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Thermal Acid Hydrolysis Pretreatment, Enzymatic Saccharification and Ethanol Fermentation from Red Seaweed, *Gracilaria verrucosa*

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The seaweed, *Gracilaria verrucosa*, was fermented to produce bioethanol. Optimal pretreatment conditions were determined to be 12% (w/v) seaweed slurry and 270 mM sulfuric acid at 121°C for 60 min. After thermal acid hydrolysis, enzymatic saccharification was carried out with 16 U/ml of mixed enzymes using Viscozyme L and Celluclast 1.5 L to *G. verrucosa* hydrolysates. A total monosaccharide concentration of 50.4 g/l, representing 84.2% conversion of 60 g/l total carbohydrate from 120 g dw/l *G. verrucosa* slurry was obtained by thermal acid hydrolysis and enzymatic saccharification. *G. verrucosa* hydrolysate was used as the substrate for ethanol production by separate hydrolysis and fermentation (SHF). Ethanol production by Candida lusitaniae ATCC 42720 acclimated to high-galactose concentrations was 22.0 g/l with ethanol yield (Y_{EtOH}) of 0.43. Acclimated yeast to high concentrations of specific sugar could utilize mixed sugars, resulting in higher ethanol yields in the seaweed hydrolysates medium.

Keywords: Thermal acid hydrolysis, enzymatic saccharification, ethanol fermentation, *Gracilaria verrucosa, Candida lusitaniae*

Introduction

In this study, the red seaweed *Gracilaria verrucosa* was used as a bioethanol producing biomass. *G. verrocosa* has a high content of easily degradable carbohydrates, making it a potential substrate for the production of liquid fuels [8]. The carbohydrates in *G. verrucosa* can be categorised according to their chemical structures: alginate, carrageenan, and agar. Carrageenan and agar, which are plentiful in the seaweed, can be used as a source of galactose and glucose.

Various pretreatment techniques have been introduced to enhance the overall hydrolysis yield, and can be categorized into physical, chemical, biological, enzymatic or a combination of these [1]. Dilute acid hydrolysis is commonly used to prepare seaweed hydrolysates for enzymatic saccharification and fermentation for economic reasons [13]. However, thermal acid hydrolysis pretreatment for 3,6-anhydrogalactose from *G. verrucosa* have produced 5-hydroxymethylfurfural (HMF), an inhibitory compound for ethanol production [8].

One of the problems encountered in *G. verrucosa* fermentation has been high concentrations of NaCl due to its origin from sea water [3]. High-salt stress to yeasts is a significant impediment on the production of ethanol from seaweed hydrolysates. Salt stress in yeasts results in two phenomena: ion toxicity and osmotic stress [11]. Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion [20].

The preferential utilization of glucose over non-glucose sugars by yeast often results in low overall ethanol production and yield. When yeast grows on a mixture of glucose and galactose, the glucose is metabolized first, whereas

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the galactose is not metabolized until the glucose has been exhausted by glucose repression of *GAL* repressor genes [17]. To overcome the problems caused by the mixed sugars of seaweed hydrolysates, the acclimation of yeast to high concentrations of specific sugar has been developed [4, 6]. Thus, acclimated yeast to a high concentration of specific sugar before fermentation is the method emplloyed for mixed sugar utilization boosting the efficiency of sugars uptake in order to obtain high ethanol production.

A number of yeasts and several bacterial strains have been studied for cellulose, glucose, cellobiose, and/or xylose fermentations. *Candida* (*Clavispora*) *lusitaniae* has been used in various fermentation studies for ethanol production. Liu *et al.* [9] reported that the temperature and inhibitor tolerant strain of *Clavispora* Y-50464 produces sufficient native β-glucosidase to grow on cellobiose as the sole source of carbon and energy fermenting it to ethanol. *C. lusitaniae* displays a fast initial ethanol production rate and attains high ethanol conversions [15]. The objective of this study was to optimize the pretreatment conditions, such as thermal acid hydrolysis, enzymatic saccharification and ethanol fermentation for the red seaweed *G. verrucosa*.

