Thesis for the Degree of Master of Engineering

Studies on bioethanol production from seaweed, *Gelidium amansii*using acclimated yeasts

by

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(우뭇가사리로부터순치효모를이용한바이오에탄올생산에관한연구)

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우뭇가사리로부터 순치 효모를 이용한 바이오 에탄올 생산에 관한 연구

조 혜 영

부 경 대 학 교 대 학 원 생물공학과

요 약

산업혁명이후화석연료의사용량이급속도로증가하여현재전세계에너지사용량의약86%에 달하고있으며, 이러한화석연료의사용으로인해지구온난화등의환경문제가전세계적으로 부각되고있다. 따라서이러한범지구적온난화현상을막기위해재생가능한바이오연료에대 한관심이증가하고있으며, 이중바이오에탄올은액체연료인휘발유를대체할수있는유력한 대체연료로서세계적으로그생산량이급증하고있다. 홍조료는1세대바이오매스인옥수수, 감자등의전분질계, 2세대인목질계에비해생산효율이훨씬높다는점에서바이오연료대량생 산의길을넓힐것으로기대된다. 특히우뭇가사리는발효가능한탄수화물의함량이목질계에 비해1.5~2배정도높은데다목질계원료에서반드시제거해야하는리그닌성분이없어제조공 정이간편하다는큰장점이있다. 더욱이홍조류는연간4~6회수확이가능할정도로생장속도 가빠르고, 별도의비료나농업용수를필요로하지않는다. 같은양의여타바이오매스에비해 훨씬높은생산성과환경친화성을가지고있다는의미이다. 따라서, 본연구에서는우뭇가사 라를바이오매스로한산촉배열가수분해와효소당화의최적화, 그리고글루코오스에대한갈

락토오즈소비저해문제를해결하기위한고농도갈락토오즈순치를통한바이오에탄올생산을 수행하였다. 당화최적화를위하여91mM H_2SO_4와8% (w/v)의슬러리파우더를45분간열처리하여25.6g/l의갈락토오즈와Celluclast 1.5L과Viscozyme L의복합효소를사용하여7.6g/l의글루코오즈를생산할수있었으며, 당화수율의경우산촉매열가수분해와효소당화를이용하여각각97.2%, 72.8%의당화수율을얻을수있었다. 효모의클루코오즈에의한갈락토오즈 소비저해문제를해결하기위해120g/l의고농도의갈락토오즈가포함된배지에서순치를수행하였다. 순치효모로는*Saccharomyces cerevisiae* KCCM 1129 와*Pichia stipitis* KCTC 7228을이용하였으며, 이순치효모를이용하여14.6g/l, 16.6g/l의에탄올을생산하였다. 효모를순치하여에탄올발효를하였을때, 에탄올수율이증가하는것으로보아해조류우뭇가사리를이용한바이오에탄올생산에있어산업적활용이가능할것으로사료된다.

Chapter1. Ethanol production from the seaweed,*Gelidium amansii*using specific sugar acclimated yeasts

Ⅰ. INTRODUCTION

Seaweed biomasshas been regarded as an alternative to fossil fuel. As a renewable and eco-friendly biomass [2, 22],seaweed is a third-generation biomass that can be used for bioenergy production.Seaweed grows quickly, is lignin-freehowever it is not used as a primary food crop [2, 6]. Especially, *Gelidium amansii*,red seaweed, hasthe advantage of high carbohydrate contents.*G. amansii* is composed of cellulose and agar (galactan). Agar iscomposed of galactose and 3,6-anhydrogalactose (AHG) [7].In ethanol production using*G. amansii*, thermal acid hydrolysis and enzymatic hydrolysis have been usedforthe saccharification.The agar is hydrolyzed to galactose and 3,6-AHG by thermal acid hydrolysis. Thermal acid hydrolysis is a simple processthat has a short reaction time. Enzymatic saccharification has been used for the saccharification of cellulose in order to overcomethe low yield of glucose production [9, 10, 11].Therefore, galactose and glucose are monosaccharides for ethanol fermentationfrom*G. amansii*. However, glucose of hydrolysates causesthe repression of galactose uptake.Because the repression decreasesthe yield of ethanol production, the repression has to be overcome for successful fermentation. The acclimation of galactose allows simultaneous utilization of glucose and galactose [5]. In many studies on mixed

sugar fermentation, the yeastused has been improved by acclimation to a highconcentration of sugar for a short time to enhance ethanolproduction.Therefore, acclimation of galactose is the key process of fermentation when *G. amansii* is used [5, 12, 14].

