연구논문

Ethanol Production from Seaweed, *Enteromorpha intestinalis*, by Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) with *Saccharomyces cerevisiae*

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Abstract: Ethanol productions were performed by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes using seaweed, *Enteromorpha intestinalis* (sea lettuce). Pretreatment conditions were optimized by the performing thermal acid hydrolysis and enzymatic hydrolysis for the increase of ethanol yield. The pretreatment by thermal acid hydrolysis was carried out with different sulfuric acid concentrations in the range of 25 mM to 75 mM H_2SO_4, pretreatment time from 30 to 90 minutes and solid contents of seaweed powder in the range of 10~16% (w/v). Optimal pretreatment conditions were determined as 75 mM H_2SO_4 and 13% (w/v) slurry at 121°C for 60 min. For the further saccharification, enzymatic hydrolysis was performed by the addition of commercial enzymes, Celluclast 1.5 L and Viscozyme L, after the neutralization. A maximum reducing sugar concentration of 40.4 g/L was obtained with 73% of theoretical yield from total carbohydrate. The ethanol concentration of 8.6 g/L of SHF process and 7.6 g/L of SSF process were obtained by the yeast, *Saccharomyces cerevisiae* KCTC 1126, with the inoculation cell density of 0.2 g dcw/L.

Keywords: Bioethanol, Fermentation, *Enteromorpha intestinalis*, *Saccharomyces cerevisiae*

1. Introduction

Biomass can serve as the excellent resource to meet the present and future energy demands [1]. Biofuels including bioethanol, biodiesel, biohydrogen and biogas can provide the alternative transportation fuels. Bioethanol is a promising transportation fuel as it can replace the fossil fuels.

Current production of bioethanol relies on ethanol from starch and sugars but there has been considerable debate about its sustainability [2]. Lignocellulosic biomass has been regarded as the one of the most prospective biomass because it could be used as inexpensive biomass and there is no competition with food directly or indirectly. However, ethanol production from lignocellulosic biomass tends to require pretreatment under severe conditions due to lignin degradation. Lignin is an aromatic polymer synthesized from phenylpropanoid precursors and it is one of the drawbacks in fermentation, as it makes lignocellulosic biomass resistant to chemical and biological degradation [3].

Seaweed is a third generation biomass for the bioethanol production since it has been known for the lignin-free composition and high carbohydrate content. Seaweed can be grown rapidly comparing to land-based biomass and have been harvested in worldwide. *Enteromorpha* (sea lettuce) are green macroalgae distributed widely in inter-tidal zones and its growth rate can reach 12.7% per day [4]. *Enteromorpha* also features the richness in polysaccharides containing a large amount of glucose, xylose, galactose, glucuronic acid, and rhamnose [4]. Thus, the green algae, *Enteromorpha*, is great potential resource for bioethanol production.

Bioethanol production requires three major processes for the conversion of polysaccharides into ethanol through pretreatment, saccharification and fermentation. The first-step is thermal acid pretreatment which alter structural integrity of biomass and increase the enzymatic accessibility prior to enzymatic hydrolysis. Enzymatic hydrolysis is second process to hydrolyze the cellulose to oligomeric and monosaccharide as well as decrease the viscosity of hydrolysates, which enables easier

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pumping and stirring [5]. The enzymatic hydrolysis currently has monomeric sugar yields of 75~80% and improvements are still projected to 85~95% [3]. After the pretreatment and the enzymatic hydrolysis, fermentation was carried out to produce ethanol by yeasts. When two processes, enzymatic hydrolysis and fermentation, were separately performed, it is referred to the separate hydrolysis and fermentation (SHF). However, each process can be simultaneously carried out, which is referred to simultaneous saccharification and fermentation (SSF) process. SSF process has been regarded as the major option due to high overall yields and process integration [6]. SSF process is favorable because it requires low energy consumption and short process time. The researchers implement both SHF and SSF processes to compare the ethanol production efficiency.