Materials and Methods

Culture of *C. lusitaniae* ATCC 42720 and Medium

The culture of *C. lusitaniae* ATCC 42720, purchased from the American Type Culture Collection (ATCC), was grown in YPD medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose. The culture was incubated with agitation at 150 rpm for 24 h at 30°C. The acclimation of yeasts was developed to improve the uptake of specific monosaccharides, and ethanol production from the mixed monosaccharides, in seaweed hydrolysates. Thus, 5 ml of culture was transferred to 50 ml of Yeast extract, Peptone and High Galactose (YPHG) medium composed of 10 g/l yeast extract, 20 g/l peptone and 120 g/l galactose, and cultured under the same conditions. The cells were centrifuged at 1390 × g for 10 min to remove the YPHG medium and transferred to a 250 ml Erlenmeyer flask containing 100 ml of 0.2 μm filtered seaweed hydrolysate.

G. verrucosa was obtained from Wan-do, Jeonnam, Korea. The seaweed was grounded by hammer mill and separated with a 200-mesh sieve prior to pre-treatment. The composition and analyses of *G. verrucosa* were carried out by the Feed and Foods Nutrition Research Center (FFNRC, Busan, Korea).

Thermal Acid Hydrolysis and Enzymatic Saccharification

Optimal conditions for thermal acid hydrolysis pretreatment of *G. verrucosa* were evaluated. The determination of optimal treatment conditions was carried out by 90-450 mM of sulfuric acid, 8-18% of seaweed slurry and 30-150 min of thermal hydrolysis time at 121°C. *G. verrucosa* hydrolysates were then neutralized to pH 5.0 using 5 N NaOH. The enzymatic saccharification of thermal acid hydrolysate was carried out by the addition of 16 U/ml of single and mixed enzymes using Spirizyme Fuel (862 amyloglucosidase unit (AGU)/ml, Novozymes, Bagsvaerd, Denmark), Viscozyme L (121 β-glucanase unit (FBG)/ml, Novozymes) and Celluclast 1.5 L (854 endo-glucanase unit (EGU)/ml, Novozymes) with a proper dilution of single enzyme, or dilution with 1:1 ratio of mixed enzymes, to 100 ml of seaweed slurry. A previous study reported [16] 16 U/ml as an optimal enzyme activity for enzymatic saccharification. Thus, enzyme saccharifications by Viscozyme L, Celluclast 1.5 L and Spirizyme Fuel were carried out with 16 U/ml of equal activities with single or mixed enzymes, respectively. Spirizyme Fuel contains amyloglucosidase that hydrolyzes α-(1,4) or α-(1,6) *linkages in D-glucans.* Viola *et al.* [18] reported on starch metabolism in red aglae. Red algae (Rhodophyceae) are photosynthetic eukaryotes that accumulate starch granules (floridean starch) outside of their plastids. Therefore, Spirizyme Fuel was used in this study. Viscozyme L contains endo-β-glucanase that hydrolyzes (1,3)- or (1,4)-linkages in β-D-glucans with side activities of xylanase, cellulase and hemicellulose. Celluclast 1.5L contains cellulase that hydrolyzes (1,4)-β-D-glucosidic linkages in cellulose and other β-D-glucans [7, 10]. The reaction was carried out with a shaking incubator at 45°C, 150 rpm for 60 h. The efficiency of pretreatment and saccharification were calculated as follow:

$$E_{ps}\,(\%) = \frac{\Delta S_{ps}}{TC} \times 100$$

In which E_{ps} is the efficiency of thermal acid hydrolysis pretreatment and enzymatic saccharification (%), ΔS_{PS} is monosaccharide increase (g/l) during thermal acid hydrolysis pretreatment and enzymatic saccharification, and TC is the total carbohydrate (g/l) in seaweed *G. verrucosa.*

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Ethanol Fermentation

Ethanol fermentation was performed with 100 ml of *G. verrucosa* hydrolysate in a 250 ml Erlenmeyer flask under semi-anaerobic conditions. Acclimated *C. lusitaniae* ATCC 42720 to high galactose concentrations was pre-cultured for 48 h and 7.58 g dcw/l of yeast were inoculated into 100 ml of *G. verrucosa* hydrolysate. The seaweed hydrolysates were fermented to produce ethanol at 30°C and 150 rpm with acclimated yeast. Samples were taken periodically and stored at -20°C prior to the measurements of ethanol, residual sugars, and HMF concentrations. The ethanol yield (Y_{EtOH}, g/g) was defined as the maximum ethanol concentration (g/l) relative to the total initial fermentable sugar (galactose + glucose) concentration at the onset of fermentation (g/l).

