Thesis for the Degree of Master of Engineering

Strain improvement by CSTR culture and ethanol production from seaweed, *Undaria pinnatifida*

by

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The Graduate School

Pukyong National University

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(연속배양을 통한 균주개량과 미역을 이용한 바이오에탄올 생산)

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요 약

화석에너지의 고갈에 대한 우려와 지구온난화 등의 환경문제가 전 세계적으로 부각됨에 따라 바이오에너지에 대한 관심이 증대되고 있다. 이 중 무공해 청정대체에너지로 주목받고 있는 바이오에탄올의 생산은 고유가시대에 있어 매우 중요한 부분으로 그 생산량이 증가하고 있다. 해조류는 당질계, 목질계에 이은 제 3세대 바이오매스로서 이 중 갈조류는 성장이 빠르고, 단위 면적당 생산성이 매우 높으며 탄수화물 함량이 높기 때문에 우리나라에 적합한 바이오매스로서 각광받고 있다.

따라서, 본 연구에서는 미역을 바이오매스로 한 산촉매 열 가수분해 조건의 최적화와 연속배양을 통한 균주개량을 통해 바이오에탄올 생산을 수행하였다. 당화최적화를 위하여 75mM H_2SO_4를 13% (w/v)의 슬러리 파우더에 60분간 처리하여 총 28.65 g/L의 단당을 얻을 수 있으며, 산촉매만으로 42.3%의 당화수율을 달성할 수 있었다. 효모의 단당류 이용효율을 높이기 위하여 고염에서도 활성을 가지는 균주를 연속배양 하였다. 인위적으로 염(NaCl)을 추가시킨 배지에서 *Pichia angophorae* KCTC 17574를 배양시켰으며, 90psu에서 연속배양한 *Pichia angophorae* KCTC 17574를 산촉매 열가수분해 한 슬러리액에서 배양하였을 때 9.4 g/L의 바이오에탄올을 생산하여 총 탄수화물에서 이론적 수율의 27.3%를 달성하였다.

해조류를 이용한 바이오에탄올 생산은 당화 및 발효를 통한 공정의 확립이나 연구가 미약한 실정으로 본 연구결과는 해조류 미역(*Undaria pinnatifida*)을 이용한 바이오에탄올 생산에 있어 산업적으로 활용될 수 있을 것으로 사료된다.

Ⅰ. INTRODUCTION

The depletion of fossil fuels and increasing problem of the CO_2 emissions have led to find alternative sources for the biomass such as corn stover, lignocellulosic biomass, forest products residue and the seaweed (Saxena *et al*., 2009: Alvira *et al*., 2010). Biomass is renewable primary energy resource providing transportation fuels such as bioethanol or biodisel (Sun *et al*., 2002). Bioethanol has been regarded as major substitute to replace the liquid fossil fuels among them (Dias *et al*., 2009). Bioethanol could be contributed to the reduction of CO_2 emission and lower dependency on the import of oil for non-oil producing countries (Nikolic *et al*., 2009).

Bioethanol has been produced from agricultural feedstock and lignocellulosic biomass in many countries (Sun *et al*., 2002; Dias *et al*., 2009). Sugarcane has been already used as the biomass for ethanol production with a commercial scale in Brazil. However, agricultural feedstock has caused moral problems and price instability. Lignocellulosic biomass is a second generation biomass since it is cheap and available in large quantities (Millati *et al*., 2002). However, hydrolysis using lignocellulosic biomass is difficult because lignin blocks celluloses and hemicelluloses to access enzymes as barrier (Mansfield *et al*., 1999). Therefore, biomass selection represents one of the main processes for the feasible bioethanol production.

The seaweed has been considered for the third generation biomass for the bioethanol production (Ge *et al*., 2011). The seaweed has high productivity per unit area per year and there is no competition with food crops. The seaweed can be grown rapidly compared to land-based biomass. *Undaria pinnatifida* (Sea mustard), *Saccharina japonica* (Sea tangle) and *Porphyra tenera* (Purple laver) are the main species grown along the Korean coasts. *Undaria pinnatifida* has been spread on worldwide and grown rapidly with the maximum length of 3 m (Hay *et al*., 1987). In this study, *Undaria pinnatifida* was used as biomass for the ethanol production.

