Thesis for the Degree of Master of Engineering

Studies on bioethanol production from macroalgae, *Saccharina japonica* and *Eucheuma spinosum*

by

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August 2013

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(거대 조류, 다시마와 유큐마로부터 바이오 에탄올 생산에 관한 연구)

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A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

In Department of Biotechnology, The Graduate School,
Pukyong National University

August 2013

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August 2013

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거대 조류, 다시마와 유큐마로부터
바이오 에탄올 생산에 관한 연구

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요 약

화석연료의 과다 사용으로 지구 온난화 및 환경오염 등의 문제가 발생하여 세계 각국은 새로운 재생 가능한 에너지원 개발에 많은 노력을 기울이고 있다. 이러한 상황에서 바이오 에너지 (bioenergy)는 급등하는 유가로 인한 경제문제와 화석연료의 사용으로부터 발생하는 환경문제를 줄일 수 있는 대안으로 대두되고 있다. 특히, 바이오 에탄올은 화석연료와 혼합하거나 대체하여 사용할 수 있으며, 이산화탄소의 순환으로 친환경적이고 지속 가능한 에너지 생산이 가능하다. 바이오 에탄올을 생산하기 위해 곡류, 목재 등 다양한 바이오 매스가 사용 되고 있는데, 곡류는 단가가 비싸고 식량이라는 도덕적 측면에서 문제가 되고 있고, 목재는 아직도 리그닌에 의한 셀룰로스의 분해 저해 및 바이오 매스 확보에 문제가 있다. 제 3 세대 바이오 매스로서 해조류는 성장이 빠르며, 육지 식물과 비교하여 단위 면적당 생산량이 많다는 장점이 있다.

본 연구에서는 다시마를 바이오매스로 한 산촉매 열 가수분해 조건의 최적화와 이스트 순치를 통한 5L 발효기에서 바이오에탄올 생산을 수행하였다. 당화최적화를 위하여 37.5mM 황산을 10% (w/v)의 슬러리 파우더에 60 분간 121℃

열 처리하였다. 50%의 당화수율을 얻었으며, 점도는 17.6cp 를 나타내었다. 최적전처리 조건을 설정하고 5L 발효기에서 에탄올 생산을 수행하였다. YPD 배지에서 효모를 배양 하고 에탄올 생산을 하였을 때 130 시간이 지나도 3 g/L 의 에탄올을 생산하였다. 그래서 다시마에 주 탄수화물인 만니톨에 미리 효모를 순치하여 에탄올 생산을 수행하였다. 그 결과 YPM 배지에서는 96 시간 동안 5 g/L 의 에탄올을 생산하였고, 고농도 YPM 배지에서 순치한 효모를 이용한 발효에서는 14 g/L 의 에탄올을 생산하였다. 효모를 순치를 하여 에탄올 발효를 하였을 때 에탄올 생산을 하였을 때 에탄올 생산 수율이 증가하는 것을 볼 수 있었다.

인도네시아에서 생산이 되는 *Eucheuma spinosum* 을 이용한 바이오에탄올 생산을 하였다. 당화최적은 150mM 황산에 11% (w/v)의 슬러리 파우더를 첨가하여 40 분간 121℃에서 열처리를 하였다. 당화 결과 7 g/L 의 글루코오스와 30 g/L 의 갈락토오스가 생산되었다. 그리고 발효저해물질 5-HMF 가 5.7 g/L 가 생산되었다. 발효저해물질 5-HMF 를 제거하기 위해 6% (w/v) 활성탄을 사용하여 30 분간 실온에서 반응 했을 때 0.8 g/L 로 제거되었다. 활성탄으로 5-HMF 를 제거하고 *Kluyveromyces marxianu* 를 이용하여 에탄올 생산을 하였을 때 17 g/L 의 에탄올이 생산되었다. 5-HMF 유무에 따라 바이오에탄올 생산에 영향을 미치는 것을 알 수 있었다.

Chapter. 1. Bioethanol production from *Saccharina japonica* with mannitol acclimation *Pichia angophorae*

1.1. INTRODUCTION

Bioethanol has attracted attention as an alternative to petroleum-derive fuel. Many studies have been performed on the production of ethanol from lignocellulosic materials, such as wood, sugarcane bagasse, switchgrass, rice straw and wheat straw [1]. Marin biomass is one of the most promising candidates to be able to replace agriculture feedstocks. They have high productivity, high CO_2 capture capacity and lignin-free composition; moreover, they would not compromise food supply and cause a serious environmental issue since they do not require any arable land [2]. Seaweeds are classified into three groups : brown, green and red and contain various types of monosaccharide. Brown seaweed especially the species *Saccharina japonica* (Sea tangle, Dasima) is extensively cultured in China, Japan and Korea [3]. *S. japonica* may have a high content of alginat, laminaran and mannitol [4]. Alginate is composed of D-mannuronate and its C5-epimer, L-guluronate, which linked by β-(1,4) glycosidic linkage [5]. Laminaran is a linear poly saccharide of β-(1,3)-D-glucose in which the chain terminates with D-mannitol with low levels of branching at β-(1,6)-glycosidic linkages [6]. Mannitol, a sugar alcohol derived from mannose, is also one of the main sugar components of brown seaweed [3]. Using the fermentation major carbohydrate is mannitol

of *S. japonica*. Mannitol can be easily dissolved from *S. japonica*, so that there would be no need of saccharification or pretreatment [7]. Mannitol, the sugar alcohol corresponding to mannose, however this is not readily fermented. The *Pichia angophorae* have a broad substrate range and can convert mannitol to ethanol. Jang *et al,*.[3] reported that *P. angophorae* produced 7.7 g/L ethanol and 33.3 % theoretical ethanol yield of total carbohydrate from *S. japonica*. This result shows *P. angophorae* can uptake mannitol and ethanol can be produced from *S. japonica*. It is suitable organism for ethanol production from brown seaweed. Also it can utilize both mannitol and glucose, and is not inhibited before the substrate is consumed [8]. However ethanol fermentation from mannitol is different ethanol production from glucose. It oxidized to fructose by mannitol dehydrogenase, a reaction that generates NADH. Regeneration of NAD^+ requires oxygen (active electron transport chain) or transhydrogenase, which converts NADH (Fig. 1). Thus fermentation of sugar alcohols by yeast requires a supply of oxygen [9], while a too high aeration resulted in the production of organic acid [8].

The purpose of this work was ethanol production from mannitol and glucose of brown algae *S. japonica* in 5L bioreactor. First pretreatment was carried out with sulfuric acid and saccharifiacation was used Celluclast 1.5L. Fermentation was performed that yeast *P. angophorae* can uptake mannitol and glucose. To improve ethanol production in 5L bioreactor from mannitol, yeast was already acclimated in YPM and high concentration YPM.

