Thesis for the Degree of Master of Engineering

# Development of pretreatment and fermentation for bioethanol production using macroalgae

by

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# (해양거대조류의 전처리 및 발효를 통한 바이오에탄올 생산 연구)

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A dissertation

by

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# 해양거대조류의 전처리 및 발효를 통한 바이오에탄올 생산 연구

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## 요 약

최근 화석연료의 고갈로 인한 불안정한 유류 가격과 이산화탄소에 의한 환경 문제로 인해 신재생 자원으로 만든 바이오연료의 개발이 각광받고 있다. 전통적으로 옥수수, 밀 그리고 사탕수수를 사용하여 바이오연료를 만들어왔지만 원료의 높은 가격과 식용작물이라는 점에서 적합하지 못한 바이오매스였다. 따라서 대체 원료로서 짚과 나무 등 농업 폐기물이 고려되었지만 lignocellulosic biomass의 특징인 리그닌을 쉽게 분해하지 못한다는 단점을 아직도 해결하지 못하고 있는 실정이다.

해조류는 적은 경작지 사용, 빠른 성장률이라는 장점을 가지고 있다. 또한 높은 탄수화물 함량과 lignocellulosic biomass보다 훨씬 쉽게 단당으로 전환된다. 해조류는 광합성을 통한 대기 중의 이산화탄소의 감소와 산소의 증가로 환경 문제도 해결할 수 있다.

따라서, 본 연구에서는 부레옥잠과 켈프를 바이오매스로 사용하여 초고온 열산 가수분해 전처리 최적화와 효소 당화 최적화, 다양한 효모를 사용하여 바이오에탄올 생산을 수행하였다. 첫 번째로 부레옥잠을 사용하여 바이오에탄올을 생성하는 연구에서는 초고온 열산 가수분해 전처리를 통해 200 mM의 황산, 160도씨, 20분, 8% (w/v)의 부레옥잠 건조분말이 최적으로 선정되었고 생산된 당의 양은 22.2 g/L로 그 수율은 45.1%였다. 효소 당화 실험에서는 Viscozyme L이 xylose를 많이 생성하고 Cellic CTec2가 glucose를 많이 생성하는 특징을 보이며 이 두 효소를 혼합하여 20 U/ml를 사용하여 48.2 g/L의 가장 많은 단당을 생성하는 것을 확인하였고 그 수율은 97.8%였다. 발효에서는 *S. cerevisiae*, *P. stipitis* 그리고 *C. lusitaniae*를 사용하였으며 그 중에서도 xylose를 거의 소비하여 22.7 g/L의 에탄올을 생성한 *C. lusitaniae*를 최적 효모로 선정하였으며 그 수율 ($Y_{EtOH}$)은 0.47이었다.

두 번째로 켈프를 사용하여 바이오에탄올을 생성하는 연구에서는 초고온 열산 가수분해와 반응표면방법론을 사용하여 황산, 염산, 질산의 전처리 효과를 비교하였다. 그 결과 2% 질산, 8% (w/v)의 켈프 건조분말, 157도씨, 20분을 처리했을 때 17 g/L 의 가장 많은 단당이 생성되는 것을 확인하였다. 효소 당화는 Cellic CTec2와 Viscozyme L을 단일 혹은 혼합하여 사용하였으며 Hanes-Woolf plot을 통해 효소와 기질의 친화도를 Km값으로 나타내어 최적을 선정하였다. 그 결과, 12 U/ml의 혼합효소가 Km값이 0.5122로 가장 낮아서 최적으로 선정하였으며 27.3 g/L의 당이 생성되었다. 발효에서는 순치하지 않은 것과 고농도의 mannitol 배지에서 순치한 *P. stipitis*, *P. angophorae*를 사용하였으며. 그 결과 순치한 *P. angophorae*를 사용하였을 때 13.6 g/L의 에탄올을 생산하였고 수율 ($Y_{EtOH}$)은 0.50으로 나타났다.

# Chapter Ⅰ.

# Ethanol production from water hyacinth (*Eichhornia crassipes*) using hyper thermal (HT) acid hydrolysis and enzymatic saccharification by yeasts

## 1.1. Introduction

Greenhouse gas already has been exceeding to high levels of 450 ppm $CO_2$, therefore, the need for the development of alternative energy has already prompted many research projects around the world [1,2]. Many hope on renewable energy will be developed as an alternative to fossil fuels, with special attention being paid to bio-ethanol blending in gasoline [3-5].

Water hyacinth (*Eichhornia crassipes*), a water plant, is wide spreaded in the Mekong River and covers vast areas of its surface. Mechanical smearing of this water plant does not completely solve the problem since its grows every year again and the removed plant has been the cause of pollution. Water hyacinth (*Eichhornia crassipes*) biomass (WHB) has been proven as a suitable biomass for bio-ethanol production [6]. The biomass from water hyacinth has about 48% hemicellulose, 18% cellulose 3.5% lignin [7]. Though there is a significant amount of variability in composition reported by different labs. However, the biomass is considered to be rich in hemicellulose with very less lignin content, in general. The biomass productivities of this plant has been very high [8] and there is abundant availability of this plant in certain parts of the world making it a suitable feedstock for the ethanol production. Also aquatic biomass has the advantages without competition to food crops [9].

Dilute acid hydrolysis is commonly used to prepare macroalgal hydrolysate for enzymatic saccharification and fermentation [10]. One of the prerequisites for the pretreatment of *E. crassipes* is that polysaccharides from this species are hydrolyzed to monosaccharides without sugar degradation to inhibitors such as 5-hydroxymethylfurfural (HMF) and weak acids such as levulinic, acetic, and formic acids. These inhibitors can retard yeast growth and reduce ethanol productivity during fermentation [11]. Therefore, hyper thermal (HT) acid hydrolysis was evaluated to minimize the degradation of sugars into byproducts such as 5-HMF, formic acid, and levulinic acid.

In this study, bioethanol was produced from the water hyacinth (*Eichhornia crassipes*). Pretreatment was used by hyper thermal acid hydrolysis and enzymatic saccharification. Fermentation was carried out with various yeasts to evaluate the optimal fermentation conditions.