The carbohydrate contents of seaweed are in a range of 30-70%. It depends on the species and culture conditions. The brown seaweed can be a promising substitute due to well developed cultivation method and mass production in Korea. Carbohydrate of brown seaweed consists of alginate, laminaran, fucoidan and mannitol (Tang *et al*., 2009). Degradation of these polysaccharides is slow and requires specific enzymes. Thus, it is essential to develop suitable pretreatment methods based on biomass properties.

Pretreatment is recognized as one of the main processes to obtain high ethanol production. Dilute-acid hydrolysis is the most commonly used for the pretreatment of lignocellulosic biomass (Lenihan *et al*., 2010). Enhanced xylose production from hemicelluloses of corn stover was obtained by appling dilute sulfuric acid method (Lu *et al*., 2009).

Salt condition is important for the ethanol production because salt could act as inhibitor, resulting in reductions of cell growth and fermentation yield when salt concentration is over certain concentration. The seaweed must be considered this factor because the origin of seaweed is from seawater which contains a lot of salts. And more salts which produced after thermal acid hydrolysis and neutralization processes have an effect on the fermentation. Several salt-tolerant yeasts can produce a liquid fuel (ethanol) and various polyols (glycerol, i-erythritol, arabitol, mannitol) which can be used in a number of industrial applications. *Debaryomyces hansenii* and *Zygosaccharomyces rouxii* have been used as tolerant yeasts at high salt concentrations (Groleau *et al*., 1995; Khroustalyova *et al*., 2001). However, most yeasts used for the ethanol production do not have the ability to tolerate a high salt condition. Thus, yeast strain improvement at high salinity condition was acclimated to produce ethanol from seaweed, *Undaria pinnatifida*.

This study was carried out to increase the monosaccharide contents by the optimization of thermal acid hydrolysis conditions and improve fermentation yield by yeast strain improvement at high salt concentration with *Undaria pinnatifida*.

Ⅱ. MATERIALS AND METHODS

1. Raw material

The seaweed, *Undaria pinnatifida* (harvested in October 2009) was obtained from Gijang fisheries local market in Busan, Korea. The seaweed was dried with sunlight or hot-air and ground by hammer mill. Ground seaweed was sieved by mesh and used for analysis. Crude protein, crude lipid, carbohydrate and ash of seaweed were analyzed by AOAC method. Crude lipid was determined by using an ether extraction procedure (Soxtec System 1046, foss, Hoganas, Sweden) after freeze-drying for 12 hrs.

2. Thermal acid hydrolysis

For the pretreatment of the seaweed, different sulfuric acid concentrations and solid contents of slurry were varied with ranges of 37.5 - 187.6 mM and 10-20% (w/v) in 100 ml Erlenmeyer flask with a working volume of 30ml, respectively. The slurry was then autoclaved for the ranges of 15-60 min at 121℃. Monosaccharide contents, viscosity and salinity were measured.

3. Yeast inoculums and CSTR culture

Yeast, *Pichia angophorae* KCTC 17574 was obtained from KCTC and prepared on YPD agar plate. YPD agar plate containing yeast extract 20 g/L, peptone 10 g/L, dextrose 20 g/L and agar 5% (w/v) was used for the yeast cultivation. A single colony was purified and the seed culture was performed with working volume of 5 ml in YPD (Yeast extract 20 g/L, peptone 10 g/L, dextrose 20 g/L) medium. The second culture was prepared with the inoculation of 5% (v/v) of seed culture in 250 ml Erlenmeyer flask with a working volume 100ml and incubated in a shaking incubator at 30℃ with 220 rpm for 12 hrs. Continuous stirred tank reactor (CSTR) culture was carried out with a working volume of 2.5 L and YPD medium was used for the yeast cultivation. Temperature and agitation rate were adjusted to 32℃ and 200 rpm. The salinity concentration of medium in the reactor was artificially increased by addition of sodium chloride. The medium salinities were increased at the rate of 5 psu (practical salinity unit) up to 100 psu and each cultivation medium was maintained for 72 hrs. The medium was replaced by 250 ml fresh YPD medium after the cultivation of 72 hrs. Cultured yeast strain was periodically sampled for the measurement of colony forming unit (CFU), pH and fermentation.