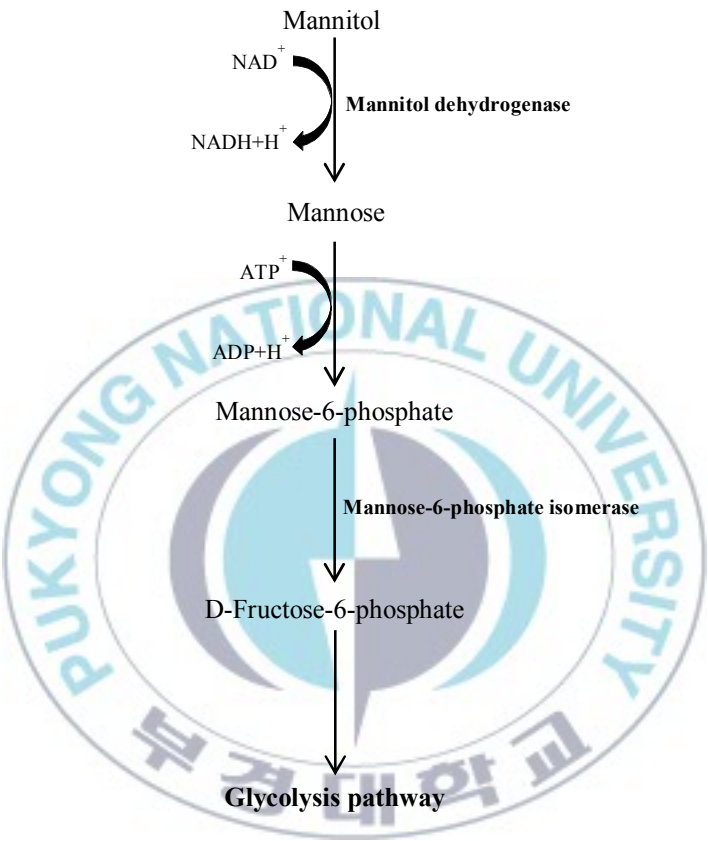

Fig. 1. Proposed pathway of mannitol metabolism for into the glycolysis

1.2. MATERIALS & METHODS

1.2.1. Raw material

The seaweed, *Saccharina japonica* was obtained from Gijang Local Products Co., Ltd.in Busan, Korea. Sample were dried at natural sun drying and ground by hammer mill. The powder was filtered through a 200-mesh sieve prior to pretreatment. Composition and proximate analysis of *S. japonica* was done by the Feed and Foods Nutrition Research Center at Pukyung National Univerity in Korea. *S. japonica* was contained carbohydrate of 54.2%, fiber of 5.7%, Lipid of 1.8%, protein of 10.1%, ash of 23.5% and water of 4.8%. As a result total carbohydrate was 59.9%

1.2.2. Thermal acid hydrolysis

Thermal acid hydrolysis was used sulfuric acid. The optimal condition of thermal acid hydrolysis was evaluated with various slurry contents (10-20%, w/v) and various sulfuric acid concentrations and hydrochloric acid (37.5-187.5 mM) at 121℃ for 60 min. Reducing sugar was measured DNS method and viscosity was used Brookfield viscometer.

1.2.3 Saccharification

Saccharification was used commercial enzyme with Celluclast 1.5L (8.4 FPU/ml, Novozymes. Inc., Denmark). The thermal acid hydrolysis without enzyme treatment was carried out 10% (w/v) seaweed slurry and 37.5 mM H_2SO_4 at 121℃ for 60 min. After

thermal acid hydrolysis was done, Media was adjusted to pH 4.8 by 10N NaOH. Celluclast 1.5L of 8.4 FPU/ml was added to 1% (w/v) in seaweed slurry. Enzyme reaction was carried out at 45℃ for 24 h.

1.2.4. Non-acclimated and acclimated *P. angophorae* culture

Yeast *Pichia angophorae* KCTC 17574 was used for ethanol fermentation. Non-acclimated yeast was grown in YPD broth (dextrose 20.0 g/L, yeast extract 10 g/L, peptone 20 g/L). Acclimated yeast was grown in YPM broth (mannitol 20.0 g/L, yeast extract 10 g/L, peptone 20.0g/L) and High concentration YPM broth (mannitol 120.0 g/L, yeast extract 10 g/L, peptone 20.0g/L). YPM was added to mannitol instead of dextrose. Also high concentration YPM was added to 120 g/L mannitol. The yeast cells for fermentation was harvested by centrifugation at 4℃ and 20 min and washed with distillation water.

1.2.5. Separate hydrolysis and fermentation (SHF) by 5L-fermentation

Ethanol fermentation was carried out by SHF in 5L bioreactor with working volume 2.5L. After the thermal acid hydrolysis with 10% (w/v) slurry and 37.5 mM H_2SO_4 at 121℃ for 60 min, the seaweed slurry was neutralized with 10 N NaOH. Saccahrification was performed with 1% Celluclast 1.5L at pH 4.8 and 45℃ for 24 h. When saccharification was done, seaweed slurry was adjusted to pH 6.8. And fermentation for cell growth was added to nutrient: yeast extract 5 g/L, $(NH_4)_2SO_4$ 7.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.75 g/L, K_2HPO_4 3.5 g/L,

$CaCl_2 \cdot 2H_2O$ 1 g/L. Non-acclimated yeast was cultured in YPD broth and acclimated yeast was cultured in YPM and high concentration YPM, respectively. Fermentation was inoculated with 0.5 g dcw/L *P. angophorae* at 30℃ and 200 rpm. Fermentation was carried out anaerobic condition. However, mannitol fermentation needed micro oxygen. Therefore aeration was injected with 0.06 vvm during fermentation.

1.2.6. Analysis

Reducing sugar was measured DNS method and viscosity was used Brookfield viscometer. Monosaccharide and ethanol was determined by HPLC (Agilent 1100 Series, Agilent. Inc., USA). Samples were filtered through a 0.2 ㎛ filter. HPLC instrument equipped with an Agilent G1362A refractive index detectoer (RID) detector. A Bio-rad Aminex HPX-87H column (300 x 7.8 mm) and Superguard C610H column (50 x 4.6 mm) were used with filtered and degassed 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min and a column temperature of 60℃.