In this study, reducing sugar production was optimized by thermal acid hydrolysis and/or dual enzyme treatment. And ethanol production by SHF and SSF processes were compared to demonstrate the potential of alternative process to produce ethanol from seaweed, *E. intestinalis*.

2. Materials and Methods

2.1. Raw materials and characterization
The seaweed, *E. intestinalis,* harvested in October 2010, was obtained from Wan-do in Jeon-nam, Korea. Samples were oven-dried at 55°C and milled by hammer mill. The crude protein content of seaweed was estimated by Kjeldahl method. Crude lipid was determined by using an ether extraction procedure (Soxtec System 1046, foss, Hoganas, Sweden) after freeze-drying for 12 h. Determination of crude ash was done by incineration at 550°C for 3 h. The total carbohydrate content was determined by the calculation.

2.2. Thermal acid hydrolysis
The thermal acid hydrolysis condition of *E. intestinalis* was optimized with three factors of sulfuric acid concentration (37.5, 75 mM), treatment time (30~90 min) and solid contents (10~16%, w/v) [10]. The slurry was treated by autoclave at 121°C in accordance with each condition and the treatment was performed in triplicate. Samples were filtered and stored at 4°C for the determination of reducing sugar content and viscosity.

2.3. Enzymatic hydrolysis of pretreated seaweed slurry
Commercial enzymes, Celluclast 1.5 L and Viscozyme L were purchased from Sigma (Novozymes, Bagsvaerd, Denmark) [14]. The pH of pretreated seaweed slurry was adjusted to 4.5 before the treatment of enzyme mixture of Celluclast 1.5 L (8.4 filter paper unit FPU/ml) and Viscozyme L (1.2 endo-glucanase unit

EGU/ml). Enzymatic hydrolysis was carried out at 55°C in water bath shaker with 120 rpm for 54 h. Enzymatic hydrolyzates were evaluated by reducing sugar contents measurement using DNS method [7].

2.4. Yeast cultivation
Yeast, *Saccharomyces cerevisiae* KCTC 1126 obtained from KCTC was used for the fermentation in this study. The inoculum culture was prepared on YPD agar plate containing yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L and agar 5% (w/v). Yeast suspension was incubated at 30±2°C with 220 rpm in a shaking incubator.

2.5. Ethanol fermentation
2.5.1. Separate hydrolysis and fermentation (SHF)
Pretreatment of seaweed slurry was performed with sulfuric acid and enzymatic hydrolysis. The pH hydrolysate slurry was then adjusted to 5.5 for the fermentation. The fermentation medium was supplemented with 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$ and 1.0 g/L yeast extract [17]. Fermentation was carried out by the inoculation of yeast suspension with a final cell concentration of 0.2 g dcw/L. Samples were withdrawn at 12 h of interval during the experiment and used for the analysis of sugars, ethanol and organic acid.

2.5.2. Simultaneous saccharification and fermentation (SSF)
SSF was carried out in 250 ml flask with working volume 100 ml of pretreated seaweed slurry. Nutrients were added to the medium; 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$ and 1.0 g/L yeast extract. Fermentation was started by the addition of enzymes mixture and yeast, *S. cerevisiae* KCTC 1126 suspension (0.2 g dcw/L). Fermentation was carried out at 30±2°C for 120 h and the stirrer speed of 220 rpm. Sampling and analysis were performed as described above.

2.6. Analysis of reducing sugar, monosaccharide and viscosity
Reducing sugar contents were determined by the 3, 5-dinitrosalicyclic acid (DNS) method with glucose (Sigma Chemical Co. USA) as the standard [7]. Sugars, ethanol and other organic acid were analyzed by HPLC (Agilent 1100 Series, Agilent. Inc., USA) fitted with Agilent G1362A refractive index detector (RID). Ethanol, acetic acid and 5-hydroxyl methyl furfural (5-HMF) were analyzed with Bio-rad Aminex HPX-87H column (300×7.8 mm) and Superguard C610H column (50×4.6 mm) at 65°C with eluent of 5 mM sulfuric acid and the flow rate of 0.6 ml/min. Samples were filtered through a 0.2 μm filter prior to analysis to remove particles.