## 1.2. Materials and Methods

### 1.2.1. Water hyacinth biomass and composition analysis

Water hyacinth was obtained from Mekong river in Vietnam. Water hyacinth was dried to a constant weight at 60°C, ground using a roller mill, and sieved with a 200-mesh sieve prior to pretreatment. The composition analysis of water hyacinth was conducted by the Feed and Foods Nutrition Research Center at Pukyong National University in Korea, according to the AOAC method [12].

### 1.2.2. Hyper thermal acid hydrolysis and enzymatic saccharification

Optimization of pretreatment was carried out hyper thermal (HT) acid hydrolysis and enzymatic saccharification. HT acid hydrolysis focused on the effects of four factors: $H_2SO_4$ concentration, temperature, hydrolysis time and slurry content. HT acid hydrolysis was carried out using $H_2SO_4$ concentration ranging from 100-400 mM at 160°C for 10 min. Temperature was optimized, using the optimal condition of $H_2SO_4$ concentration ranging from 140-200°C for 45 min. Treatment time was optimized, using the optimal $H_2SO_4$ concentration and optimal temperature ranging from 5-30 min. Finally, slurry contents was optimized, using the optimal $H_2SO_4$ concentration, optimal temperature and optimal hydrolysis time ranging from 6-16% (w/v).

The enzymatic saccharification of water hyacinth was evaluated after finding the optimal conditions for HT acid hydrolysis using 8% (w/v) slurry concentration. The pH level of acid hydrolysates was adjusted to pH 5 with 10 N NaOH. Various enzymes were added at a level of 16 U/mL in 100 mL working volume in a 250-mL flask including Celluclast 1.5L (854 endo-glucanase unit (EGU)/mL), Viscozyme L (121 β-glucanase unit (FBG)/mL) and Cellic CTec2 (120 filter paper unit (FPU)/mL). Three enzymes with highly efficient saccharification were selected for mixed enzyme experiments to identify the optimal condition for the enzymatic saccharification. The saccharification reaction was performed at 50°C on a shaking incubator at 150 rpm. The efficiency of hyper thermal acid hydrolysis and enzymatic saccharification was calculated using Eq. (1) as follows:

$$E_{ps}\ (\%) = \frac{\Delta S_{ps}\ (g/L)}{TC\ (g/L)} \times 100 \qquad \text{Eq. (1)}$$

in which Eps is efficiency of hyper thermal acid hydrolysis and enzymatic saccharification (%), $\Delta S_{ps}$ is monosaccharide increase (g/L) during hyper thermal acid hydrolysis and enzymatic saccharification, TC is total carbohydrate (g/L) in water hyacinth.

### 1.2.3. Ethanol fermentation

*Saccharomyces cerevisiae* KCTC 1126 and *Pichia stipitis* KCTC 7228 were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea); *Candida lusitaniae* ATCC 42720 was obtained from the American Type Culture Collection (ATCC, Monassas, USA). These yeasts were grown in YPD medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose as a seed culture. The culture was incubated with agitation at 150 rpm for 24 h at 30°C. Each cultured yeast strains were sampled to determine the dry cell weight through the optical density ($OD_{600}$) using the standard curves of dry cell weight and $OD_{600}$.

Fermentation was evaluated in 250-mL flasks with a working volume of 100 mL. Following HT acid hydrolysis, neutralization to pH 5.0 and enzymatic saccharification were carried out. Next, nutrients were added to the fermentation medium: 2.5 g/L of $NH_4Cl$, 5 g/L of $K_2HPO_4$, 0.25 g/L of $MgSO_4$ and 2.5 g/L of yeast extract. Fermentation was carried out with *S. cerevisiae*, *P. stipitis* and *C. lusitaniae*. The fermentation with the selected yeast was performed at 30°C and 150 rpm for 72h. The yield of ethanol was calculated using Eq. (2) as follows:

$$Y_{EtOH}\ (g/g) = \frac{[EtOH]_{max}}{[Sugar]_{ini}} \qquad \text{Eq. (2)}$$

in which $Y_{EtOH}$ is ethanol yield (g/g), $[EtOH]_{max}$ is maximum ethanol concentration achieved during fermentation (g/L), $[Sugar]_{ini}$ is total initial fermentable sugar (glucose, xylose) concentration (g/L) [13].

### 1.2.4. Analytical methods

Cell growth was determined based on the optical density at 600 nm ($OD_{600}$) using an ultraviolet-visible spectrophotometer (Amersham Biosciences Ultrospec 6300 Pro, Biochrom, Cambridge, England). $OD_{600nm}$ was converted to the dry cell weight (dcw) using a standard curve of dry cell weight and $OD_{600nm}$. The pH was measured using a pH-meter (Meltler-Toledo AG, CH-8603, Schwerzenbach, Switzerland). Glucose, xylose, 5-HMF, formic acid, levulinic acid and ethanol concentrations were determined using HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID). A Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) was used with filtered and degassed 5 mmol/L $H_2SO_4$ as an eluent at a flow rate of 0.6 mL/min and a temperature of 65°C.

## 1.3. Results and Discussion

### 1.3.1. Optimization of the hyper thermal acid hydrolysis

The composition of Water hyacinth was analyzed by the AOAC method and found to contain 38.7% carbohydrate, 12.5% crude protein, 0.36% crude lipid, 19.4% crude ash, and 29.0% fiber. The total carbohydrate content of the water hyacinth used in this study was 67.7%.

The optimal conditions for $H_2SO_4$ concentration, temperature, treatment time and slurry content are shown in Fig. 1. The determination of optimal conditions was carried out with $H_2SO_4$ concentration of 100-400 mM, temperature of 140-200℃, treatment time of 5-30 min and 6-16% (w/v) slurry content.