4. Fermentation with pretreated slurry

Fermentation was carried out in 250 ml Erlenmeyer flask with a working volume of 100ml. The seaweed slurry pretreated by thermal acid hydrolysis at optimal conditions was neutralized to pH 7 by 5N NaOH. A colony from CSTR culture was purified and cultivated in YPD medium containing salinity for 24 hrs. The second culture was prepared with the inoculation of 5% (v/v) of pre-culture at each salinities medium. And then yeast strains of 0.1 g dcw/L, *Pichia angophorae* KCTC 17574 at each salinity medium were added to the pretreated seaweed slurry. The medium was incubated on 30 ± 2℃ for 72 hrs and sampled to determine the concentration of ethanol and acetic acid.

5. Analysis

Cell growth was measured by UV-Vis spectrophotometer at OD_{600} and converted to dry cell weight using standard curve. The slurry of thermal acid hydrolysis was analyzed to determine the concentration of monosaccharide and organic acid by high performance liquid chromatography (HPLC) (Agilent 1100 Series, Agilent. Inc., USA) equipped with refractive index detector (RID). Bio-rad Aminex HPX-87H column (300.0 x 7.8 mm) was used with degassed 5mM sulfuric acid as the flow rate of 0.6 ml/min and the temperature of 65℃. The samples of fermentation were prepared by centrifugation for 8 min at 12,000 rpm and the supernatant were

filtered with 0.2 μm prior to analysis. Viscosity was measured by Brookfield viscometer (BROOKFIELD DV-Ⅲ Rheometer v3.1, Brookfield Eng. Inc., USA) equipped with spindle No. ULA, SC4-18 and SC4-34 at temperature of 30℃. Salinity was measured by salinometer (Salinity Refractometer, ATAGO. Inc., Japan).

6. Response surface methodology (RSM)

The influences of three variables were determined by a response-surface methodology (RSM) using SAS 9.1 (SAS Institute, Cary, NC, USA). The experiments were designed by Central Composite Design (CCD) based on 2^3 factors. Seventeen experiments were set-up at each range of three variables and analyzed.

Ⅲ. RESULTS AND DISCUSSION

1. The composition of *Undaria pinnatifida*

The seaweed was primarily ground to the powder by hammer mill for experiment. The composition of *Undaria pinnatifida* is showed in Table 1. *Undaria pinnatifida* contained 48.5% of carbohydrate, 18.2% of crude protein, 1.8% of crude lipid, 28.0% of crude ash and 3.5% of crude fiber. Total carbohydrate content was determined as 52.0% including crude fiber as cellulose on dry solid basis in this study.

Table 1. Composition of *Undaria pinnatifida* on dry basis

Component	Dry basis (%, w/w)
Crude protein	18.2
Crude lipid	1.8
Crude carbohydrate	48.5
Crude ash	28.0
Crude fiber	3.5

2. Thermal acid hydrolysis

Thermal acid hydrolysis was evaluated on effects of three factors, acid concentration, treatment time and solid content of slurry, for the degradation of carbohydrate in *Undaria pinnatifida*. Acid concentration, treatment time and solid content were coded to X_1, X_2 X_3 for the analysis by RSM (response surface methodology) on the yield of monosaccharide and viscosity coded to Y_y and Y_v as response, respectively. The quadratic model was calculated and equation (1) was obtained on the yield of monosaccharide.

$$Y_y = -94.388X_1^2 - 0.008X_2^2 - 0.118X_3^2 - 0.414X_1X_2 + 1.469X_1X_3 - 0.020X_2X_3 + 82.704X_1 + 1.482X_2 + 3.562X_3 - 58.367 \quad (1)$$

The regression coefficients and significant level are given in Table 2. Table 2 showed that all regression coefficients in quadratic model were significant ($P<0.05$) and all variables have an important effect on yield of monosaccharide. F-value (11.25) and p-value (<0.01) also demonstrated that the model was statistically significant. R^2 was determined to be 0.925 and it showed that 92.5% of variation could be explained well in the model.

The saccharification of carbohydrate was related with acid concentration and treatment time as shown in Fig. 1(a). Effects of acid concentrations at 121℃ were evaluated. The yield of monosaccharide increased from 27.9% to 42.5% with increase of acid concentrations from 7.5 mM up to 93.0 mM