Viscosity was measured by Brookfield viscometer (BROOK-

FIELD DV-III Rheometer v3.1, Brookfield Eng. Inc., USA) equipped with spindle No. ULA, SC4-18 and SC4-34 at 30°C. Yeast cell growth for the inoculation was measured by UV-Vis spectrophotometer at OD_{600} and converted to dcw using standard curve.

3. Results and Discussions

### 3.1. Composition and characterization of *E. intestinalis*

The composition of *E. intestinalis* was determined. *E. intestinalis* contains 31.6% of crude protein, 1.3% of crude lipid, 24.3% of crude ash and 42.8% of total carbohydrate. In other studies with green seaweeds, *Ulva* and *Enteromorpha* carbohydrate contents were approximately 30~40% as glucose, xylose, galactose, rhamnose and glucuronic acid [4].

### 3.2. Effect of pretreatment condition

Seaweed, *Enteromorpha*, requires pretreatment in order to make the carbohydrate accessible to enzymes. The pretreatment of seaweed slurry with sulfuric acid increases the enzymatic digestibility of biomass, giving the higher sugar yield than that without sulfuric acid treatment [8]. Therefore, three different variables of solid content, treatment time and sulfuric acid concentration, were selected as the determinant factors for the pretreatment condition.

The results of reducing sugar yield (g/L) by 37.5 mm and 75.0 mm H_2SO_4 concentrations at 121°C for 30 min in *E. intestinalis* are shown in Fig. 1(a). Yield of reducing sugar (g/L) increased with the increases of sulfuric acid concentration and slurry content in pretreatment. The increase of sulfuric acid concentration from 37.5 mM to 75 mM produced higher reducing sugar content of 9.2 g/L and the maximum yield of 16.5% was achieved with the 75 mM H_2SO_4 at the slurry content of 13% (w/v).