Acid concentration is an important parameter to increase monosaccharides. The results of HT acid hydrolysis under conditions of 10% (w/v) slurry, 160℃, and treatment time of 10 min while changing the $H_2SO_4$ concentration, are shown in Fig. 1(A). When the hydrolysis reaction was carried out with an increasing $H_2SO_4$ concentration, it was noted that monosaccharide and $E_{ps}$ after treatment with 100-400 mM $H_2SO_4$ concentration were not greater than those with 200 mM $H_2SO_4$ concentration. Moreover, the levels of inhibitory compounds such as 5-HMF, formic acid, and levulinic acid slightly increased when the acid dosage increased beyond 200 mM $H_2SO_4$ concentration. Therefore, 200 mM $H_2SO_4$ concentration was selected as the suitable acid concentration with $E_{ps}$ of 30.7% for HT acid hydrolysis.

Fig. 1(B) shows that the effects of various temperatures were evaluated with 10% (w/v) slurry, 200 mM $H_2SO_4$ concentration and treatment time of 10 min. An increase in temperature from 140 to 160℃ resulted in an increase in the concentration of glucose. The highest glucose concentration of 16.3 g/L, and $E_{ps}$ of 30.7% including initial xylose of 4.5 g/L, were obtained. However, upon further increasing the temperature to 200℃, the glucose concentration and $E_{ps}$ (including initial xylose) were decreased to 10.3 g/L and 19.5%, respectively. This was probably caused by the conversion of monosaccharides to other chemicals such as 5-HMF and subsequently HMF into formic acid and levulinic acid. At high temperatures, there was a significant correlation between the degradation of monosaccharides and the formation of inhibitory compounds, as shown above 160℃. Therefore, 160℃ was chosen as the suitable temperature in this study.

Fig. 1(C) shows the effect of treatment time on monosaccharide production from water hyacinth biomass. When HT acid hydrolysis was conducted at 160℃, with 10% (w/v) slurry and 200 mM $H_2SO_4$ concentration, the monosaccharide concentration was increased with an increase of treatment time to 20 min, and then was not increased significantly after 20 min. The highest monosaccharide concentration of 24.5 g/L and $E_{ps}$ of 36.2% were obtained by HT acid hydrolysis for 20 min.

As shown in Fig. 1(D), monosaccharide concentration increased with increasing slurry content, and the maximum monosaccharide concentration at 16% (w/v) slurry content was 31.1 g/L with Eps of 32%. However, increasing the slurry content during HT acid hydrolysis resulted in the decrease of $E_{ps}$ from 45% to 32%. Therefore, 8% (w/v) of slurry content with $E_{ps}$ of 45% was selected for ethanol production.

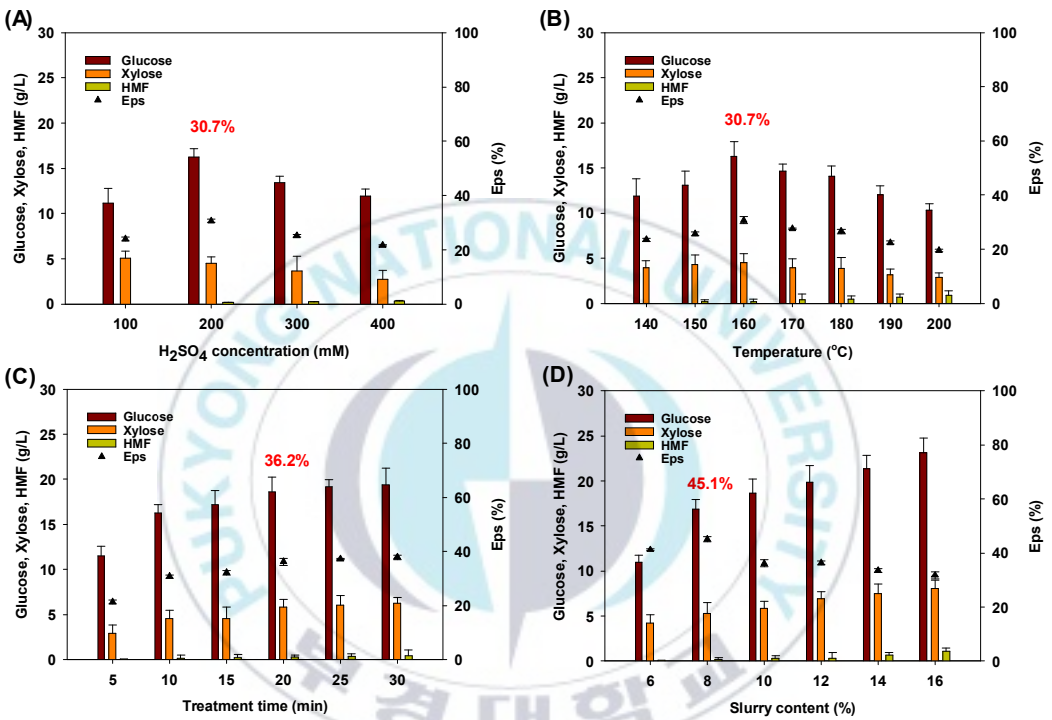


Fig. 1. Effect of optimal conditions of hyper thermal (HT) acid hydrolysis and enzyme saccharification on the degradation of inhibitory compound and monosaccharides : (A) $H_2SO_4$ concentration, (B) temperature, (C) treatment time, (D) slurry contents

### 1.3.2. Optimization of enzymatic saccharification

Enzymatic saccharification was assessed to increase the monosaccharide concentration before fermentation. Enzymatic saccharification with various enzymes and enzyme units were carried out to evaluate optimal enzymatic saccharification conditions. The effects of single enzymes (Celluclast 1.5L, Viscozyme L, Cellic CTec2) and their mixture are shown in Fig. 2(A). The maximum monosaccharide content of 41.7 g/L was obtained when an enzyme mixture was added to the pretreated hydrolysate. With the enzyme mixture, the effects of enzyme amounts were examined with 8-24 units/mL to optimize the enzyme concentration (Fig. 2(B)). Monosaccharide concentration did not show a significant difference above 20 units/mL of enzyme. Thus, 20 units/mL of the enzyme mixture was used as the optimal enzyme concentration for the enzymatic saccharification of pretreated water hyacinth slurry. Monosaccharide concentration of 48.2 g/L was obtained with 38.0 g/L glucose, and 10.2 g/L xylose. Thus, fermentation was carried out with 48.2 g/L of monosaccharide.