1.3. RESULTS & DISCUSSION

1.3.1. Thermal acid hydrolysis from *S. japonica*

Pretreatment was carried out using *S. japonica* for ethanol. Seaweed with different sulfuric acid concentrations and slurry contents of solid were pretreated at ranges of 37.5 mM-187.5 mM and 10%-20% (w/v) for 60min at 121℃. The reducing sugar yield of sulfuric acid and hydrochloric acid treatment was related to acid concentration and slurry contents as shown in Fig. 2. Reducing sugar average yield of 42.2% and the highest yield of 50% at 20% (w/v) slurry and 150 mM sulfuric acid was obtained with sulfuric acid treatment. However reducing sugar yield of hydrochloric acid treatment was obtained average 26% and the highest yield of 35%. Reducing sugar yield of sulfuric acid was 16% higher than hydrochloric acid. Sulfuric acid was dissolved better than hydrochloric acid on characteristic of biomass structure. When *Gelidium amansii* treated with sulfuric acid and hydrochloric acid, reducing sugar was sulfuric acid treatment higher than hydrochloric acid treatment. However sulfuric acid treatment effected better than hydrochloric acid because of organic acid was increased by extending the treatment time and increasing hydrochloric acid concentration [10]. Also viscosity was measured after sulfuric acid and hydrochloric acid treatment as shown in Fig. 1(A) and Fig. 1(B). Viscosity was under 50 cP on 10% (w/v) and 15% (w/v) slurry with treated sulfuric acid. However viscosity from was increased at 20% (w/v) *S. japonica* slurry. Viscosity of using hydrochloric acid pretreatment was much higher than using sulfuric acid pretreatment. Viscosity was important factor on fermentation using

S. japonica. Because high viscosity of alginate from *S. japonica*, ethanol fermentation could be influenced by stir inhibition. Therefore pretreatment considered high reducing sugar yield and low viscosity condition. Pretreatment condition was determined with 10% (w/v) slurry and 37.5 mM sulfuric acid at 121℃ for 60 min. This condition was obtained reducing sugar yield of 44% and viscosity of 17 cP (Table 1.).

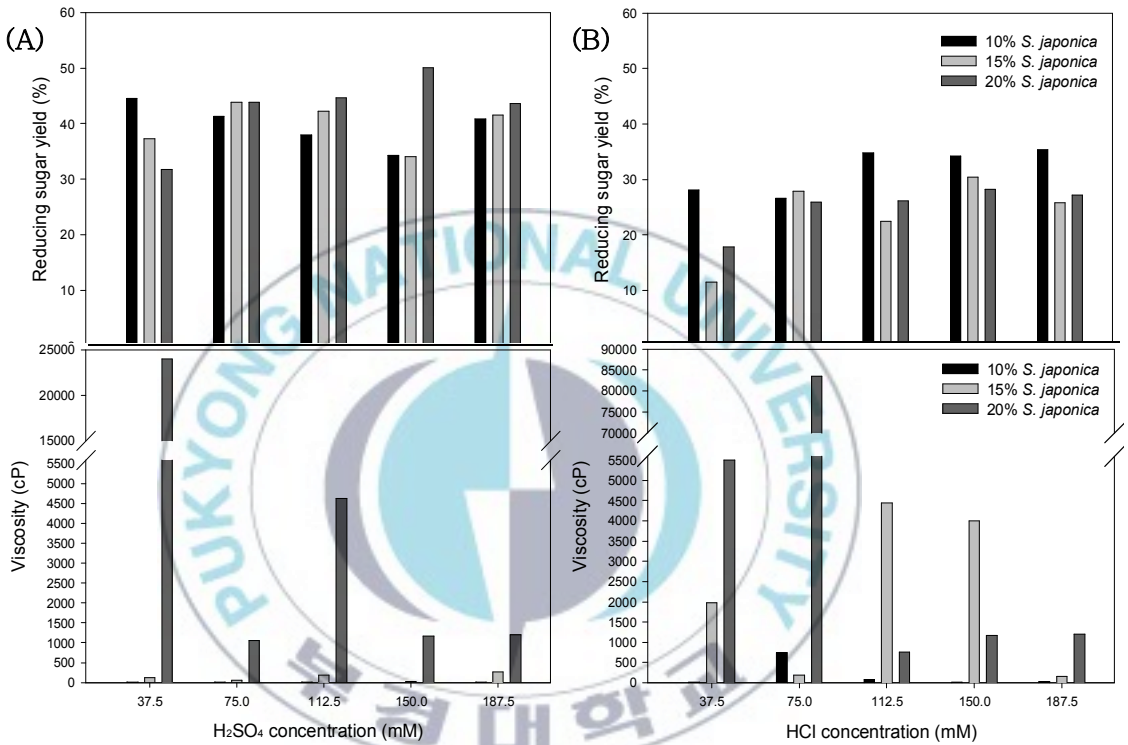

Fig.2. Effect of sulfuric acid and hydrochloric acid concentrations and slurry contents on reducing sugar yields and viscosities after thermal acid hydrolysis: (A) reducing sugar yields and viscosities treated by H_2SO_4 (B) reducing sugar yields and viscosities treated by HCl

Table 1. Summary of reducing sugar yield and viscosity by thermal acid hydrolysis

Acid	Concentration (mM)	Reducing sugar yield (%)			Viscosity (cP)		
		10%	15%	20%	10%	15%	20%
H_2SO_4	37.5mM	**44.5**	37.3	31.7	**17.6**	117.4	23994.8
	75mM	41.3	43.8	43.8	17.6	58.8	1052.7
	112.5mM	37.9	42.2	44.6	8.1	190.7	4631.1
	150mM	34.8	34.1	50.0	4.8	23.0	1166.0
	187.5mM	40.8	41.5	43.6	8.3	260.9	1196.0
HCl	37.5mM	28.1	11.5	17.8	24.9	1979.5	5506.8
	75mM	26.5	27.8	25.9	740.2	189.9	83502.1
	112.5mM	34.8	22.4	26.1	77.0	4439.0	761.8
	150mM	34.1	30.3	28.2	18.87	4007.1	1166.7
	187.5mM	35.3	25.7	27.1	27.8	162.9	1196.7