Table 2. Significance of regression coefficients on yield of monosaccharide

	Regression coefficient	Standard Error	t-value	P-value
Intercept	-58.367	15.153	-3.85	0.0063
Acid concentration	82.705	24.373	3.39	0.0115
Treatment time	1.482	0.396	3.74	0.0073
Slurry content	3.563	1.189	3.00	0.0200
Acid concentration ×Acid concentration	-94.388	21.0878	-4.48	0.0029
Acid concentration × treatment time	-0.414	0.272	-1.52	0.1714
Treatment time × treatment time	-0.008	0.004	-2.27	0.0571
Solid content × acid concentration	1.469	0.815	1.80	0.1144
Solid concent × treatment time	-0.020	0.011	-1.85	0.1074
Solid content × solid content	-0.118	0.034	-3.45	0.0107

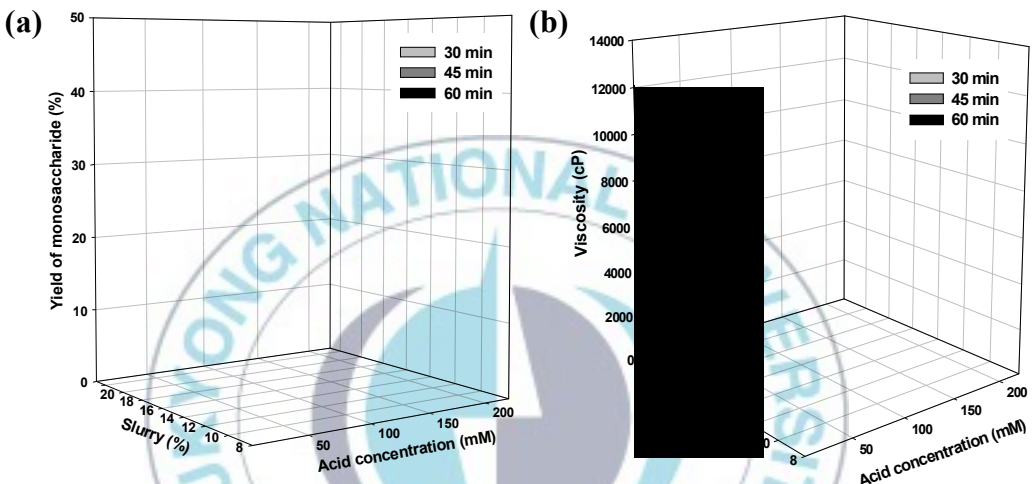

Fig. 1. Response-surface (a) Effect of acid concentration (mM), slurry (%) and treatment time on the yield of reducing sugar contents (b) Effect of acid concentration (mM), slurry (%) and treatment time on viscosity

and decreased over 93.0 mM of acid concentrations. High acid concentrations generally produced high amounts of monosaccharide achiving high reaction rate (Redding *et al*., 2010). However, high acid concentration caused sugar decomposition and formed into inhibitors such as furfural and HMF. And these inhibitor concentrations depend on the raw materials and harshness of pretreatment (Redding *et al*., 2010). It has been reported that carbohydrate was degraded into furfural and HMF under harsh conditions with the high concentrations of acids (Sun *et al*., 2002; Redding *et al*., 2010). Therefore, proper acid concentration should be applied to minimize inhibitor production for the fermentation. A maximum monosaccharide content of 28.75 g/L was produced with 75 mM sulfuric acid at 121℃ with low furfural and HMF concentrations. (data not shown)

The effects of pretreatment times were also studied. Fig. 1(a) showed that the increase of treatment time from 30 min to 60 min increased the yield of monosaccharide production from 29% to 42% at the same acid concentration of 75mM, respectively**.** High monosaccharide content was obtained by extending the treatment time referring the previous report (Lu *et al*., 2009).

Solid contents of slurry were also changed with various acid concentration and treatment time. The viscosity was dependent on the amounts of the solid content as shown in Fig. 1(b). High solid contents of slurry could produce more monosaccharide contents by the conversion of polysaccharide to monosaccharide

(Lu *et al*., 2009). However, high solid contents of slurry caused high viscosity which make medium difficult to be handled (Rosgaard *et al*., 2007). Due to this reason, solid content of slurry was set at 13% (w/v). The quadratic model on viscosity was obtained following equation (2).

$$Y_v = 6234.916X_1^2 - 0.282X_2^2 + 20.483X_3^2 + 115.428X_1X_2 - 730.283X_1X_3 - 9.250X_2X_3 - 1794.101X_1 + 110.075X_2 + 286.998X_3 - 5416;382 \quad (2)$$

The regression coefficients and significant level are shown in Table 3. Although this model did not explain the effect of each factor and interactions among variables well, it showed the tendency between factors. This quadratic model was significant (<0.0001) with a high R^2 value (0.929).