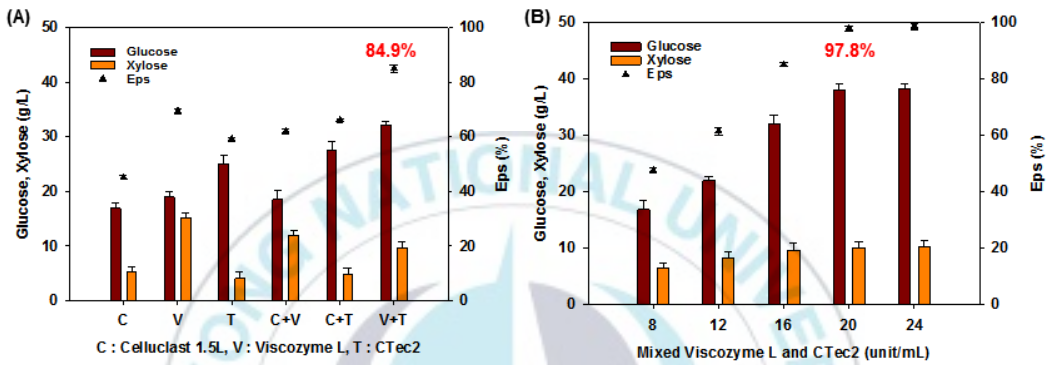


Fig. 2. Effects of single, mixed enzyme treatments (A) and various enzyme activities (B) on monosaccharides release of *E. crassipes* hydrolysate at 8% (w/v) slurry after HT acid hydrolysis at pH 5.0, 50°C for 48 h

*The initial glucose and xylose were 16.9 g/L and 5.3 g/L after HT acid hydrolysis

### 1.3.3. Ethanol fermentation

Thermal acid hydrolysis and enzymatic saccharification with a slurry content of 8% (w/v) water hyacinth were carried out to produce monosaccharides. Fermentation was carried out with *Saccharomyces cerevisiae* KCTC 1126, *Pichia stipitis* KCTC 7228 and *Candida lusitaniae* ATCC 42720 as shown in Fig. 3(A), (B) and (C), respectively.

Fermentation with *S. cerevisiae* KCTC 1126 is shown in Fig. 3(A). Among the monosaccharides, glucose was completely consumed within 48 h, however, xylose was not consumed. The maximum ethanol concentration of 15.3 g/L was produced with a $Y_{EtOH}$ of 0.32. Fermentation with *P. stipitis* KCTC 7228 as shown in Fig. 3(B). *P. stipitis* has been reported to produce ethanol from xylose and glucose. Once the fermentation started, glucose was consumed until 24 h. However, xylose was consumed very slowly until 72 h, and 6.79 g/L xylose remained. The ethanol concentration after 72 h of fermentation with *P. stipitis* was 19.5 g/L with $Y_{EtOH}$ of 0.41. Fermentation with *Candida lusitaniae* ATCC 42720 as shown in Fig. 3(C). Glucose and galactose were completely consumed within 36 and 72 h, respectively. Also, 8 g/L of xylose was utilized for the ethanol production. *C. lusitaniae* produced 22.7 g/L ethanol with $Y_{EtOH}$ of 0.47.

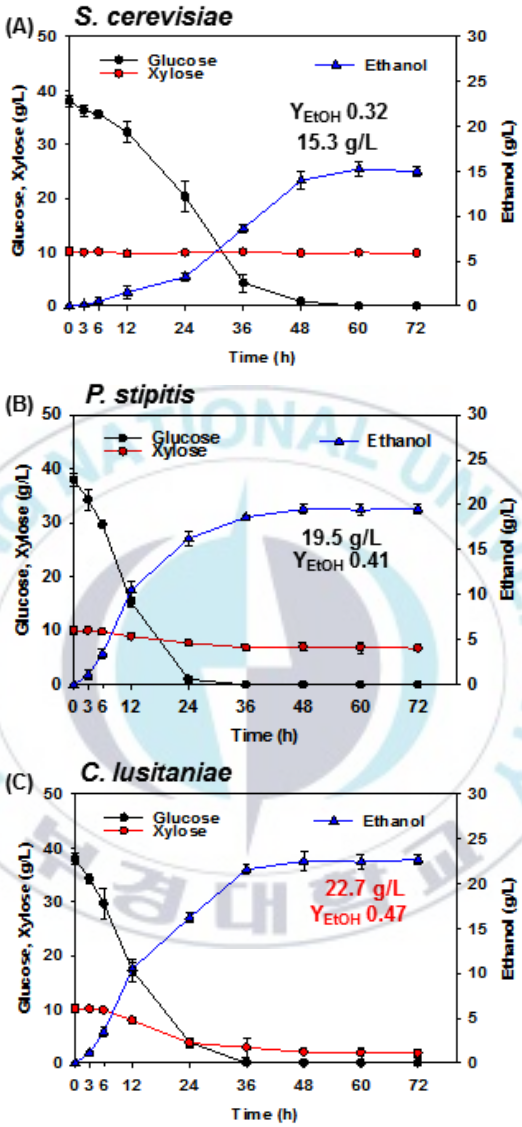


Fig. 3. Ethanol production by *S. cerevisiae* (A), *P. stipitis* (B) and *C. lusitaniae* (C) with 8% (w/v) *E. crassipes* hydrolysates at 30℃, 150 rpm for 72 hours

## 1.4. Conclusions

Water hyacinth is a promising biomass resource for the bioethanol production. The optimal HT acid hydrolysis conditions for water hyacinth were 200 mmol/L $H_2SO_4$, with 8% (w/v) slurry at 160ºC for 20 min and the optimal condition for saccharification was 20 U/mL mixture of Viscozyme L and Cellic Ctec2 at 50ºC for 48 h. The maximum ethanol concentration with *C. lusitatniae* was 22.7 g/L with $Y_{EtOH}$ of 0.47.

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# Chapter Ⅱ.