1.3.2. Separate hydrolysis and fermentation (SHF) using *Pichia angophorae*

Ethanol fermentation was carried out with non-acclimated and acclimated *P. angophorae* in 5L bioreactor. Non-acclimated *P. angophorae* was cultured in YPD broth. On the other hands, acclimated *P. angophorae* was cultured in YPM and high concentration YPM which is obtained sugar of *S. japonica*. Also ethanol fermentation was injected air of 0.06 vvm. Because mannitol become mannose by mannitol dehydrogenase that NAD^+ was reduced NADH. When NAD^+ was reduced NADH needed oxygen for regeneration of NAD^+. And mannose was changed D-fructose-6-phosphate by mannose-6-phosphate isomerase, D-fructose-6-phosphate was entered glycolysis pathway (Fig. 1). Thus mannitol fermentation is important oxygen concentration. Ethanol fermentation from *S. japonica* was carried out with non-acclimated *P. angophorae* (Fig. 3.). It was uptake glucose of 6 g/L in 12 h and produced ethanol of 3 g/L during 132 h. *P. angophorae* not consumed mannitol of 20 g/L during 132 h. Ethanol production was only 3 g/L by monosaccharide of 16 g/L. The theoretical ethanol yield of total carbohydrate was obtained 9.8% (Table 2). To ethanol concentration, ethanol fermentation was carried out with mannitol acclimated *P. angophorae* in 5L bioreactor. Previous report showed that adaptation of these yeasts to wood hydrolysate solutions by recycling resulted in improved substrate utilization and ethanol production [11]. Ethanol fermentation was performed with mannitol acclimaed *P. angophorae* (Fig. 4.). Mannitol acclimated *P. angophorae* produced 5.2 g/L ethanol during

96 h. Also mannitol uptake rate was faster than non-acclimated *P.angophorae*. However mannitol *P. angophorae* was not completely consumed mannitol. Therefor to improved ethanol production ability, ethanol fermentation was carried out with high concentration YPM acclimated *P. angophorae*. Ethanol fermentation with high concentration mannitol acclimated *P. angophorae* was obtained ethanol of 14 g/L that used monosaccharide of 30 g/L (Fig. 5). Ethanol production and sugar utilization was improved when yeast adapted in specific sugar. It is possible that acclimatization of cells to the fermentation medium would have shortened the lag phase, facilitating early entry of adapted cell into the logarithmic phase [12]. Acclimation of cells to the medium conditions and stresses caused by the presence of inhibitors in the medium plat an important role, as the cells acclimatize to the fermentation conditions with time [13]. Some authors have proposed that adaptation of a microorganism under such circumstances might be ascribed to the synthesis of new enzymes or cofactors that allow more efficient sugar metabolism and reduce the effects of any inhibitors [14]. As the result, theoretical ethanol yield of total carbohydrate of acclimated *P. angophorae* in YPM and high concentration YPM was obtained 16.4% and 46.7% (Table 2.). This study showed that acclimation was increased sugar utilization and ethanol production ability.

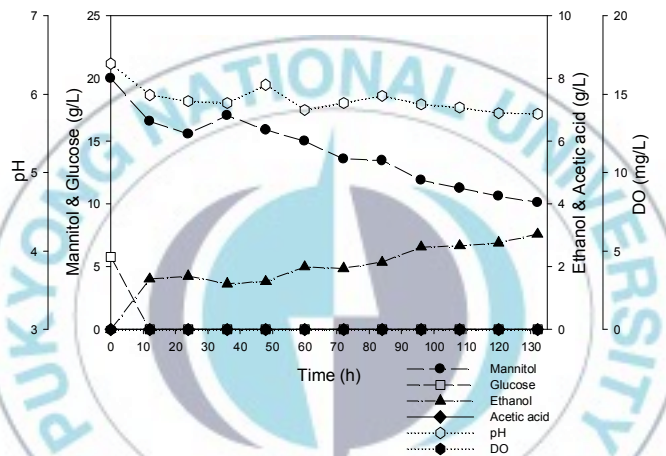

Fig. 3. Ethanol production from *Saccharina japonica* by SSF with non-acclimated *P. angophorae* in 5L bioreactor; Mannitol (●), Glucose (□), Ethanol (▲), Acetic acid (◆) , pH (◇), DO (♦)

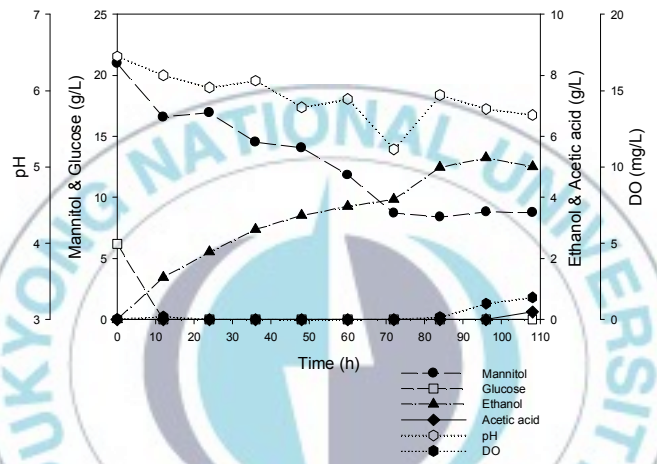

Fig. 4. Ethanol production from *Saccharina japonica* by SSF with mannitol acclimated *P. angophorae* in 5L bioreactor; Mannitol (●), Glucose (□), Ethanol (▲), Acetic acid (◆) , pH (◇), DO (♦)

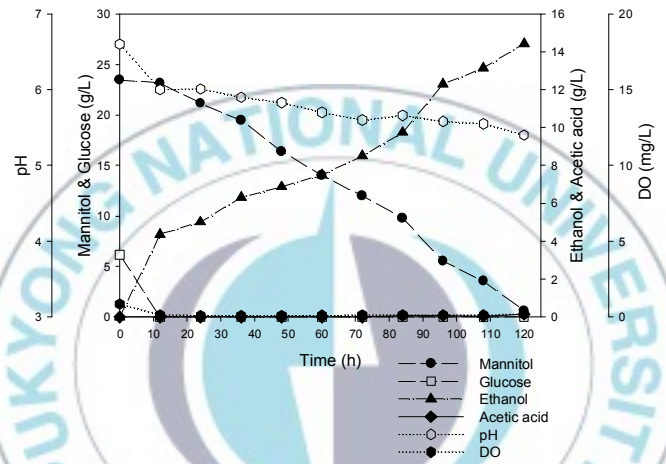

Fig. 5. Ethanol production from *Saccharina japonica* by SSF with high concentration mannitol acclimated *P. angophorae* in 5L bioreactor; Mannitol (●), Glucose (□), Ethanol (▲), Acetic acid (◆) , pH (◇), DO (♦)

Table 2. Ethanol theoretical yield of total carbohydrate

Fermentation	Non acclimated *P. angophorae*	YPM acclimated *P. angophorae*	High YPM acclimated *P. angophorae*
Ethanol theoretical yield of total carbohydrate (%)	9.8	16.4	46.7