However, the increase of slurry content decreases reducing sugar production causing difficulty with rotation. Fig. 1(b) shows the effect of slurry contents and sulfuric acid concentrations on viscosity of slurry. The viscosities of slurry were decreased when solid contents decreased and sulfuric acid concentration increased. It has been reported that increased slurry content led to high viscosity which inhibited the cell growth and medium handling although high solid content provided economic advantages [9,10]. High solid content also results in processing problems including high power consumption and reduced conversion [11]. In this experiment, 13% (w/v) solid content showed maximum hydrolysis yield, however, further increase in solid content decreased the hydrolysis yield. Therefore, 13% (w/v) slurry content was used for the ethanol production process.
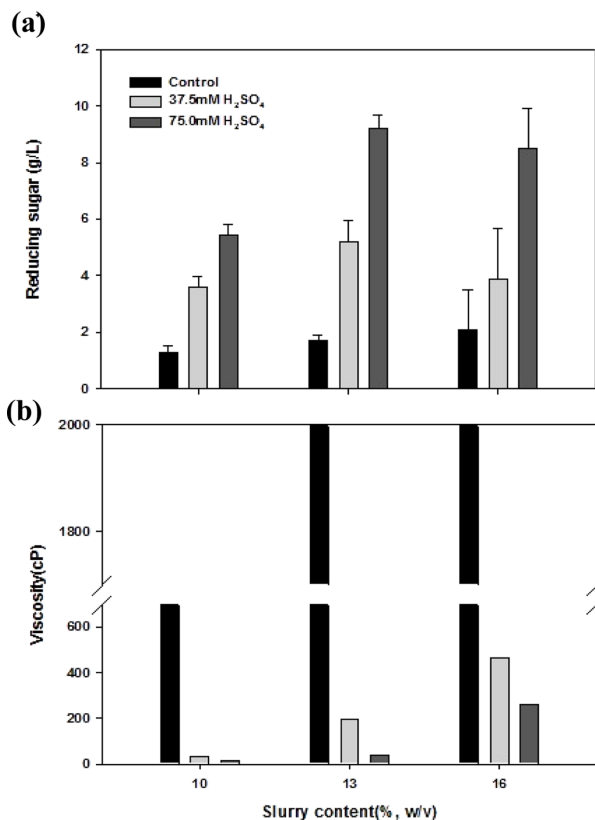
Fig. 1. Evaluation of optimal condition for the thermal acid hydrolysis using concentrations of H_2SO_4 and seaweed slurry contents. (a) Production of reducing sugar (g/L) and (b) viscosity (g/L) depending on concentrations of H_2SO_4 and seaweed slurry contents (w/v).
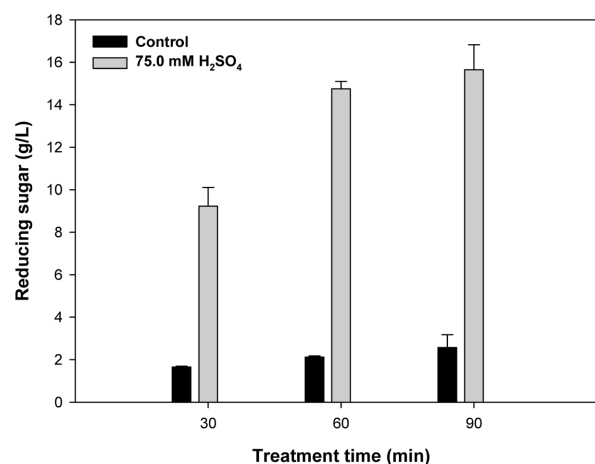
Fig. 2. Production of reducing sugar (g/L) treated with 75 mM H_2SO_4 and 13% (w/v) *E. intestinalis* slurry depending on the treatment time.
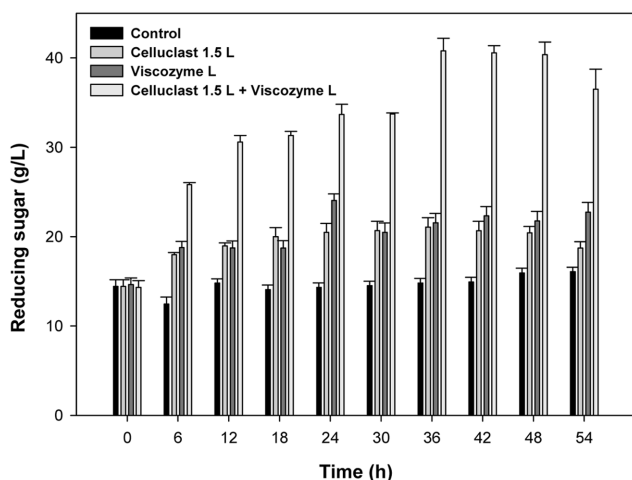
Seaweed, *E. intestinalis*, slurry were treated with time variation and the result of reducing sugar yield were shown in Fig. 2. Using the solid content of 13% (w/v), the yield of reducing sugar increased from 9.2 g/L to 14.7 g/L by increasing the treatment time from 30 min to 60 min. However, a slight increase

of reducing sugar yield was observed when treatment time was extended from 60 min to 90 min. Lu *et al*. (2007) reported that the increase of treatment time led to the decrease of reducing sugar production which caused sugar decomposition with production of furfural and 5-HMF [10]. Considering the pretreatment efficiency and cost, 13% (w/v) of solid contents, 75 mM of H_2SO_4 and treatment time of 60 min were selected for further experiments.

3.3. Hydrolysis of *E. intestinalis* by commercial enzymes

Enzymatic hydrolysis of green seaweed, *E. intestinalis*, with commercial enzyme mixture was performed after the thermal acid hydrolysis. *E. intestinalis* was further hydrolyzed by the addition of commercial enzymes, Celluclast 1.5 L and Viscozyme L, to increase the saccharification yield of seaweed.