The optimal conditions considering these variables were determined with 13% (w/v) slurry with 75mM sulfuric acid at 121℃ for 60 min. A maximum monosaccharide content of 28.75 g/L with 42% of yield by conversion of total carbohydrate was obtained with low viscosity of 33.19 cP.

Table 3. Significance of regression coefficients on viscosity

	Regression coefficient	Standard Error	t-value	P-value
Intercept	-5416.382	7978.345	-0.68	0.5190
Acid concentration	-1794.101	4969.931	-0.36	0.7288
Treatment time	110.075	169.918	0.65	0.5378
Slurry content	286.998	604.474	0.47	0.6494
Acid concentration ×Acid concentration	6234.916	2183.236	2.86	0.0245
Acid concentration × treatment time	115.428	55.570	2.08	0.0764
Treatment time × treatment time	-0.282	1.553	-0.18	0.8611
Solid content × acid concentration	-730.283	166.710	-4.38	0.0032
Solid concent × treatment time	-9.250	4.446	-2.08	0.0760
Solid content × solid content	20.482	13.973	1.47	0.1861

3. Effect of salinity on cell viability

Dried seaweed contains high amounts of salt and its amount varies with the species. Some of the problems encountered during anaerobic digestion of seaweed are these high concentrations of SO_4^{2-}, NaCl and heavy metals (Nkemka *et al*., 2010). Moreover, more salts were produced resulted from thermal acid hydrolysis and neutralization processes in seaweed. If this salt concentration was presented in wrong ratio, these salts make the fermentation process slow down (Klinke *et al*., 2004). It has been reported that alkali salts and heavy metal salts presented in lignocellulosic hydrolysate also caused the difficulties in process (Klinke *et al*., 2004).

In order to maintain cell growth and fermentation activity of yeast, continuous stirred tank reactor (CSTR) culture was applied to change the metabolic activities of *Pichia angophorae* strain. The cell, *Pichia angophorae* KCTC 17574, adaptation to salinity concentration in a range of 50-100 psu was carried out and the results are shown in Fig. 2. The initial salinity concentration containing in YPD medium is nearing 50 psu. Cell numbers of 10^{14}-10^{15} CFU/ml were observed at the medium salinities from 50 to 70 psu and then decreased with the salinity concentration increase. The cell numbers were significantly reduced up to 10^{8}-10^{9} CFU/ml with salinity concentration of 75 psu and colony shape was also changed due to osmotic pressure. (data not shown) Although cell numbers decreased at high salt

concentration up to 100 psu, these cells obtained from CSTR culture grew well in the YPD medium containing high salt concentration of 100 psu. It is thought that these yeast strains possess distinctive salt tolerance which allows them to survive under high salt concentration, indicating improved yeast strains are not halo-philc, but salt-tolerant.

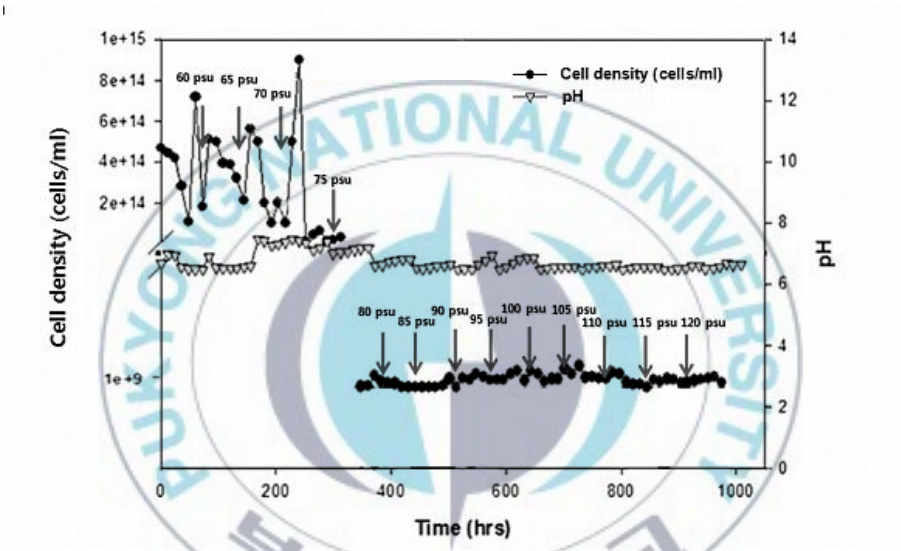

Fig. 2. Cell viability variation of mutant strains on *Pichia angophorae* at each salinity concentration with CSTR