Separated hydrolysis and fermentation (SHF) is a separated process of saccharification and fermentation. The main advantage of SHF is to separately optimize the process steps. Especially, enzymatic saccharification and fermentation need different optimal pH and temperature. Therefore, the SHF process is more efficient for high ethanol yield than simultaneous saccharification and fermentation (SSF) process when *G. amansii* is used [1, 7, 13].

In order to produce ethanol using seaweed as the source material, this study conducted a thermal acid hydrolysis and enzymatic saccharification of*G.amansii*. *Pichia stipitis* has ethanol yield about 82 % and able to ferment most of sugars including glucose, galactose and cellobiose. And *Saccharomycescerevisiae* can generate a high yield of ethanol about 90 % of glucose [16]. Therefore, fermentations were carried out using *Pichia stipitis* and *Saccharomyces cerevisiae.*The yeasts were acclimated to produce the high concentration of ethanol and minimize thefermentation timeby the prevention of repression on galactose uptake.

Ⅱ. MATERIALS AND METHODS

1.Raw materials and composition analysis

Gelidium amansii was obtained from the Gijang fisheries market in Busan, Korea. A composition analysis of *G. amansii* was conducted at the Feed & Foods Nutrition Research Center ofPukyongNational University in Busan, Korea.

2. Thermal acid hydrolysis

Milled *Gelidium amansii* was added to91 mM H_2SO_4 to make solid/liquid (S/L)contents of 8 % (w/v). Then,a thermal acid hydrolysis was carried out in the autoclave at 121℃ for 45 min [6, 21]. The efficiency of thermal acid hydrolysis was calculated as follow:

$$E_{TAH} = \frac{\Delta S_{gal}}{[Gal]_{max}} \times 100$$

in which E_{TAH} is efficiency of thermal acid hydrolysis (%), ΔS_{gal} is galactose increase (g/l) during thermal acid hydrolysis and $[Gal]_{max}$ is thetheoretical maximum galactose concentration in pretreated *G. amansii*[20].

3. Enzymatic saccharification

The enzymatic saccharification of acid hydrolysate was performed by adding 8.4 EGU/mlofCelluclast 1.5 L (Novozyme) and1.2 FBG/mlof Viscozyme L (Novozyme) with 2 % (w/v) sodium azideto inhibit microbial growth during the enzymatic hydrolysis after adjusting pH 4.5 with 10N NaOH [9, 24]. Viscozyme L has endo-beta-glucanase that hydrolyzes (1,3)- or (1,4)-likages in beta-D-glucans with side activities ofxylanase, cellulase and hemicellulose.Celluclast 1.5L has cellulase that hydrolyzes (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans.

The reaction was carried out in water bath at 45℃, 30 rpm for 60 h [13].The efficiency of enzymatic saccharification was calculated as follow:

$$E_{ES}(\%) = \frac{\Delta S_{glu}}{[Glu]_{max}} \times 100$$

in which E_{ES} is efficiency of enzymatic saccharification (%), $\Delta S_{glu}$is glucose increase (g/l) during enzymatic saccharification and $[Glu]_{max}$ is thetheoreticalmaximum glucose concentration from fiber of *G. amansii*[20].

4. Seed culture and fermentation medium

Stocked *Pichia stipitis* KCTC 7228 and *Saccharomyces cerevisiae* KCCM 1129 were cultured in YPG agar plate composed of 10 g/l yeast extract, 20 g/l peptone, 20 g/l galactose and 15 g/l agar for 24 h[19]. Each colony of yeasts was inoculated with 15 ml YPG (Yeast extract, Peptone and Galactose) medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l galactose. The mixture was cultured at 30℃, 30 rpm for 24 h. Five ml ofcultures were transferred to 50 ml to YPG medium and culturedunder thesame condition. The OD_{600} of *P. stipitis* and *S. cerevisiae* reached 23.6 and 27.3, respectively. The acclimation of *P. stipitis* and *S. cerevisiae* to high concentration of galactose was carried out. Five ml ofyeastswereinoculated50 ml YPHG (Yeast extract, Peptone and High concentration of galactose) medium composed of 10 g/l yeast extract, 20 g/l peptone, 120 g/l galactose and cultured for 18 h until OD_{600} of *P. stipitis* and *S. cerevisiae*reached 26.2 and 24.8 [19].The eachcell of 25 mlwas centrifuged at 3000 rpm for 5 min to remove the YPG medium and cell pellets were inoculated, respectively.