The enzymatic hydrolysis with commercial enzymes is shown in Fig. 3. The reducing sugar of 20.9 g/L with 37.7% of theoretical yield was obtained using Celluclast 1.5 L only. Viscozyme L treatment showed hydrolysis, showing maximum reducing sugar content of 24.2 g/L with 43.7% of theoretical yield. Viscozyme L, a multi-enzyme complex including arabinase, cellulase, beta-glucanase, hemicellulase and xylanase could degrade diverse polysaccharides containing in *E. intestinalis* whereas Celluclast 1.5 L degraded cellulose. The hydrolysis efficiency of the mixed two enzymes, Celluclast 1.5 L and Viscozyme L, was higher than that of individual enzyme treatment. A maximum reducing sugar of 40.4 g/L corresponding to 73.0% of theoretical yield was obtained at the incubation of 36 h with the mixture of two enzymes. It has been reported that mixed enzymes treatment increased the release of reducing sugar content comparing to individual enzyme treatment [12].

3.4. SSF and SHF

Fermentation was carried out using SHF and SSF methods. In order to compare SHF and SSF, fermentation yields were calculated by assuming that all monosaccharides in the slurry are available for the fermentation. Based on the assumption, ethanol concentration of 8.6 g/L, corresponding to 30.5% of theoretical case was obtained at 48 h in SHF process as shown in Fig. 4(a). The ethanol concentration of 7.6 g/L with an ethanol yield of 26.9% of theoretical case was observed by SSF process as shown in Fig. 4(b). Fig. 4 also shows the time course of reducing sugar consumption, ethanol and inhibitor productions in each process.

In the SHF, reducing sugar was rapidly decreased from 40 g/L to 14.7 g/L during 24 h of fermentation. During the same period, a rapid increase of ethanol concentration was observed. However, 10 g/L of reducing sugar was still remained at the end of the SHF process. This indicates that non-fermentable sugar monomers in *E. intestinalis* were not utilized by *S. cerevisiae* during fermentation since the glucose was consumed as a carbon source by the yeast. Similar pattern of reducing sugar consumption and ethanol production was also observed in case

Fig. 4. Fermentation of reducing sugars from thermal acid hydrolysis and enzyme treated *E. intestinalis* to produce bioethanol Changes of reducing sugar, ethanol, acetic acid and HMF concentrations (g/L) in (a) SHF (b) SSF processes.
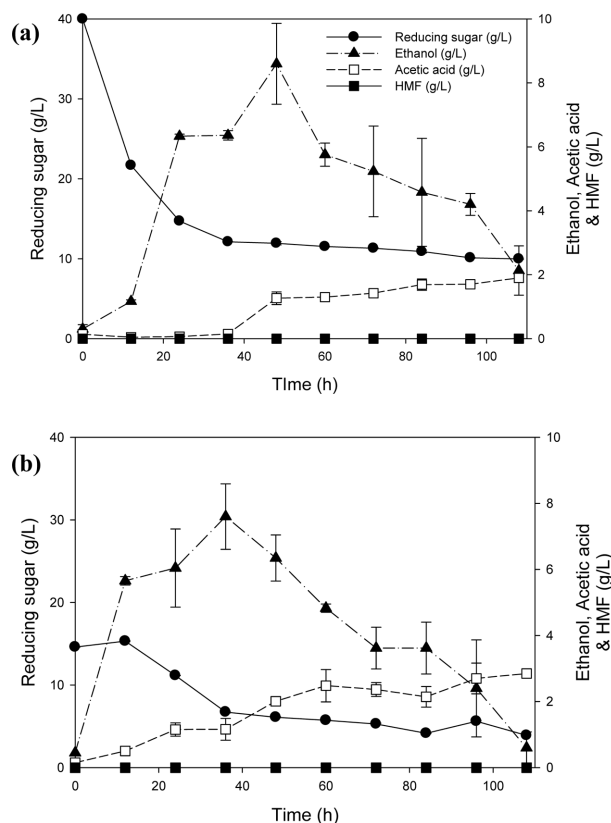
Fig. 3. Reducing sugar production (g/L) by enzymatic hydrolysis of pretreated *E. intestinalis* slurry with enzymes, Celluclast 1.5 L and/or Viscozyme L.

