used for fermentation without the concentration step, approximately 24.5–27.7 g ethanol/l could be produced. However, in an economic analysis, at least 50 g/l ethanol concentration is desirable for industrialization of the bioethanol production process [13]. Therefore, some measures are needed to produce more concentrated bioethanol to meet the economical standard, such as using HCSS and multiple feeding of the sugar solution during the fermentation process. Addition of HCSS at the beginning of fermentation results in a decrease in both bioethanol production yield and efficiency. Therefore, in this study, we tried to produce a high ethanol concentration to meet the economical standard by employing the fed-batch culture method for ethanol fermentation.

Using the HCSS of HR (240 g RS/l) obtained from HR biomass containing 8% solid content through EH/VE, fermentation started at a concentration of approximately 40 g RS/l (corresponding to 33.5 g glucose/l) in the culture medium, and the RS concentration was maintained by feeding HCSS three times at 16, 24, and 32 h in the fermentation process, corresponding to concentrations of glucose remaining of 0.10, 0.14, and 6.7 g/l, respectively. The concentrations of ethanol reached up to 49.12, 53.70, and 54.33 g/l after 2, 4, and 5 days, respectively (Fig. 3). The RS remaining was 29 g/l and it did not decrease even

when the fermentation time was extended up to 120 h. It may be because some RSs (including disaccharides, oligosaccharides, and polysaccharides belong to the reducing group) can not be utilized by the microorganism, and a small part of measurement error was caused by some compound that has an open-chain form with an aldehyde group or a free hemiacetal group.

According to several previous studies, it seems to be that studies on ethanol production have been carried out more often with macroalgae than microalgae, because macroalgal biomass is easy to harvest in a large scale. In addition, it can be grown directly in the open sea, including offshore farms and near-shore coastal farms [4, 41, 42]. However, the overall bioethanol production from macroalgae fell short of our expectation because of the relatively complex carbohydrate composition, high content of sugars that are hard to be fermented (rhamnose, 3,6-anhydroxygalactose, *etc.*) in certain species, and poor availability or lack of specific enzymes for degradation of various polysaccharides existing in macroalgae. Nevertheless, a possibility of bioethanol production at the concentration of 55 g/l was reported once using *Geledium elegans* [13]. However, in this case, 30% solid content was used for the hydrolysis, and the ratio of ethanol yield to biomass used was 18.33 g/100 g DM. In comparison, our study showed that the ratio of ethanol yield to biomass used was 24.9–28.6 g/100 g DM,

higher than the previous report [13].

Few research cases have been reported for bioethanol production using microalgae, because most researchers seem to focus on biodiesel production using species containing a high lipid content [43, 44]. Table 3 shows ethanol production from microalgae and macroalgae based on the results in the literature and this study. The highest ethanol concentration reported so far among studies on bioethanol production from microalgae was 11.73 g/l using *C. reinhardtii* UTEX 90 [7], which is much lower than the 54.3 g/l reported in this study, even though the ethanol yield is not much significantly different (24.9 and 23.5 g ethanol/100 g DM). The highest ethanol concentration obtained from macroalgae was 55 g/l, corresponding to 18.33 g/100 g DM. Moreover, in the other previously reported cases, microalgal biomasses were all derived from unicellular microalgae, which have a disadvantage of a high energy cost for the harvesting process. The HR used in this study belongs to filamentous green algae with an advantage of a low energy cost for the simple harvesting process.

Recently, there have been extensive studies on bioethanol production from lignocelluloses among other non-food biomass [45]. Although lignocellulosic biomass has several advantages, such as a high carbohydrate content and easy access to biomass, it also has disadvantages, such as the

Table 3. Comparison of bioethanol production using macroalgae and microalgae.

Bioresources	Microorganism	S/L (%)	Saccharification	Ethanol (g/100 g)	Ethanol (g/l)	References
Macroalgae						
G. elegans	*S. cerevisiae* IAM 4178	30.0	Acid/enzyme	18.33	55.0	[13]
Sargassum spp.	*S. cerevisiae*	-	Acid/enzyme	-	2.79	[16]
K. alvarezii	*S. cerevisiae* (NCIM 3523)	-	Acid	-	24.6	[17]
G. verrucosa	*S. cerevisiae*	-	Acid	3.8	15.0	[18]
G. amansii	*B. custersii*	15.0	Acid	-	27.6	[19]
L. japonica	*E. coli* KO11	18.0	Acid/enzyme	16.11	29.0	[21]
Microalgae						
C. reinhardtii UTEX 90	*S. cerevisiae* S288C	5.0	Enzyme	23.5	11.73	[2]
C. vulgaris FSP-E	*Z. mobilis*	5.0	46.1% Glucose	23.3	11.66	[30]
C. reinhardtii mutant cw15	*S. cerevisiae*		Acid	-	8.7	[46]
S. abundans PKUAC 12	*S. cerevisiae*	5.0	Acid/enzyme	10.3	5.15	[47]
S. obliquus CNW-N.	*Z. mobilis*	4.0	Acid	21.3	8.55	[48]
S. obliquus YSW15	Anaerobic bacteria/consortia	2.12	Ultrasonication	31.6	6.7	[49]
Chlorococcum sp.	*S. cerevisiae*	1.5	Acid	-	7.2	[50]
C. reinhardtii UTEX 90	*S. cerevisiae* S288C	5.0	Hydrothermal acid	29.2	14.6	[51]
H. reticulatum	*S. cerevisiae* KCTC 7107	6.0	Enzyme*	24.9	54.3	This study
		8.0	Enzyme	14.92	11.53	This study

S/L: Solid/liquid; *: using enzymatic hydrolysis, then vacuum enrichment.

relatively high cost of bioethanol production caused by the absolute requirement for pre-treatment and sugar concentration, which makes it hard to establish an eco-friendly production process. Taken together, HR has several advantages, such as low lignin content, high carbohydrate production rate, high yield of fermented sugars due to its high content of glucose and mannose in the carbohydrate composition, and above all, a much simpler harvesting process than other microalgae. On the other hand, HCSS can be prepared only by EH/VE from HR without a particular pre-treatment step. Additionally, this study revealed that an economical ethanol concentration, more than 5% of ethanol, could be achieved through the fed-batch culture method with multiple feeding of a low concentrated sugar solution. These characteristics and our results indicate that bioethanol production using HR may require a lower production cost than lignocellulosic biomass or other algal biomasses.

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