Analytical Methods

The glucose, galactose, HMF and ethanol concentrations in the samples were determined by HPLC (Agilent 1100 Series, Agilent. Inc., Santa Clara, CA, USA) equipped with a refractive index detector. The Bio-Rad Aminex HPX-87H column (300.0 × 7.8 mm) was maintained at 65°C and samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Salinity was measured using a salinometer (Salinity Refractometer, ATAGO Inc., Japan).

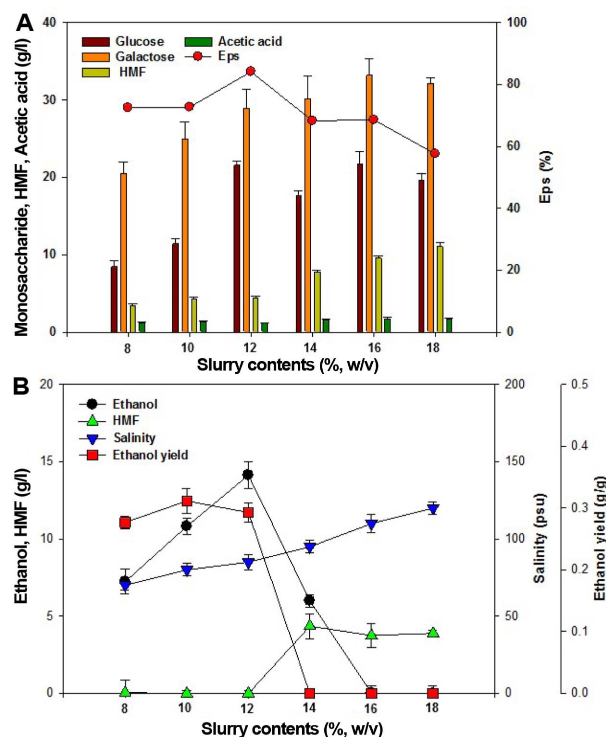

Fig. 1. Effect of thermal acid hydrolysis using various slurry contents on (A) pretreatment and saccharification and (B) ethanol production using *C. lusitaniae* ATCC 42720 (experimental conditions: slurry content = 8-18% (w/v), H_2SO_4 = 270 mM, thermal hydrolysis = 121°C, 60 min).

Results and Discussion

Chemical Composition of *G. verrucosa*

The composition of *G. verrucosa* was analyzed by the AOAC method which revealed 66.9% carbohydrate, 10.6% crude fiber, 9.5% crude protein, 0.6% crude lipid, and 12.4% crude ash among other minor components. The weight ratio of galactose to AHG in the agar content was reported to be 0.51:0.49 [5]. The maximum galactose content was calculated as 0.38 g/g in *G. verrucosa*: (0.669 g agar/g *G. verrucosa*) × (0.51 g galactose unit/g agar) × (180 g galactose/162 g galactose unit). The maximum glucose content was calculated as 0.12 g/g in *G. verrucosa*: (0.106 g cellulose/g *G. verrucosa*) × (180 g glucose/162 g glucose unit of cellulose). Therefore, the total fermentable monosaccharides of galactose and glucose in *G. verrucosa* were 0.38 + 0.12 = 0.50 g/g.

Optimization of Seaweed Slurry Contents

As shown in Fig. 1A, thermal acid hydrolyses with various slurry contents were carried out using 100 ml of 8-18% (w/v) seaweed slurry in a 250 ml Erlenmeyer flask with 270 mM H_2SO_4 at 121°C for 60 min. Fig. 1A shows that the monosaccharide and HMF concentrations increased with increasing slurry contents. The maximum E_{ps} with monosaccharide concentration of 50.4 g/l was obtained with 12% (w/v) slurry content. Increasing the slurry contents to 18% during thermal acid hydrolysis and enzymatic saccharification resulted in the decrease of E_{ps} from 84.2 to 57.6%.