5. Separated hydrolysis and fermentation (SHF)

The fermentation was carried out in 500 ml flasks with a working volume of 250 ml [17]. Afterenzymatic saccharification and final neutralization to pH 6.4 were carried out. Fermentations were carried out with acclimated and non-acclimatedyeasts, *P. stipitis* and *S. cerevisiae.*

Thefermentation was carried out at 30℃, 30 rpm for 96 h.The efficiency of ethanol yield was calculated as follow:

$$Y_{EtOH} = \frac{[EtOH]}{[Sugar]_{ini}}$$

in which Y_{EtOH} is ethanol yield (g/g), [EtOH] is the ethanol concentration achieved during fermentation (g/l). $[Sugar]_{ini}$ is total initial fermentable sugar (galactose+glucose) concentration at onset fermentation (g/l). $Y_{EtOH}$of 0.51 is the theoretical maximumethanol yield [19].

6. Analytical methods

The cell concentrations were determined by the optical density (OD) measurement of the cells using a UV-Vis spectrophotometer and converted to dry cell weight. The concentrations of glucose, galactose, ethanol and 5-HMF were measured by HPLC (Agilent 1100 Series, Agilent. Inc., USA) equipped with Agilent G1362A refractive index detector. A BioradAminex HPX-87H column and Supelguard C610H column were used with filtered and degassed 5 mM H_2SO_4 as eluent at the flow rate of 0.6 ml/min and a column temperature of 65℃.The activities of cellulaseand β-glucosidase were determined according to the procedure described in Mandels et al. and Kubicek et al. [15, 18].

Ⅲ. RESULTS AND DISCUSSION

1.Composition of *G. amansii*

G. amansii isred algae and has the highest carbohydrate contents among seaweeds[11]. The analytical results indicated that the total carbohydrate content was 74.4 % (w/w). Agar and fiber in the total carbohydrate were 62.8 % (w/w) and 11.6 % (w/w), respectively.The ratio of galactose to 3,6-anhydrogalactose was reported as 1:1.13 (0.47:0.53) [8].Therefore, the maximum galactose content was calculated as 33 %; (0.628g agar/g *G.amansii*) x (0.47g galactose unit/g agar) x (180g galactose/162g galactose unit) x 100. The maximum glucose content was calculated as 13 %; (0.116g cellulose/g *G.amansii*) x (180g glucose/162g unit of cellulose) x 100.The total initial fermentable sugar contentwas calculated as 46 % of *G. amansii*. Therefore, the maximum galactose concentration can reach 26.4 g/l; (80g *G.amansii* /l liquid) x (0.33 g galactose/g *G.amansii*)and glucose concentration can reach 10.4 g/l; (80g *G.amansii* /l liquid) x (0.13 g glucose/g *G.amansii*)from 80 g/l of slurry[19]. Also, the components in *G. amansii* were comprised of 18.1 % (w/w) crude protein, 0.2 % (w/w) crude lipid and 7.3 % (w/w) crude ash as shown in Fig. 1.

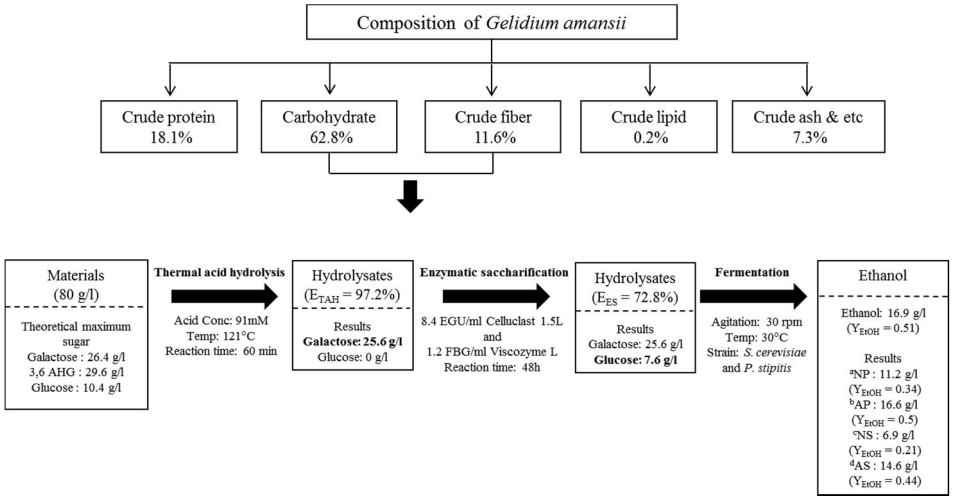

Fig. 1. Compositional analysis data and mass balance flow chart of bioethanol production process from *Gelidium amansii*