4. Ethanol fermentation

The slurries pretreated by optimal thermal acid hydrolysis conditions were used for the fermentation medium. Yeast strains, *Pichia angophorae* KCTC 17574 at various salinity concentrations media were used to produce ethanol from seaweed, *Undaria pinnatifida*. *Pichia angophorae* could produce ethanol using fermentable monosaccharide of D-mannitol, D-glucose, D-xylose and laminaran (Horn *et al.*, 2000). These saccharides of D-mannitol, D-glucose and laminaran are major components in brown seaweed, *Undaria pinnatifida*.

In this study, yeast strains cultured at a salinity range of 80-100 psu were used for ethanol fermentation because the salinity of medium becomes 85-90 psu after the thermal acid hydrolysis and neutralization processes. The fermentation was carried out by addition of 0.1 g dcw/L of yeast strain dispersion to the pretreated slurry. The fermentation medium was incubated at 30 ± 2℃ with 220 rpm for 72 hrs.

The activities of high salt tolerant yeast were evaluated and the result of ethanol production at various salinity concentrations was shown in Fig. 3. The ethanol concentration of 9.42 g/L and the volumetric productivity of 0.26 g/L/h were obtained with strain cultured at 90 psu after 36 hrs. And the ethanol concentration of 5.84 g/L and the volumetric productivity of 0.49 g/L/h were observed with strain

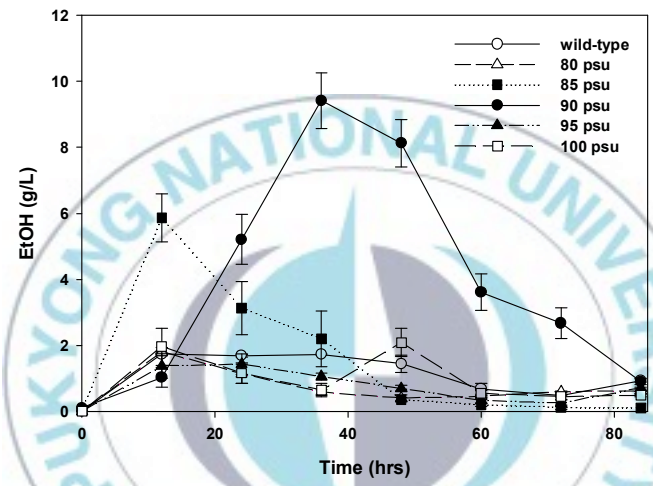

Fig. 3. Ethanol production of *Undaria pinnatifida* with improved strains cultured at 80 psu (△), 85 psu (■), 90 psu (●), 95 psu (▲) and 100psu (□) including wild-type strain (○)

cultured at salinity concentration of 85 psu after 12 hrs. Yeast strains cultured at 80, 95, 100 psu and wild-type *Pichia angophorae* strain produced the ethanol concentration less than 2 g/L. These results were probably due to the suitability of salinity conditions between culture and fermentation medium. Cells cultured at 85 and 90 psu may be activated well under fermentation medium conditions which were similar to that of CSTR culture in medium. The volumetric productivities of 0.49 g/L/h and 0.26 g/L/h were obtained by using strain cultured at 85 and 90 psu, respectively. It suggested that strain cultured at 85 psu are subject to faster approach to reaction completion than strain cultured at 90 psu. And these results offer the possibility of using strain cultured at 85 psu effectively for industrial applications.

The highest ethanol concentration was observed using improved strain cultured at 90 psu and the time course of ethanol production is shown in Fig. 4. The ethanol concentration of 9.42 g/L was obtained with a 27.3% of theoretical yield of total carbohydrate at 36 hrs of incubation. The fast ethanol production rate by improved strain at high salt concentration was obtained within the first 36 hrs and then ethanol concentration decreased slowly. Two days are enough to produce ethanol and then ethanol degraded into other products. The initial pH of fermentation medium was 6.5 and pH decreased to 5 due to the by-product such as CO_2 produced by ethanol fermentation. After the reaching highest ethanol production,