As shown in Fig. 1B, the effect of seaweed slurry contents on ethanol production was evaluated at 30°C and 150 rpm in various *G. verrucosa* hydrolysates using wild type of *C. lusitaniae* ATCC 42720 after enzymatic saccharification. High slurry content increased the practical salinity unit (psu) of the seaweed hydrolysate from 70 to 120 psu as illustrated in Fig. 1B. Use of seaweed slurry over 12% (w/v) decreased ethanol production due to the increase of salt concentrations. Omori *et al.* [12] reported high salt concentrations resulted in the rapid inhibition of cell growth and ethanol fermentation. Therefore, 12% (w/v) seaweed con-

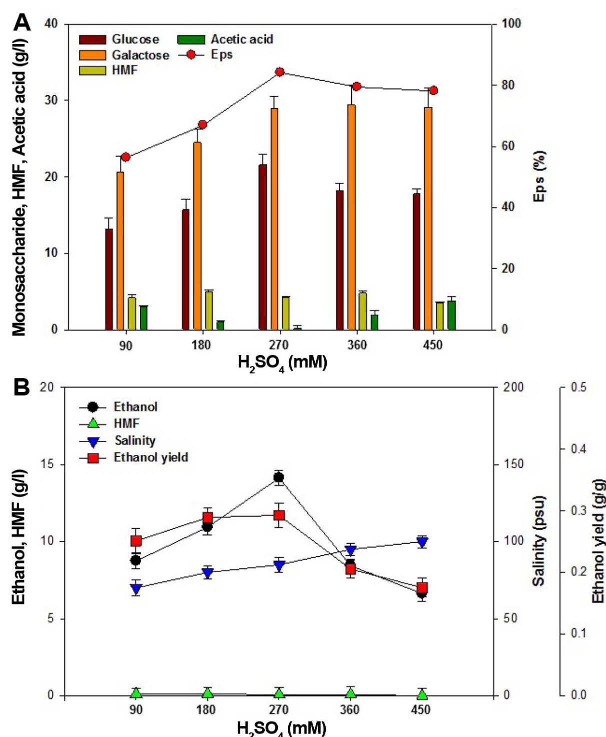

Fig. 2. Effect of thermal acid hydrolysis using various acid concentrations on (A) pretreatment and saccharification and (B) ethanol production using *C. lusitaniae* ATCC 42720 (experimental conditions: slurry content = 12% (w/v), H_2SO_4 = 90-450 mM thermal hydrolysis = 121°C, 60 min).

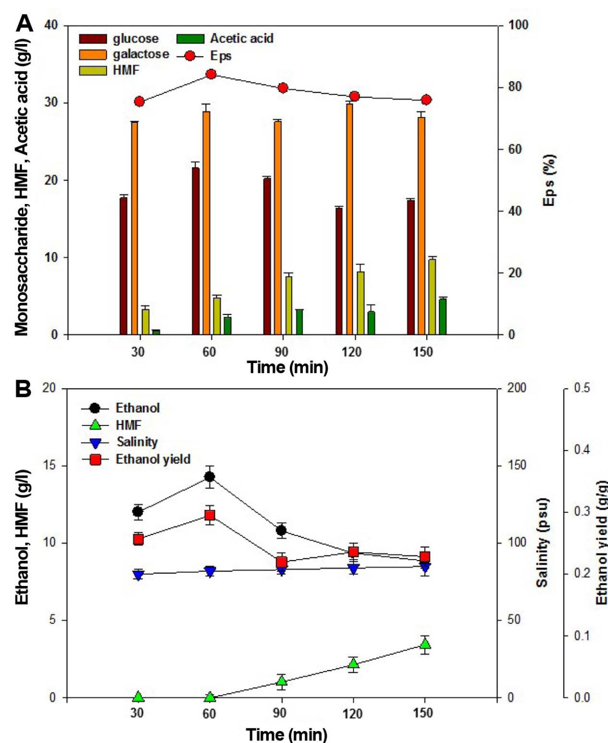

Fig. 3. Effect of thermal acid hydrolysis using various thermal hydrolysis durations on (A) pretreatment and saccharification and (B) ethanol production using *C. lusitaniae* ATCC 42720 (experimental conditions: slurry content = 12% (w/v), H_2SO_4 = 270 mM, thermal hydrolysis = 121°C, 30-150 min).