(E_{TAH} = Efficiency of thermal acid hydrolysis: $E_{TAH} = \frac{\Delta S_{gal}}{[Gal]_{max}} \times 100$

; E_{ES} = Efficiency of enzymatic saccharification: $E_{ES}(\%) = \frac{\Delta S_{glu}}{[Glu]_{max}} \times 100$

; Y_{EtOH} = The efficiency of ethanol yield: $Y_{EtOH} = \frac{[EtOH]}{[Sugar]_{ini}}$

; [a]NP = Non-acclimated *P. stipitis*; [b]AP = Acclimated *P. stipitis*; [c]NS = Non-acclimated *S. cerevisiae*; [d]AS = Acclimated *S. cerevisiae*)

2. Thermal acid hydrolysis

The agar can be hydrolyzed by the acid however, cannot be hydrolyzed by the enzyme [7]. Sulfuric acid of 91 mMwas added to8 % (w/v) slurry of *G. amansii* and treated at 121℃ for 45 min. As a result, the galactose concentration of 25.6g/l was obtained and the E_{TAH} was 97.2 % as shown in Fig.1. Because the physical morphology of the agar is softer than that of cellulose, the optimal reaction conditions for thermal acid hydrolysis would be milder than that of cellulose [15]. Therefore, the glucose was not found in the thermal acid hydrolysis.When thermal acid hydrolysis was used, the 5-hydroxy-methyl furfural (5-HMF) was generated from the degradation of 3,6-anhydrogalactose due to its acid-labile character [11].In this study, 4.8 g/l of 5-HMF as inhibitors in fermentation process was found in the thermal acid hydrolysis.However, according to a previous study, the 5-HMF concentration above 5 g/l shows the crucial role of inhibiting fermentation process when using red seaweed hydrolysates [11, 20]. Therefore5-HMF concentration of thermal acid hydrolysis did not affect the fermentation process.

3. Enzymatic saccharification

For the hydrolysis of fiber, Celluclast 1.5 L, Viscozyme Land mixed enzymesof Celluclast 1.5 L and Viscozyme L were used.After a thermal acid hydrolysis, enzymatic saccharification was carried out at pH 4.5, 130 rpm, 45℃ for 60 h as shown in Fig. 2. The glucose was released until 48 h after theaddition of enzymes. Especially,the mixedenzymes (Celluclast 1.5 L + Viscozyme L) produced 7.6 g/l and 7.8 g/l glucose at 48 h and 60 h, respectively.In the case of Celluclast 1.5 Ltreatment, the glucose was releaseduntil 48h of saccharification.The final glucoseconcentration of 5.5 g/l with E_{ES} of 52.9 % was obtained. When Viscozyme L was treated, the glucose concentration of 1.7 g/l with E_{ES} of 16.3 % was obtained at 4 h. Enzyme saccharification of Celluclast 1.5 L to *G. amansii* hydrolysate was preferable than that of Viscozyme L. Among those treatment methods, the mixed enzyme treatment showed synergistic effectand maximum efficiency of enzymatic saccharification [20]. Therefore, mixed enzymes were used as an optimal saccharification of fiber to glucose for 48 h, and theglucoseconcentration of 7.6 g/lwith E_{ES}= 72.8 % was obtained as shown in Fig. 1.