of SSF. The concentration of reducing sugar remained under 10 g/L after 36 h of fermentation. In the SSF, the extent of carbohydrate conversion into reducing sugar was lower than that obtained in the SHF process. This might be the difference in process temperatures of fermentation and enzymatic hydrolysis of treatment. The overall process of SSF was carried out at temperature of 32°C, which is lower than the optimal temperature of 55°C for the action of each enzyme. Although the commercial enzymes had quite different specific activities, SSF process worked better than SHF process, considering the efficiencies of ethanol production and time required for separate enzymatic hydrolysis of 36 h and fermentation of 48 h.

The ethanol concentrations decreased after reaching maximum ethanol concentrations at 48 h and 36 h in SHF and SSF processes, respectively. The decrease of ethanol concentration might be related to the conversion of ethanol to acetic acid. It has been reported that the microbial population was adapted to consume sugar and ethanol when the ethanol was accumulated in the medium thus resulted in reduction of ethanol concentration [13]. Similar result of decline in ethanol production after maximum ethanol production was reported in other study [14].

The changes of fermentation inhibitor concentration during SHF and SSF process are shown in Fig. 4. The increase of acetic acid was observed with the decrease of ethanol concentration as shown in Fig. 4(a) and (b). Acetic acid concentration less than 3 g/L, lower than inhibitory concentration of fermentation was observed throughout all experiment in SSF and SHF processes [15]. HMF was also major by-product derived from hexoses. However, 5-HMF was not detected in both processes.

Despite the 70% of hydrolysis, results showed low ethanol production. It showed that all of reducing sugars produced from thermal acid hydrolysis and enzymatic hydrolysis were not utilized during fermentation process. The yeast, *S. cerevisiae*, can utilize C6-monosaccharides as substrate and hardly metabolize other types of saccharide that such as xylose, arabinose [5]. In this study, various types of monosaccharides hydrolyzed from *E. intestinalis* were not utilized because of the limitation of substrate uptake by the yeast. Hsu *et al*. [5] reported that xyolse in lignocellulosic biomass cannot be efficiently utilized due to the same reason. However, our result shows that the ethanol production amount and yield were higher than other study although the ethanol fermentation was carried out with only *S. cerevisiae* not combination with other yeasts which can metabolize C5-monosaccharides [16]. Another reason related to low ethanol conversion was due to rhamnose and glucuronic acid which were handly converted into ethanol by fermentation. Thus, improvement of yeast strains capable of fermenting both hexose and pentose sugars should be applied for the improvement of ethanol production from seaweeds.

4. Conclusions

Seaweed, *E. intestinalis*, is an attractive biomass for bioethanol production due to lignin-free composition and its high carbohydrate contents. The study of bioethanol production process and improvement of saccharification step from *E. intestinalis* was described in this paper. The application of dual-enzymes, Celluclast 1.5 L and Viscozyme L, can improve enzymatic digestibility and the conversion of polysaccharide to reducing sugar reached about 73.4% after 36 h. This results are based on the further studies to increase saccharification yield from seaweed, *E. intestinalis*.

The highest ethanol concentration of 8.6 g/L corresponding to 30.5% of theoretical yield was achieved in SHF process whereas 7.6 g/L of ethanol concentration corresponding to 26.9% in SSF process was obtained with *S. cerevisiae*. It was concluded that SSF process showed a better efficiency than SHF process, considering fermentation yield and time required for all process.

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