tent with E_{ps} of 84.2% was selected as the optimal seaweed slurry content for ethanol production.

Evaluation of Sulfuric Acid Concentrations in Pretreatment

As shown in Fig. 2A, B, the effects of sulfuric acid concentrations were evaluated with 12% (w/v) slurry content at 121°C for 60 min. The determination of optimal conditions was carried out with sulfuric acid concentrations of 90-450 mM.

Redding *et al*. [14] reported that high acid concentrations resulted in the release of high amounts of monosaccharide. However, E_{ps} after treatment with 360-450 mM sulfuric acid was not greater than that with 270 mM sulfuric acid with 12% (w/v) slurry content, as shown in Fig. 2A.

As shown in Fig. 2B, the effect of sulfuric acid concentration on ethanol production was evaluated using the wild type of *C. lusitaniae* ATCC 42720 after enzymatic saccharification. The usage of sulfuric acid over 270 mM decreased ethanol production due to the increase in salt concentration. Therefore, 270 mM sulfuric acid concentration was chosen as the optimal thermal acid hydrolysis and ethanol production condition.

Optimization of Thermal Acid Hydrolysis Time

Efficiencies of pretreatments with 12% (w/v) slurry and 270 mM sulfuric acid with various thermal hydrolysis times are shown in Fig. 3A. Monosaccharide concentrations increased slightly with increasing thermal hydrolysis time. Thermal hydrolysis times of 30-150 min at 121°C showed similar E_{ps} of 75.3-84.2%. However, E_{ps} reached a maximum within 60 min of thermal hydrolysis, as shown in Fig. 3A.

As shown in Fig. 3B, the effects of pretreatment and saccharification on ethanol production were assessed using the wild type of *C. lusitaniae* ATCC 42720. Use of thermal hydrolysis times of over 60 min decreased ethanol production due to the inhibitory effect of HMF. Therefore, a 60 min thermal hydrolysis time was used in subsequent experi-

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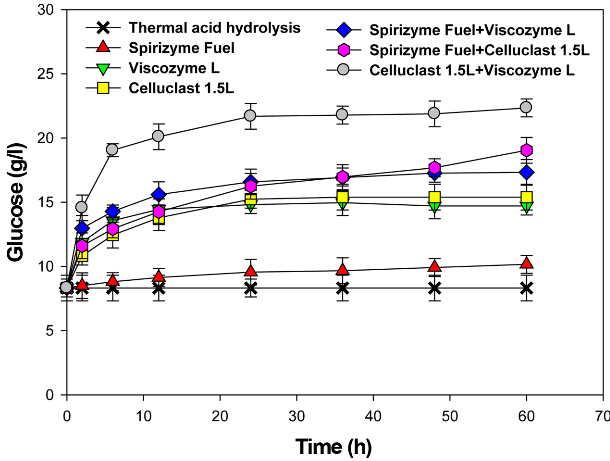

Fig. 4. Effect of enzyme treatments on glucose release from *G. varrucosa* hydrolysate pretreated with 12% (w/v) slurry after thermal acid hydrolysis. The initial glucose was 8.3 g/l after thermal acid hydrolysis pretreatment. 16 U/ml of single and mixed enzymes with Spirizyme Fuel, Celluclast 1.5 L and Viscozyme L were added to 120 g dw/l of seaweed slurry.

ments. From these results, the optimal acid hydrolysis conditions for ethanol production were obtained with 12% (w/v) slurry content, 270 mM sulfuric acid, at 121°C for 60 min.