1.4. CONCLUSION

The seaweed, *Saccharina japonica*, is a promising substitute biomass for the bioethanol production due to its rapid growth and high productivity. For the pretreatment by thermal acid hydrolysis, acid concentration and the solid contents of slurry affecting the monosaccharide yield and viscosity were studied. The thermal acid hydrolysis conditions were 10% (w/v) slurry and 37.5 mM sulfuric acid at 121℃ for 60 min. Reducing sugar yield of 44% and low viscosity of 17 cP were obtained. *Pichia angophorae* KCTC 17574 was acclimated to mannitol for enhanced ethanol production. The highest ethanol concentration of 14 g/L corresponding to 46.7% of theoretical yield of total carbohydrate was achieved in SHF by high mannitol acclimated *P. angophorae*. Whereas *P. angophorae* that cultured YPD was obtained 3 g/L of ethanol concentration corresponding to 16.4% of theoretical yield of total carbohydrate. Therefore, yeast acclimation to high concentration of specific sugar was effective in producing ethanol from seaweed.

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Chapter. 2. Bioethanol production from *Eucheuma spinosum* and reduction of toxicity using active charcoal

2.1. INTORDUCTION

Bioethanol has attracted attention as an alternative to petroleum-derived fuel. The efficient production of ethanol from inedible biomass that people do not find palatable has long been considered a crucial requirement for the effective utilization of these materials [1]. Bioethanol, a clean and renewable energy source, which can be produced through fermentation from renewable biomass, has drawn much attention from the government and researchers [2]. Also ethanol blended gasoline has the potential to contribute significantly to reduce these emissions [5]. Macroalgae, an abundant and carbon-neutral renewable resource, are now considered as a third-generation biomass that can be used bioenergy [3]. Generally, macroalgae grows faster compared area for mass cultivation [6]. This biomass, with several species rich in carbohydrates and is known to contain low lignin or no lignin at all, is suitable for bioethanol production [4]. Red seaweeds, especially the species *Eucheuma spinosum* is used as a feedstock for bioethanol production, the estimated bioethanol yields could reach up to 110,000 tons annually [7]. The most common polysaccharides from seaweeds are galactans (agar and carrageenan) from red seaweeds [8]. Galactans consist

entirely of galactose and 3, 6-anhydrogalactose. The substitution pattern of the sulfate groups and the amount of 3, 6-anhydrogalactose vary in different general, influenced by ecological conditions [9]. In general, bioethanol production from biomass involves pretreatment, enzymatic hydrolysis, fermentation and distillation. The saccharification of macroalgae is important prior to ethanol fermentation. Various physical, chemical and biological pretreatments have been studied to increase the saccharification efficiency [10]. Especially, Dilute-acid hydrolysis has been proven to be a fast and cheap method for producing the sugars. One problem associated with dilute-acid hydrolysis is the formation of toxic compounds such as furans and phenolic compound [11]. Furfural and 5-hydroxymethyl furfural (HMF) are two furan derivatives which are formed by further hydrolysis of the sugars, pentose and hexoses, respectively. These compounds damage microorganisms by reducing enzymatic and biological activities, breaking down DNA, and inhibiting protein and RNA synthesis [12]. Also inhibitive effect of HMF extended lag phase during the growth of microorganism cells [13]. Inhibition of these inhibitors can be avoided either by different detoxification methods prior to fermentation and by or in situ detoxification by yeast [15]. Ethanologenic microbes have developed a mechanism to relieve HMF inhibition by reducing aldehydes to the less toxic alcohols, furfuryl alcohol and 5-hydroxymethyl furfural alcohol [14]. Various detoxification methods have been studied such as extraction with organic solvents, overliming, evaporation, steam stripping, sulfite treatment, ion-exchange, enzyme treatment, aeolite treatment and activated carbon

treatment. An effort was made to develop active charcoals to remove the inhibitors of the fermentations in the hydrolysates for enhancing the fermentability [16].

In this study monosaccharide production from *E. spinosum* was optimized by thermal acid hydrolysis and enzyme hydrolysis. Active charcoal treatment was carried out to remove HMF. Yeast, *Kluyveromyces marxianus* KCTC 7150, was used for ethanol fermentation. Ethanol production by SHF process was compared to existence or not existence HMF. And fermentation was carried out by 5L bioreactor.

2.2. MATERIALS & METHODS

2.2.1. Raw material

The seaweed, *Eucheuma spinosum* was obtained from Indonesia. Sample were dried at natural sun drying and ground by hammer mill. The powder was filtered through a 200-mesh sieve prior to pretreatment. Composition and proximate analysis of *E. spinosum* was done by the Feed and Foods Nutrition Research Center at Pukyong National University in Korea. *E. spinosum* was contained carbohydrate of 64.7%, fiber of 4.7%, lipid of 4.6%, protein of 4.5%, ash of 18.1% and water of 17.3%. As a result total carbohydrate was 69.4% (Table 1.)

Table 1. Composition of *Eucheuma spinosum*

Carbohydrate	Fiber	Lipid	Protein	Ash	Water
64.7%	4.7%	4.6%	4.5%	18.1%	17.3%

2.2.2. Thermal acid hydrolysis

Thermal acid hydrolysis was used sulfuric acid. The optimal condition of thermal acid hydrolysis was evaluated with various slurry contents (5-11%, w/v) and various sulfuric acid concentrations (37.5-150 mM) at 121℃ for 40 min. Sample was centrifuged at 12000 rpm for 10min. The supernatant obtained after centrifugation was then analyzed for galactose, glucose and HMF by HPLC.

2.2.3. Detoxification

After pretreatment, active charcoal (Samchun., Inc.) was used for remove HMF. Particle size was 4-8 mesh and pore size was 1.5-4 nm. Test for remove HMF was added with active charcoal (3-6%) and stirred for 2hr. The mixture was centrifugated at 12000 rpm for 10 min. Sugar and 5-HMF was estimated before and after detoxification process by HPLC. The treated hydrolysate was then used for the fermentation studies.

2.2.4. Saccharification

Saccharification was used commercial enzyme with Celluclast 1.5L and Viscozyme L (Novozymes. Inc., Denmark). The thermal acid hydrolysis without enzyme treatment was carried out 11% (w/v) seaweed slurry and 150 mM H_2SO_4 at 121℃ for 40 min. After thermal acid hydrolysis was done, Media was adjusted to pH 4.8 by 10N NaOH. Celluclast 1.5L of 8.4 FPU/ml and Viscozyme L of 1.2 EGU/ml were added to 1% (w/v) in seaweed

slurry. Enzyme reaction was carried out at 45℃ for 24 h.