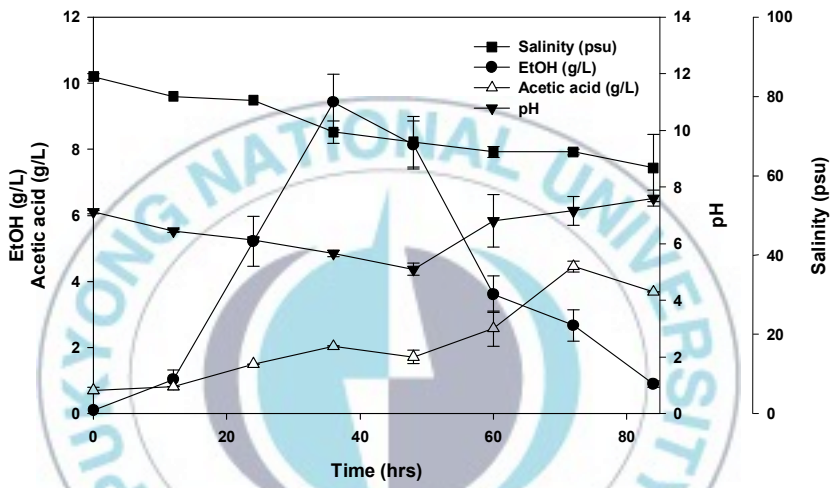

Fig. 4. Ethanol and acetic acid production, pH and salinity (psu) of *Undaria pinnatifida* with salt-tolerant yeast strain obtained by CSTR culture at 90 psu at optimal pretreatment condition: Ethanol concentration (●), Salinity (▼), pH (■) and Acetic acid concentration (△)

acetic acid was produced during the fermentation process. Most of by-products resulting in conversion of sugars to ethanol are normally stated to be glycerol, succinic acid and acetic acid (Taherzadeh *et al*., 1997). The acetic acid concentration was increased by the decrease of ethanol concentration and maximum acetic acid concentration of 4.46 g/L was produced at 72 hrs of incubation as shown in Fig. 4. This result showed that ethanol was oxidized and turned into acetic acid probably due to acetic acid bacteria after 36 hrs of incubation. It has been reported that acetic acid is produced due to degradation of different components which constitute biomass and can act as potential fermentation inhibitors when this exceeds acetic concentration of 6 g/L in the lignocellulosic biomass (Larsson *et al*., 1999; Oliva *et al*., 2006). Our result showed that acetic acid concentration of 4.46 g/L was produced at the end of the fermentation and does not affect ethanol fermentation in our experiments.

Our studies show that strain, *Pichia angophorae* KCTC 17574 improvement by CSTR culture results in high ethanol production compared with wild-type strain and these strain can have the activity at high salt concentration. In order to improve the fermentation yield, strains which can utilize various seaweed components will be developed and other factors affecting seaweed fermentation is also considered. Studies on wet-milling using wet macroalgae are carried out to reduce unit

operation cost like milling, grinding etc and these improved strains could apply for the development of ethanol production. .

Ⅳ. CONCLUSION

The seaweed, *Undaria pinnatifida* is a promising substitute for the bioethanol production due to rapidly growth and high productivity. For the pretreatment, three variables, acid concentration, treatment time and the solid content of slurry which affected the monosaccharide yield and viscosity were studied by thermal acid hydrolysis. The optimal thermal acid hydrolysis conditions were obtained with 75 mM sulfuric acid and 13% (w/v) slurry at 121℃ for 60 min. High monosaccharide contents of 28.75 g/L with 42.5% of theoretical yield were observed.

Salt-tolerant yeast strain, *Pichia angophorae* KCTC 17574 was improved by CSTR culture and ethanol concentration of 9.42 g/L with 27.3% of theoretical yield of total carbohydrate was obtained using yeast strain cultured at 90 psu.

V. ACKNOWLEDGMENT

양식학과를 졸업하고 생물공학과 생물고분자공학 실험실에 들어온지 2년 반이 다되어 가고 있습니다. 실험의 기초도 모르던 제가 연구를 하고 석사를 졸업할 수 있도록 끊임없이 학문의 길로 이끌어주셨던 저희 교수님, 김성구 교수님께 진심으로 감사드립니다. 그리고 어머니처럼 실험실 식구들을 걱정해주시고 신경 써주시는 강향숙 사모님께 감사의 말을 전합니다.