Effect of Enzymatic Saccharification on Glucose Release

Glucose content after enzymatic saccharification of *G. verrucosa* was determined and is displayed in Fig. 4. Spirizyme Fuel, Viscozyme L and Celluclast 1.5 L treatments of thermal acid hydrolysate were evaluated for glucose release from 12% (w/v) slurry of *G. verrucosa* following thermal acid hydrolysis. The initial glucose concentration was 8.3 g/l after thermal acid hydrolysis pretreatment. Fig. 4 shows the glucose conversion between single and mixed enzyme treatments. The optimum enzyme reaction time was 24 h. The increase in reaction time up to 60 h had no significant effect on enzymatic saccharification. The glucose concentration of mixed enzyme treatments was higher than that of single enzyme treatments from *G. verrucosa* hydrolysate. As shown in Fig. 4, mixtures of Celluclast 1.5 L and Viscozyme L resulted in the best result for *G. verrucosa* hydrolysate. Ahn *et al.* [2] reported that mixed enzyme treatments increased the release of glucose content when compared to single enzyme treatments. Spirizyme Fuel treatment is a dual enzyme system and it did not result in an increase of glucose content due to the small amount of starch in *G. verrucosa*. The maximum glucose concentration was 21.7 g/l with mixed enzymes of Viscozyme L and Celluclast 1.5 L from in 120 g dw/l *G. verrucosa* slurry hydrolysate.

Batch Fermentations with Wild Type and Acclimated *C. lusitaniae* ATCC 42720

A culture of four yeast strains was carried out using *C. lusitaniae*, *P. stipitis*, *S. cerevisiae* and *K. marxianus*. The highest growth observed was from *C. lusitaniae* ATCC 42720 (data not shown). Thus, ethanol fermentation was carried out using *C. lusitaniae* ATCC 42720 using *G. verrucosa* hydrolysate. Separate hydrolysis and fermentation (SHF) was carried out by the addition of *C. lusitaniae* ATCC 42720 non-acclimated (wild type), or acclimated to high-galactose concentrations, shown in Fig. 5A and 5B, respectively. Glucose was consumed for 36 h, and then galactose was consumed for 24 h. However, the galactose was not totally consumed until 120 h, and 14.0 g/l of galactose remained, as is shown in Fig. 5A. The ethanol concentra-

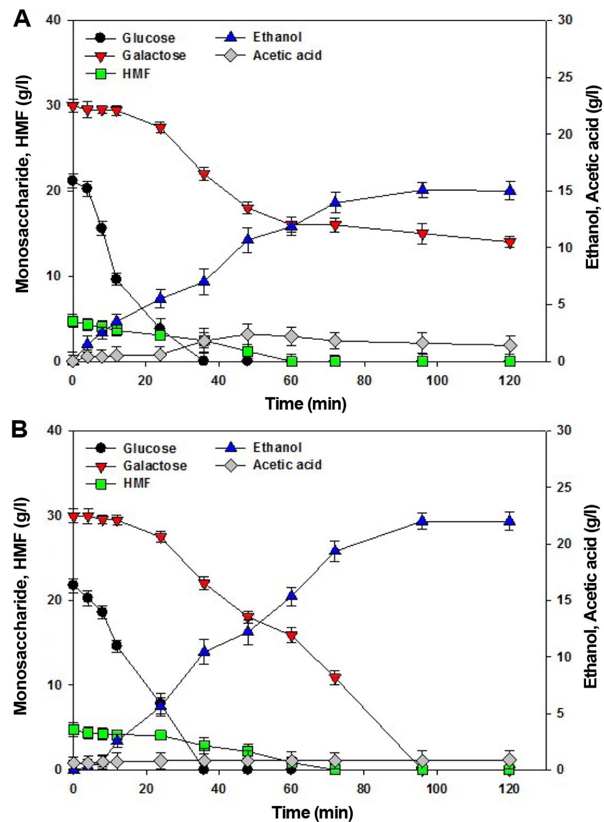

Fig. 5. Ethanol production from *G. verrucosa* by SHF using (A) non-acclimated and (B) acclimated *C. lusitaniae* ATCC 42720 to high concentrations of galactose.

tion after 96 h of fermentation with wild type *C. lusitaniae* ATCC 42720 was 15.1 g/l with Y_{EtOH} = 0.29.