2.2.5. Yeast

Yeast, *Kluyveromyces marxianus* KCTC 7150, was used for ethanol fermentation. Yeast was grown in YPD broth (dextrose 20.0 g/L, yeast extract 10 g/L, peptone 20 g/L). Single colonies of yeast from a YPD plate were used for the preparation of seed cultures and the culture was incubated in a rotary shaker at 30℃ for 12h. The second culture (30 ml YPD medium in a 100 ml Erlenmeyer flask) were prepared by inoculation with the seed cultures (5%, v/v) and the cultures of *K. marxianus* was incubated in a rotary shaker at 30℃ and 130 rpm.

2.2.6. Separate hydrolysis and fermentation (SHF)

Fermentation was performed with 11% (w/v) slurry and 150 mM sulfuric acid at 121℃ for 40 min. Nutrients were added to the medium; yeast extract 5 g/L, $(NH_4)_2SO_4$ 7.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.75 g/L, K_2HPO_4 3.5 g/L, $CaCl_2 \cdot 2H_2O$ 1 g/L. Ethanol fermentation was used for *K. marxianus*. Flask fermentation was carried out in 500 ml flask with working volume 200 ml. And ethanol fermentation was carried out with HMF and without HMF. To remove HMF, active charcoal treatment was used for 5% (w/v) active charcoal. Enzyme hydrolysis was performed with Celluclast 1.5L and Viscozyme L at 45℃ for 24h. After 500ml flask fermentation was done, fermentation was carried out by SHF in 5L bioreactor with working

volume 2.5L. Fermentation of 5L bioreactor was carried out without HMF and anaerobic condition. Sample was centrifugated at 12000 rpm for 10 min. The supernatant obtained after centrifugation was then analyzed for bioethanol and monosaccharide contents by HPLC.

2.2.7. Analysis

Monosaccharide, ethanol and 5-HMF were determined by HPLC (Agilent 1100 Series, Agilent. Inc., USA). Samples were filtered through a 0.2 ㎛ filter. HPLC instrument equipped with an Agilent G1362A refractive index detectoer (RID) detector. A Bio-rad Aminex HPX-87H column (300 x 7.8 mm) and Superguard C610H column (50 x 4.6 mm) were used with filtered and degassed 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min and a column temperature of 60℃.

2.3. RESULTS & DISCUSSION

2.3.1. Thermal acid hydrolysis

Thermal acid hydrolysis was evaluated on the basis of the effects of two factors: acid concentration and seaweed slurry as variables for the degradation of carbohydrate in *E. spinosum*. The production of galactose and glucose by thermal acid hydrolysis is shown in Fig. 1. The saccharification of carbohydrate to monosaccharide was related to acid concentration and seaweed slurry. High acid concentration and seaweed slurry showed better results of releasing high amount of monosaccharide. Galactose and glucose were increased with increasing acid concentration and seaweed slurry. The thermal acid hydrolysis produced high galactose of 30 g/L and high glucose of 7 g/L at 11% seaweed slurry and 187.5 mM H_2SO_4. However, high acid concentration caused sugar decomposition, thus, formed into fermentation inhibitors such as furfural and 5-hyroxymethyl furfural (HMF). The profiles of HMF concentration are shown in Fig. 2. The HMF by thermal acid hydrolysis produced averagely 6.5 g/L on 11% (w/v) seaweed slurry, 4.5 g/L on 8 % (w/v) seaweed slurry and 3.0 g/L on 5% (w/v) seaweed slurry, respectively. Also high acid concentration and seaweed slurry showed better results of releasing high amount of HMF. However, this study was chosen pretreatment condition of high monosaccharide amount because HMF could be removed active charcoal.

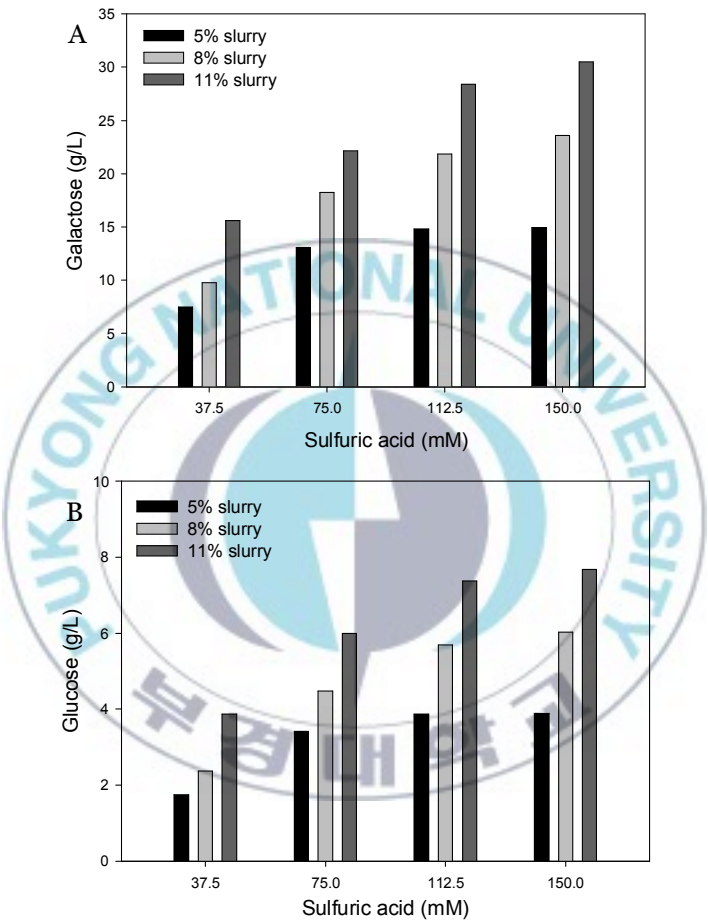

Fig. 1. Production of galactose and glucose from *Eucheuma spinosum* by thermal acid hydrolysis at 121℃ for 40 min

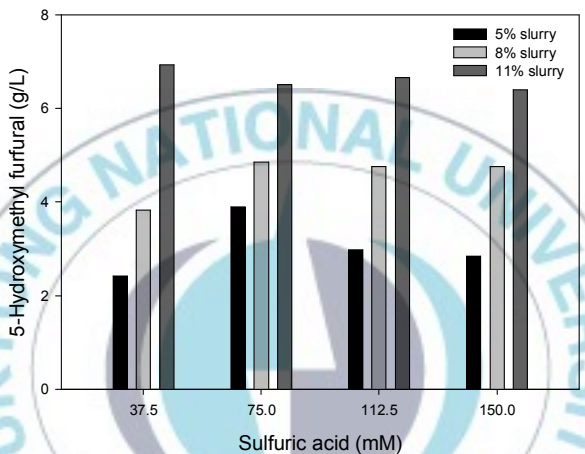

Fig. 2. Production of HMF from *Eucheuma spinosum* by thermal acid hydrolysis at 121℃ for 40 min