부족한 제가 석사생활을 하며 많은 것을 배울 수 있도록 도움을 주신 김중균 교수님, 공인수 교수님, 이형호교수님, 박남규 교수님, 홍용기 교수님 그리고 바이오에너지 연구에 많은 도움을 주셨던 정귀택 교수님에게 감사의 말씀을 드립니다. 학부생활동안 많은 전공 지식을 쌓을 수 있도록 도움을 주신 저의 지도교수님이셨던 배승철 교수님 감사합니다. 옆에서 많이 챙겨주셨던 김종명 교수님, 늘 신경 쓰고 도움을 주신 최창근 교수님 정말 감사합니다.

대학생이 되던 때부터 알게 되어 지금까지 석사생활을 잘 해나갈 수 있도록 든든한 버팀목이 되어준 수근선배, 많이 도와주고 신경 써주는 채훈선배, 자주 티격태격하지만 마음이 정말 따뜻한 석주오빠, 힘들 때마다 힘을 낼 수 있도록 격려해 준 승준선배, 늘 옆에서 도와주고 제 편이 되어준 지숙이 언니, 생물공학과에 처음 온 저를 많이 도와주었던 미란이 언니, 엄마아빠보다도 더 많은 시간을 함께 보내고 제 얘기를 많이 들어주던 탄다(김혜진)!!, 실험실의 분위기 메이커면서 무운의 종결자로 웃음을 주던 나경이, 지금은 다른 회사에 취업했지만 누구보다 열심히 에탄올 실험에 임했던 라권이, 실험실 막내로 묵묵히 그리고 열심히 바이오에탄올을 연구하는 민지, 지금은 다른 길을 갔지만 좋은 말(??)을 많이 해 준 승훈선배, 처음 실험실에 와서 어색해하던 저를 많이 도와주고 함께 해준 설희와 해리, 웃음이 매력적인 혜영이, 마음이 따뜻한 친구 진우와 모든 실험실 식구들에게 고마움을 전합니다.

힘들 때마다 이야기 다 들어주고 바로 잡아주었던(??) 희정이, 힘들고 외로울 때 버틸 수 있도록 옆에서 도와준 설이, 나랑 너무 비슷한 점이 많아 친해졌고 이제는 같은 길을 걷게 된 정미, 늘 분노하게 하지만 늘 당하는 인국이, 지금은 멀리서 나라를 지키고 있는

철한이, 학부생활 내 도와주고 좋은 말을 많이 해주고 도움을 주었던 멋진 석기선배, 완소남을 외치는 상재오빠에게도 고맙다는 말을 하고 싶습니다.

볼 때마다 구박하시지만 많은 조언을 해주신 한박사님!! 석사 2년을 잘 해낼 수 있도록 격려해주신 준영이오빠!! 힘든 고민 다 들어주고 나의 이야기 친구가 되어 주었던 준호오빠, 새신랑이 된 멋쟁이 건현이 오빠, 항상 내 걱정해주는 착한 황남용이 오빠에게도 고마움을 전합니다.

평생 내 소울메이트이자 또 다른 꿈을 향해 달려가는 멋진 내친구 은주, 바쁘다고 자주 보진 못했지만 항상 내 옆을 지켜준 하림이, 고등학교부터 지금까지 옆에서 언제나 힘이 되어 준 유진이, 못난 술버릇 다 받아주는 든든한 친구 진우, 항상 웃음을 주는 석준이, 건강 챙겨주고 도움을 많이 주는 고마운 친구 경민이, 자주 연락 못했지만 항상 보고 싶은 예슬이, 맨날 밥 사달라고 하는 승훈이, 같이 영어 수업도 들으며 늘 해피바이러스를 전파하는 진지혜! 에게도 모두 고맙다고 말하고 싶습니다.

연락도 잘 못하는 부족한 친구를 잊지 않고 늘 보고싶다고 말해주는 사랑하는 나의 친구들, 예린이, 선혜, 민정이, 경은이, 경민이에게도 고마움을 전합니다.

부족한 제가 석사생활을 잘 마무리 할 수 있도록 격려하고 또 격려해주신 사랑하는 우리 엄마, 아빠, 하늘나라에서 저를 지켜주시는 우리 할아버지와 외할아버지, 늘 저를 위해 기도해주시는 할머니와 외할머니 그 외의 가족 친척분들에게 고마움을 전합니다. 그리고 마지막으로 남매보다 더 끈끈한 정으로 힘들 때마다 옆에서 도와주는 우리 인기오빠에게도 고마움을 전합니다. ^^*

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