C. lusitaniae ATCC 42720 was found to be acclimated to high concentrations of galactose, as revealed when glucose and galactose were simultaneously consumed, shown in Fig. 5B. After the glucose was almost exhausted at 24 h, the galactose was then consumed due to the preference of glucose to galactose. When the glucose was exhausted, ethanol fermentation slowed, resulting in a lag phase known as a diauxic shift from 36 to 48 h. The fermentation using acclimated *C. lusitaniae* ATCC 42720 produced an ethanol concentration of 22.0 g/l and a yield of 0.43 after 96 h of fermentation. Ethanol production at 36 h in Fig. 5A is different from that of Fig. 5B. The preferential utilization of glucose over galactose, however, often results in a low yield and productivity for ethanol. Wikandari *et al.* [19] reported that the addition of acetic acid of up to 6 g/l still resulted in acceptable ranges for both ethanol yield and productivity. Thus, acetate concentrations of lower than 3 g/l did not significantly influence the conversion of sugar to ethanol in the overall fermentation process. Therefore, the acclimation of yeasts to high concentrations of galactose successfully improved the efficiency of the ethanol fermentation of mixed sugars as shown in Fig. 5A and 5B. Cho *et al.* [4] reported acclimated yeasts produced a higher ethanol concentration than non-acclimated (wild type) yeasts. Therefore, the acclimation of yeasts to a high concentration of galactose could facilitate the simultaneous utilization of glucose and galactose for the increased production of ethanol from the seaweed *G. verrucosa*.

In conclusion, the optimal pretreatment conditions of seaweed *G. verrucosa* were determined to be 12% of slurry and 270 mM of sulfuric acid at 121°C for 60 minutes. After thermal acid hydrolysis and enzymatic saccharification, monosaccharide was obtained at 50.4 g/l from 60 g/l total carbohydrate of 120 g dw/l *G. verrucosa* slurry. A mixture of Celluclast 1.5 L and Viscozyme L achieved the best result for the enzymatic saccharification of *G. verrucosa* hydrolysate.

Acclimated yeast with high-galactose concentrations increased the sugar uptake more than non-acclimated yeast. The ethanol production and yield from *C. lusitaniae* ATCC 42720 non-acclimated and acclimated with high-galactose concentrations were 15.1 g/l with Y_{EtOH} of 0.29 and 22.0 g/l with Y_{EtOH} of 0.43, respectively. These optimal pretreatment and saccharification conditions and fermentation profiles provide a basis for ethanol fermentation from the red seaweed, *G. verrucosa*.

Acknowledgments

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국문초록

꼬시래기 홍조류로부터 열산가수분해, 효소당화 및 에탄올 발효

라채훈, 최진규, 강창한, 선우인영, 정귀택, 김성구*
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본 연구는 해조류, 꼬시래기를 발효하여 에탄올을 생산하였다. 최적 전처리 조건은 12% (w/v) 해조류 슬러리, 270 mM 황산, 121도 60분동안 실시하였다. 열산가수분해 후에, 꼬시래기 가수분해산물에 16 U/ml의 혼합효소 Viscozyme L과 Celluclast 1.5 L를 이용하여 효소당화를 수행하였다. 50.4 g/l의 총 단당류의 농도는, 120 g dw/l 꼬시래기 슬러리로부터 열산가수분해와 효소당화에 의해 총 탄수화물 60 g/l의 전환율 84.2%를 나타내었다. 꼬시래기 가수분해산물은 분리당화발효(SHF)로 에탄올 생산을 위한 기질로 사용하였다. 고농도 galactose로 순치한 *Candida lusitaniae* ATCC42720에 의한 에탄올 생산은 0.43의 에탄올 수율(Y_{EtOH})인 22.0 g/l를 생산하였다. 특정 당에 순치한 효모는 혼합당의 흡수에 유용하며, 그 결과 해조류 가수분해산물 배지로부터 높은 에탄올 수율을 나타내었다.