# Optimization of ethanol fermentation from *Ascophyllum nodosum* (Kelp) using hyper thermal (HT) acid hydrolysis with response surface methodology and enzymatic saccharification

## 2.1. Introduction

Currently, ethanol is an alternative transportation fuel and one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by natural energy resources such as petroleum and coal. Traditionally, the major sources of ethanol have been sucrose from sugarcane [1] and glucose from corn starch [2], however this kind of biomass can also be used as food for humans and animals, which has led to concerns about morality and increasing prices. Therefore, use of agricultural waste streams as renewable resources is preferable [3, 4]. These materials are a promising carbon source for ethanol production because of their wide availability, low cost and low desirability as food. Macroalgae is among the most promising renewable resources for biofuel production such as bioethanol, biobutanol, biodiesel, biogas, and biohydrogen [5]. Bioethanol production using macroalgal biomass is advantageous since macroalgae grow faster, and fix $CO_2$ at a higher rate than terrestrial plants. In addition, macroalgae have a high level of carbohydrates without lignin, and are easier to convert to monosaccharides than lignocellulosic biomass [6].

Like most ethanol production processes, high ethanol yield and low production costs are required by the optimization of pretreatment and fermentation processes. The response surface methodology (RSM) according to a central composite design (CCD) has been used for conditions for the determination of the optimal conditions for a multi-variable system [7, 8].

Studies on the kinetics of enzymatic hydrolysis of various biomass have been reported extensively over the past decades in order to perform a real time analysis of the hydrolysis process and to develop predictive models for the performance of the enzymes on the substrates. Michaelis-Menten's equation is mostly used in describing the behaviour of the reaction due to its simplicity and the high degree of fitness to the kinetics of most enzymes. Based on the hydrolysis reaction equation, the initial rate of hydrolysis $V_0$ can be expressed by the equations;

$$V_0 = \frac{V_{max}[S_0]}{K_m + [S_0]}$$

where $V_{max}$ is the maximum rate of hydrolysis, $S_0$ is the initial substrate concentration and $K_m$ is the Michaelis-Menten constant. The constant ($K_m$) shows the affinity or strength of the binding between the substrate and enzyme. Low values of $K_m$ indicate greater affinity of the enzyme towards the substrate, hence low substrate concentrations are required to achieve a given rate. Using a Hanes-Woolf plot ([S]/V versus [S]), all the parameters can be determined.

In this study, bioethanol was produced from the kelp (*Ascophyllum nodosum*). Pretreatment was used by hyper thermal acid hydrolysis with response surface methodology. Hanes-Woolf plot was used for the analysis of enzymatic saccharification. Fermentation was carried out with non-adapted and adapted various yeasts to evaluate the optimal fermentation conditions.

## 2.2. Materials and Methods

### 2.2.1. Seaweed and composition analysis

Kelp (*A. nodosum*) was obtained from Canada. Kelp was dried to a constant weight at 60°C, ground using a roller mill, and sieved with a 200-mesh sieve prior to pretreatment. The composition analysis of Kelp was conducted by the Feed and Foods Nutrition Research Center at Pukyong National University in Korea, according to the AOAC method [9].

### 2.2.2. Hyper thermal acid hydrolysis with response surface methodology

Hyper thermal acid hydrolysis was carried out according to a statistical experimental design program. Three factors, acid concentration ($X_1$), temperature ($X_2$), and treatment time ($X_3$), were tested with various acid concentrations (0.6 ~ 3.4%), temperatures (132 ~ 188℃), and treatment times (6 ~34 min).

Hyper thermal acid hydrolysis was performed in a 250 mL Erlenmeyer flask with a working volume of 100 mL. The slurry was then adjusted to pH 5.0 with NaOH to measure monosaccharide contents using high performance liquid chromatography (HPLC). All statistical calculations were performed with response surface methodology (RSM) using the SAS software (ver. 9.4; SAS Institute, Cary, NC, USA).

### 2.2.3. Kinetics of enzymatic saccharification

Optimal conditions for the enzymatic saccharification of total carbohydrate from kelp were determined as 16 units/mL of Viscozyme L (121 β-glucanase unit (FBG)/mL) and Cellic CTec2 (120 filter paper unit (FPU)/mL), or mixed enzymes to treat 100 g/L *A. nodosum* slurry at pH 5.0, 50°C, and 150 rpm for 48 h, after the hyper thermal acid hydrolysis.

The enzymatic saccharification reaction was conducted according to the procedure outlined above and samples were taken at different times to monitor the performance of the process. The data collected after 72 h of enzymatic saccharification was used to calculate the kinetic parameters by using the Hanes-Woolf plot.

### 2.2.4. Yeasts culture and ethanol fermentation

*Pichia stipitis* KCTC 7228 and *Pichia angophorae* KCTC 17574 were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). These yeasts were grown in YPD medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose as a seed culture. The culture was incubated with agitation at 150 rpm for 24 h at 30°C. Fermentation was performed using 100 mL of *A. nodosum* hydrolysate in a 250-mL Erlenmeyer flask under anaerobic conditions.

Enzymatic saccharification was carried out after HT acid hydrolysis for bioethanol fermentation. After the enzymatic saccharification, *A. nodosum* hydrolysates were fermented at 30℃ and 150 rpm by the addition of *Pichia stipitis* KCTC 7228 and *Pichia angophorae* KCTC 17574 adapted or not adapted to a high concentration of mannitol. The yield of ethanol was calculated using Eq. (1) as follows:

$$Y_{EtOH}\ (g/g) = \frac{[EtOH]_{max}}{[Sugar]_{ini}} \qquad \text{Eq. (1)}$$

in which $Y_{EtOH}$ is ethanol yield (g/g), $[EtOH]_{max}$ is maximum ethanol concentration achieved during fermentation (g/L), $[Sugar]_{ini}$ is total initial fermentable sugar (glucose, mannitol) concentration (g/L) [10].

### 2.2.5. Analysis

Cell growth was determined based on the optical density at 600 nm ($OD_{600}$) using an ultraviolet-visible spectrophotometer (Amersham Biosciences Ultrospec 6300 Pro, Biochrom, Cambridge, England). OD600nm was converted to the dry cell weight (dcw) using a standard curve of dry cell weight and OD600nm. The pH was measured using a pH-meter (Meltler-Toledo AG, CH-8603, Schwerzenbach, Switzerland). Glucose, mannitol, 5-HMF, formic acid, levulinic acid and ethanol concentrations were determined using HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID). A Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) was used with filtered and degassed 5 mmol/L $H_2SO_4$ as an eluent at a flow rate of 0.6 mL/min and a temperature of 65℃.