2.3.2. Detoxification

To reduce the effect of microbial inhibitors caused by the thermal acid hydrolysis, active charcoal treatments was used which improved the bioconversion of the sugar into ethanol. Pretreatment was carried out with 11% (w/v) slurry and 150 mM sulfuric acid at 121℃ for 40 min. Pretreatment hydrolysate when treated with active charcoal brought about maximum reduction in HMF from 5.7 g/L to 0.8 g/L (86% removal) as shows in Fig. 3. However, galactose and glucose concentrations not reduced to 26 g/L and 7 g/L from the initial concentration during concentration process as shows in Fig. 4. Some reports showed wood charcoal are capable in selectively removing the inhibitors such as furan and phenolic compounds without removing the fermentable sugars. This absorption characteristic of the wood charcoals is preferable to obtain the high fermentabilities of the hydrolysate [16]. After detoxification was carried out, enzyme saccharification was performed with Celluclast 1.5L and Viscozyme L at 45℃ for 24 min.

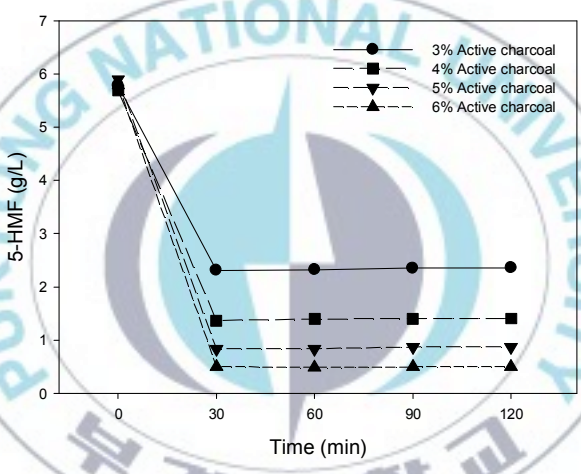

Fig. 3. Effect of active charcoal treatment on HMF contents for 120 min

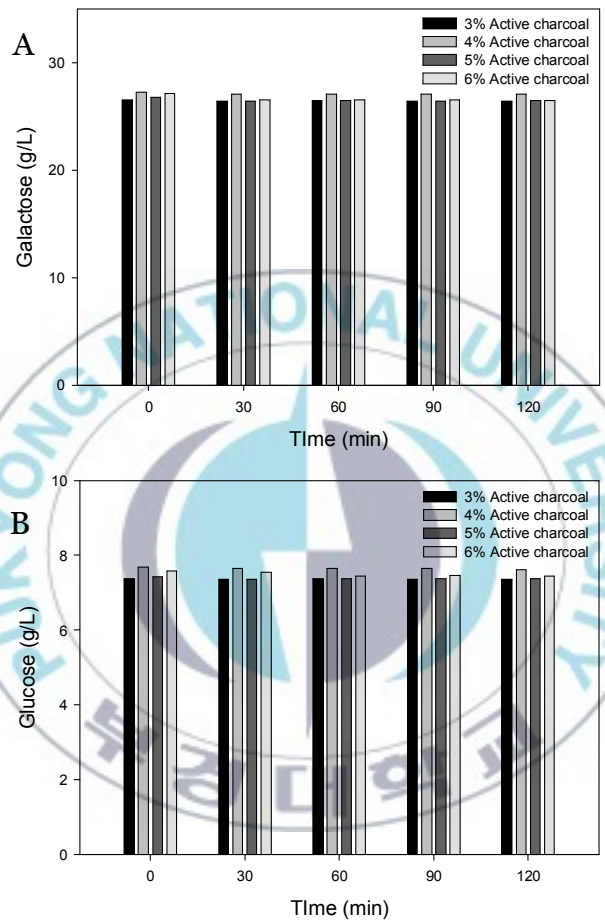

Fig. 4. Effect of active charcoal treatment on galactose (A) and glucose (B) contents for 120 min

2.3.3. Fermentation

Ethanol fermentation was carried out with existence HMF and not existence in SHF process. The optimal thermal acid hydrolysis was performed with 11% (w/v) slurry and 150 mM sulfuric acid at 121℃ for 40 min. After pretreatment produced HMF of 5.7 g/L. therefore active charcoal treatment was carried out for remove HMF. And enzyme hydrolysis was carried out with Celluclast 1.5L and Viscozyme L at 45℃ for 24 h. Fig. 5A shows ethanol fermentation was carried out with HMF of 5.7 g/L by *K. marxianus*. Galactose and glucose was consumed 15 g/L and 11 g/L. However galactose was not completely consumed during 72 h. Ethanol concentration of 10 g/L produced during 36 h and theoretical ethanol yield of total carbohydrate was 25.7 %. And HMF was reduced from 5.7 g/L to 0 g/L during 24 h. Because HMF was converted furan-2,5-dimethanol(FDM) by yeast. FDM are also furan derivatives, which appear to be less toxic to the yeast. The main routes of the reduction of HMF compete for cofactor NADH. NAP+ is regenerated from NADH to enable continued glycolysis [18]. The presence of HMF appeared to cause redox imbalance and interfere with glycolysis, cell growth, and biosynthesis. Shortage of NADH has been observed in the presence of HMF, and it appears that HMF reduction competes for NADH [19]. As the result, HMF could cause accumulation of acetaldehyde delaying ethanol production [20]. On the other hands, Fig. 5B shows ethanol fermentation was carried out without HMF. As a result ethanol produced 17 g/L during 48 h from *K. marxianus* and theoretical ethanol yield of total carbohydrate was 43.8 %. Ethanol concentration of not existence HMF produced

higher than existence of HMF. Also the theoretical ethanol yield of not existence HMF produced 18% higher than existence HMF. Ethanol fermentation was carried out without HMF in 5L bioreactor. Galactose and Glucose was produced 22 g/L and 11 g/L from pretreatment. And active charcoal treatment and enzyme hydrolysis was performed. Ethanol concentration of 17 g/L produced during 120 h. Ethanol fermentation period of 5L bioreactor was extended longer than in flask fermentation. However, ethanol fermentation in flask and 5L bioreactor produced same ethanol concentration of 17 g/L. Ethanol fermentation was improved when without HMF. In this study, confirmed that HMF was effective yeast of ethanol production pathway.