## 2.3. Results and Discussion

### 2.3.1. Pretreatment

The composition of *A. nodosum* was analyzed by the AOAC method and found to contain 66.9% carbohydrate, 5.02% crude protein, 4.18% crude lipid, 21.1% crude ash, and 2.81% fiber. The total carbohydrate content of the *A. nodosum* used in this study was 69.7%.

To optimize and evaluate the conversion of *A. nodosum* to monosaccharides, a CCD was introduced using the following three factors: HCl concentration ($X_1$), temperature ($X_2$), and treatment time ($X_3$). Table 1 and Fig. 1 shows the experimental design and the results described by monosaccharides (g/L). The highest monosaccharide of 10 g/L was obtained under treatment with 2.2% HCl at 153℃ for 21 min.

To optimize and evaluate the conversion of *A. nodosum* to monosaccharides, a CCD was introduced using the following three factors: $H_2SO_4$ concentration ($X_1$), temperature ($X_2$), and treatment time ($X_3$). Table 2 and Fig. 2 shows the experimental design and the results of monosaccharides (g/L) production by the $H_2SO_4$ treatment. The highest monosaccharide of 13 g/L was obtained under treatment with 1.8% $H_2SO_4$ at 157℃ for 17 min.

To optimize and evaluate the conversion of *A. nodosum* to monosaccharides, a CCD was introduced using the following three factors: $HNO_3$ concentration ($X_1$), temperature ($X_2$), and treatment time ($X_3$). Table 3 and Fig. 3 shows the experimental design and the results of monosaccharides (g/L) production by the $HNO_3$ treatment. The highest monosaccharide of 17 g/L was obtained under treatment with 2.0% $HNO_3$ at 157℃ for 20 min.

The optimal conditions for the thermal acid hydrolysis were determined as 8% (w/v) slurry content, 2.0% $HNO_3$, and 20 min of hyper thermal treatment at 157°C. The monosaccharide was 17 g/L including glucose and mannitol.

Table 1. Response surface level combinations of monosaccharide production in the experimental design and responses of acid ($H_2SO_4$, %), temperature (°C) and treatment time (min)

| Design point | Independent Variable | | | Dependent Variable |
|---|---|---|---|---|
| | Acid concentration (%, v/v) | Temperature (℃) | Treatment time (min) | Monosaccharide (g/L) |
| 1 | 3 | 180 | 30 | 4.09 |
| 2 | 3 | 180 | 10 | 7.29 |
| 3 | 3 | 140 | 30 | 10.98 |
| 4 | 3 | 140 | 10 | 8.87 |
| 5 | 1 | 180 | 30 | 7.28 |
| 6 | 1 | 180 | 10 | 10.36 |
| 7 | 1 | 140 | 30 | 9.50 |
| 8 | 1 | 140 | 10 | 7.50 |
| 9 | 2 | 160 | 20 | 10.42 |
| 10 | 3.4 | 160 | 20 | 8.66 |
| 11 | 0.6 | 160 | 20 | 11.10 |
| 12 | 2 | 188 | 20 | 5.00 |
| 13 | 2 | 132 | 20 | 6.39 |
| 14 | 2 | 160 | 34 | 7.39 |
| 15 | 2 | 160 | 6 | 8.42 |
| 16 | 2 | 160 | 20 | 10.44 |
| 17 | 2 | 160 | 20 | 10.07 |

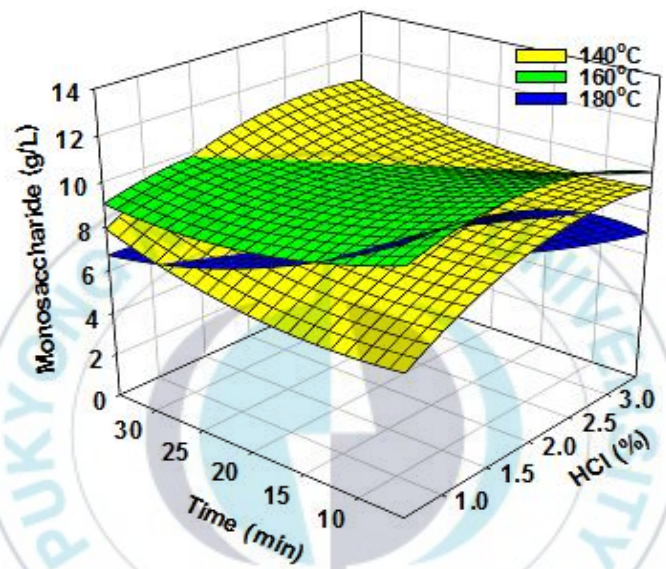


Fig. 1. Effect of sulfuric acid concentration, temperature and treatment time on monosaccharide production

Table 2. Response surface level combinations of monosaccharide production in the experimental design and responses of acid (HCl, %), temperature (°C) and treatment time (min)

| Design point | Independent Variable | | | Dependent Variable |
|---|---|---|---|---|
| | Acid concentration (% v/v) | Temperature (°C) | Treatment time (min) | Monosaccharide (g/L) |
| 1 | 3 | 180 | 30 | 3.69 |
| 2 | 3 | 180 | 10 | 4.36 |
| 3 | 3 | 140 | 30 | 7.67 |
| 4 | 3 | 140 | 10 | 6.31 |
| 5 | 1 | 180 | 30 | 4.95 |
| 6 | 1 | 180 | 10 | 6.79 |
| 7 | 1 | 140 | 30 | 8.89 |
| 8 | 1 | 140 | 10 | 9.78 |
| 9 | 2 | 160 | 20 | 13.31 |
| 10 | 3.4 | 160 | 20 | 8.20 |
| 11 | 0.6 | 160 | 20 | 7.68 |
| 12 | 2 | 188 | 20 | 3.22 |
| 13 | 2 | 132 | 20 | 4.54 |
| 14 | 2 | 160 | 34 | 10.13 |
| 15 | 2 | 160 | 6 | 11.57 |
| 16 | 2 | 160 | 20 | 13.59 |
| 17 | 2 | 160 | 20 | 13.69 |

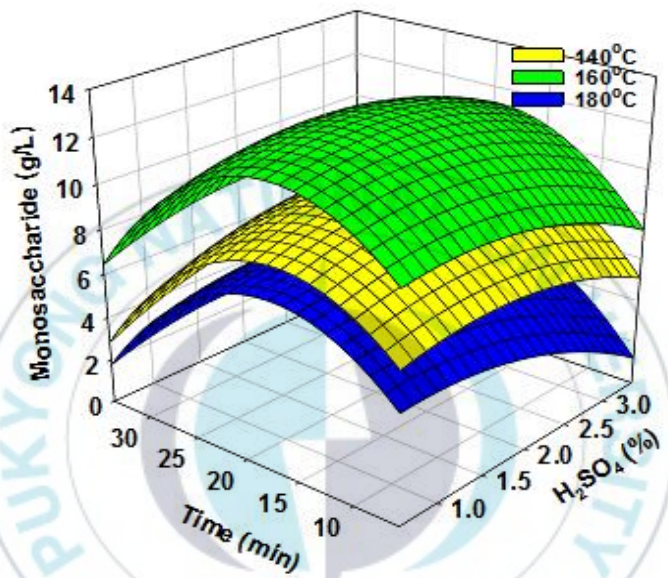


Fig. 2. Effect of hydrochloric acid concentration, temperature and treatment time on monosaccharide production

Table 3. Response surface level combinations of monosaccharide production in the experimental design and responses of acid ($HNO_3$, %), temperature (°C) and treatment time (min)

| Design point | Independent Variable | | | Dependent Variale |
|---|---|---|---|---|
| | Acid concentration (% v/v) | Temperature (°C) | Treatment time (min) | Monosaccharide (g/L) |
| 1 | 3 | 180 | 30 | 7.09 |
| 2 | 3 | 180 | 10 | 8.27 |
| 3 | 3 | 140 | 30 | 9.68 |
| 4 | 3 | 140 | 10 | 11.38 |
| 5 | 1 | 180 | 30 | 6.33 |
| 6 | 1 | 180 | 10 | 7.98 |
| 7 | 1 | 140 | 30 | 9.93 |
| 8 | 1 | 140 | 10 | 10.77 |
| 9 | 2 | 160 | 20 | 17.96 |
| 10 | 3.4 | 160 | 20 | 12.14 |
| 11 | 0.6 | 160 | 20 | 9.31 |
| 12 | 2 | 188 | 20 | 8.12 |
| 13 | 2 | 132 | 20 | 10.05 |
| 14 | 2 | 160 | 34 | 13.51 |
| 15 | 2 | 160 | 6 | 10.21 |
| 16 | 2 | 160 | 20 | 17.9 |
| 17 | 2 | 160 | 20 | 17.5 |

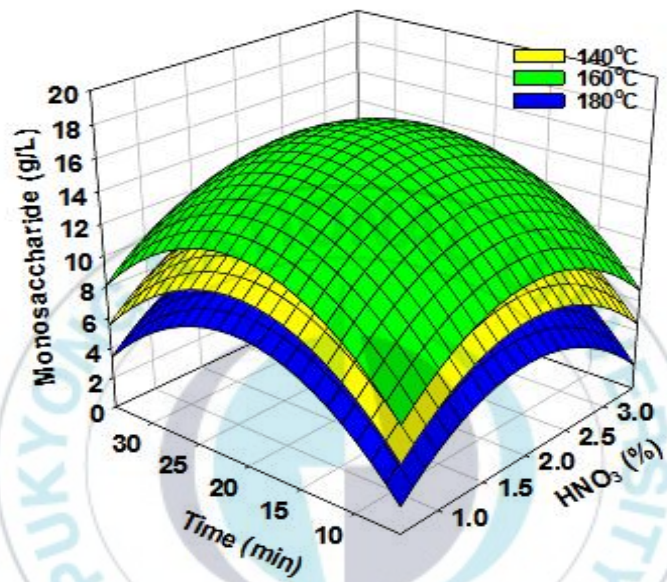


Fig. 3. Effect of nitric acid concentration, temperature and treatment time on monosaccharide production

### 2.3.2. Enzymatic saccharification

Enzymatic saccharification was evaluated to increase the monosaccharide concentration before fermentation. Enzymatic saccharification with various enzymes and enzyme units were carried out to evaluate optimal enzymatic saccharification conditions.

The kinetic parameters were calculated using Hanes-Woolf plot (Fig. 4(A)). [S] is the substrate concentration, V is the rate of the reaction, $V_{max}$ is the maximum rate and $K_m$ is the Michaelis constant. Low $K_m$ value of enzyme indicates high affinities to substrate and high $K_m$ value means weak affinities to substrate. The mixed enzyme of Viscozyme L and Cellic CTec2 with $K_m$ value was 0.5122, which is smaller than Viscozyme L with 1.6328 and Cellic CTec2 with 1.3442. Thus, enzyme mixture was selected for the further experiment.

The effects of enzyme units of enzyme mixture were determined with 4-20 units/mL to evaluate the enzyme concentration (Fig. 4(B)). Glucose concentration did not show a significant difference above 12 units/mL of enzyme. Thus, 12 units/mL of the enzyme mixture was selected as the optimal enzyme concentration for the enzymatic saccharification. Monosaccharide concentration of 27.3 g/L was obtained: 20.3 g/L glucose, and 7.0 g/L mannitol. Thus, fermentation was carried out with 27.3 g/L of monosaccharide.