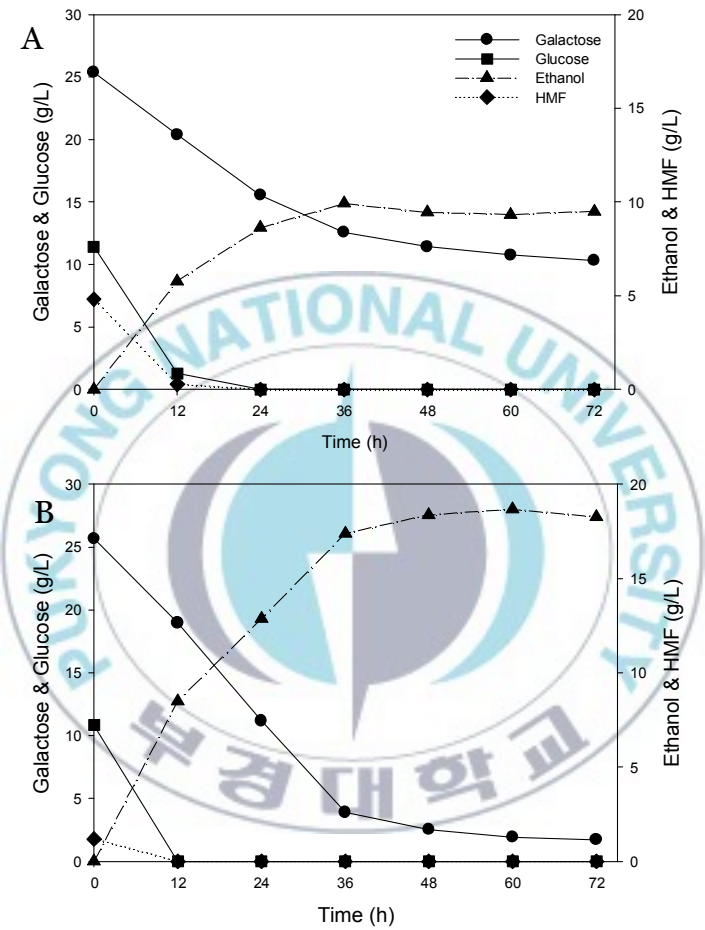

Fig. 5. Ethanol fermentation without active charcoal treatment (A) and with active charcoal treatment (B) from *E. spinosum* by SHF process with *K. marxianus*.

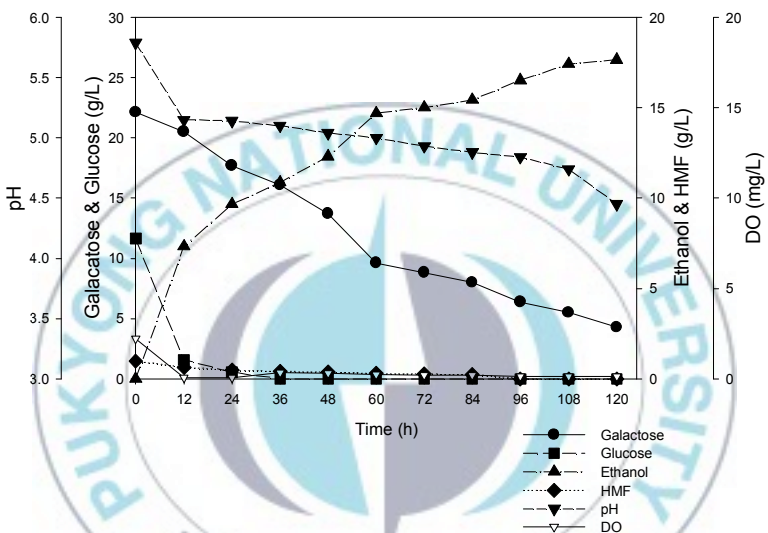

Fig. 6. Ethanol fermentation with active charcoal treatment from *E. spinosum* by SHF process with *K. marxianus* in 5L bioreactor

2.4. CONCLUSION

The Seaweeds, *E. spinosum*, is a promising substrate biomass for the bioethanol production due to its rapid growth and high productivity. In this study, saccharification of *E. spinosum* was improved by applying thermal acid hydrolysis and enzyme treatment. And active charcoal treatment was carried out for removed HMF after pretreatment. Pretreatment condition was determined with 11% (w/v) slurry content and 150 mM sulfuric acid at 121℃ for 40 min. The galactose and glucose of 30 g/L and 8 g/L were obtained after thermal acid hydrolysis. Active charcoal of 5% (w/v) was used at room temperature for 30 min to remove HMF and HMF was reduced from 5.7 g/L to 0.8 g/L during 30 min. Fermentation was carried out with *K. marxianus*. Ethanol production was 10 g/L when active charcoal not used. And ethanol concentration of 17 g/L was produced in flask and 5L bioreactor when HMF was removed by active charcoal. Ethanol fermentation was improved when without HMF.

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ACKNOWLEDGMENT

대학원을 진학한지 어느덧 2년이 지나 벌써 졸업을 하게 되었습니다. 제가 졸업을 할 수 있도록 제 옆을 항상 지켜준 우리 엄마, 아빠, 민수, 이모들 정말 감사하고 사랑합니다. 부족한 저를 끝까지 돌보아 주시고 가르쳐주신 김성구 교수님, 존경하고 감사합니다. 저희들을 친 자식처럼 아껴주신 강향숙 사모님~항상 감사합니다. 그리고 저희가 모르는 것을 물어보면 친절히 가르쳐주신 정귀택 교수님, 감사하고 존경합니다.

중학교 때부터 내 옆에 있어준 미선, 예림, 주리~ 앞으로도 싸우지 말고 잘 지내고 할머니가 되어서도 우리 떨어지지 말고 항상 같이 있자*^^* 사랑해 얘두라~ 같은 길을 가고 있어 말이 잘 통하는 혜수, 정말 고마워. 너도 꼭 무사히 졸업하길 바래. 마지막으로 제가 실험길 생활 하는 동안 가족보다 더 오랜 시간 지냈던 실험실 부원들 지숙이 언니, 유경언니, 혜진언니, 혜영이, 창한오빠, 채훈선배, 실험실 생활이 힘들 때 같이 있어 줘서 고맙고 졸업하고 나서도 잊지 못할 꺼 같습니다.

2년동안 대학원생활을 하면서 많은 것을 배웠고 이제 사회에 나가 배운 것을 잘 사용하여 앞으로도 힘든 일이 와도 이 시절의 기억을 잊지 않고 잘 헤쳐나가겠습니다.

감사합니다.