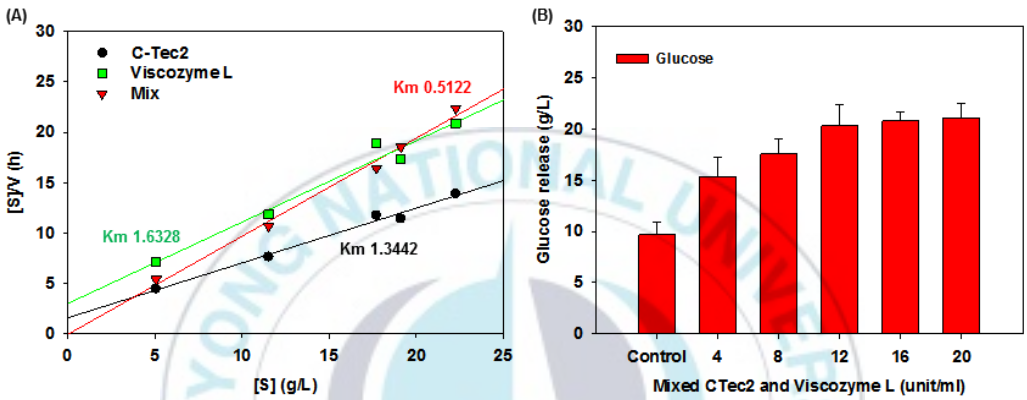


Fig. 4. Effects of single, mixed enzyme treatments (A) and various enzyme activities (B) on glucose release of *A. nodosum* hydrolysate at 10% (w/v) slurry after HT acid hydrolysis at pH 5.0, 50°C for 48 h

*The initial glucose was 9.7 g/L after HT acid hydrolysis

### 2.3.3. Fermentation

Fig. 5(A) shows the results of fermentation using non-adapted *P. stipitis*. The glucose and mannitol concentrations at the start of fermentation were 20.3 g/L and 7.0 g/L, respectively. Glucose was consumed during the initial 36 h and mannitol was not consumed until 72 h. The ethanol concentration after 72 h of fermentation with non-adapted *P. stipitis* was 8.2 g/L, with $Y_{EtOH}$ of 0.30. Fig. 5(B) shows the results of fermentation with *P. stipitis* adapted to mannitol. Glucose was consumed after 24 h, and mannitol was consumed after 36 h. The final ethanol concentration was 12.5 g/L with $Y_{EtOH}$ of 0.46. Fig. 5(C) shows the results of fermentation using non-adapted *P. angophorae*. Glucose was consumed during the initial 36 h and mannitol was not consumed until 72 h. The ethanol concentration after 72 h of fermentation with non-adapted *P. angophorae* was 9.6 g/L, with $Y_{EtOH}$ of 0.36. Fig. 5(D) shows the results of fermentation with *P. angophorae* adapted to mannitol. Glucose was consumed after 24 h, and mannitol was almost consumed after 24 h. The final ethanol concentration was 13.6 g/L with $Y_{EtOH}$ of 0.50. Therefore, the adaptation of *P. angophorae* to high concentrations of mannitol is important to increase the ethanol yield of ethanol from *A. nodosum*.

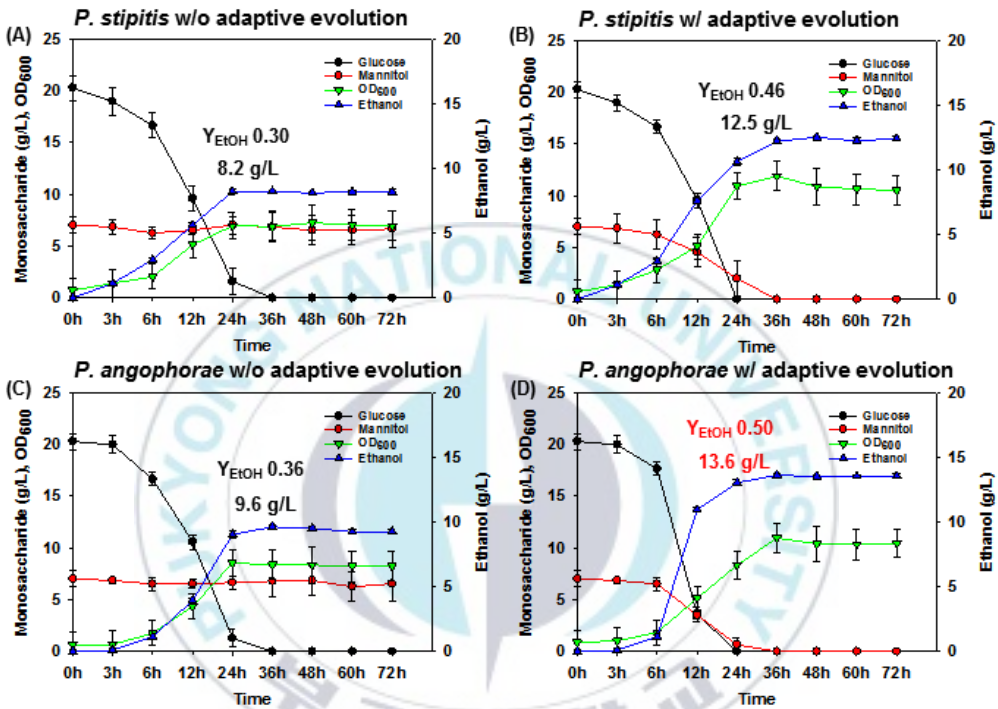


Fig. 5. Ethanol production by *P. stipitis* w/o adaptive evolution (A) and *P. stipitis* w/ adaptive evolution (B), *P. angophorae* w/o adaptive evolution (C) and *P. angophorae* w/ adaptive evolution (D) with 10% (w/v) *A. nodosum* hydrolysates at 30°C, 150 rpm for 72 hours

## 2.4. Conclusions

Kelp (*A. nodosum*) is a promising biomass resource for bioethanol production. The optimal pretreatment conditions of kelp were 2.0 mmol/L $HNO_3$ at 157ºC for 20 min and the optimal conditions for enzyme saccharification were 12 U/mL mixture of Viscozyme L and Cellic Ctec-2 at 50ºC for 48 h. *P. angophorae* adapted to high concentrations of mannitol produced high ethanol yield compared to that of the non-adapted strain. The maximum ethanol concentration with *P. angophorae* adapted to the high concentration of mannitol was 13.6 g/L with $Y_{EtOH}$ of 0.50.

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