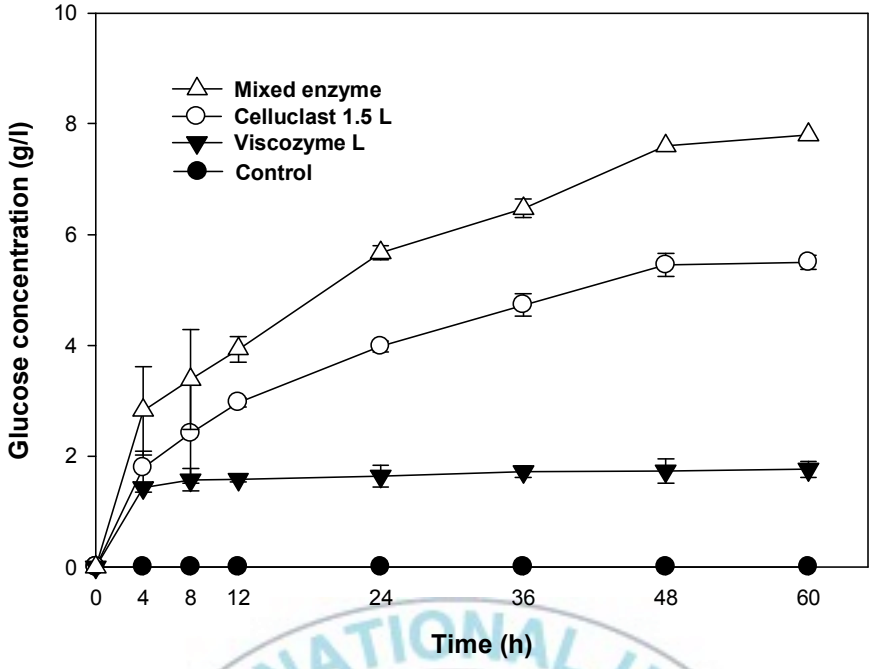

Fig. 2. Effect of various enzymes for the production of glucose from *G. amansii* by enzymatic saccharification at45℃, 30 rpm for 60 h

4. Separated hydrolysis and fermentations (SHF)

SHF was carried out by the addition of thegalactose acclimated or non-acclimated*P. stipitis*(Fig. 3)or*S. cerevisiae*(Fig. 4). Fermentation with non-acclimated*P. stipitis*to high concentration of galactose was shown in Fig. 3 (a).Glucose was consumed first as the fermentation started because glucose was the preferred substrate to galactose. Glucose was consumed in 48 h, and then galactose was consumedfor 24 h. However,galactose was not totally consumed until 96 h, and 7.1 g/l of galactose remained as shown in Fig. 3 (a). The ethanol concentration after 96 h of fermentation with non-acclimated *P. stipitis* was 11.5 g/l with Y_{EtOH} = 0.34 as shown in Fig. 3 (a).

A galactose concentration of 25.6 g/l and a glucose concentration of 7.6 g/l were consumed by high concentration of galactose acclimated *P. stipitis*as shown in Fig 3 (b). Compared to non-acclimated *P. stipitis,*glucose was totally consumed during 60 h. The galactose was consumed until 84h, and the final ethanol concentration of 16.6 g/l with Y_{EtOH} = 0.5was producedas shown in Fig. 3 (b).

The fermentation with non-acclimated *S. cerevisiae*to high concentration of galactose produced ethanol concentration of6.9 g/l with Y_{EtOH} = 0.21 for 96 h as shown in Fig. 4 (a). As the result of saccharification, 25.6 g/l galactose was hydrolyzed by thermal acid hydrolysis and 7.6 g/l glucose was obtained by enzymatic hydrolysis.*S. cerevisiae*preferred glucose to galactose. The glucose consumption rate of*S. cerevisiae*with 0.33was faster than that of*P. stipitis*with 0.16as shown in Fig. 4 (a). The glucose was consumed in 24 h,however, galactose was rarely consumed becauseof the

repression of galactose uptake by glucose. Since glucose directly enters the glycolysiswhich is the main metabolic pathway in the ethanol fermentation, glucose is taken by yeasts in preference to galactose requiring conversion to glucose prior to the use for theglycolysis[2]. Therefore, the ethanol production from the mixture of galactose and glucose was inhibited dueto the glucose repression to galactose consumption. The utilization of galactose by yeast requires the enzymes of the Leloir pathway, which catalyze the transformation of galactose to glucose-6-phosphate. These enzymes are encoded by a family of GAL gene and their expression is induced by the growth in galactose and repressed during the growth in glucose. When galactose is absent from the medium, GAL gene inhibits the function of transcriptional activator. Therefore, the acclimation of yeasts to galactose diminishes the repression of galactose consumption [3, 4]. The fermentation with acclimated *S. cerevisiae*utilized25.8 g/l galactose and 8.1 g/l glucose as shown in Fig. 4 (b). The galactose was rarely consumed due to the repression ofnon-acclimated *S. cerevisiae*as shown in Fig. 4. (a). However, when *S. cerevisiae* was acclimated to high concentration of galactose, the glucose and galactose weresimultaneously consumedas shown in Fig. 4 (b). The diauxic fermentation was observed in acclimated *S. cerevisiae* on galactose. Fermentation using glucose produced ethanol for 24 h. When glucose was exhausted, ethanol fermentation had temporarily slowed down resulting in a lag that is called the diauxic shift from 24 h to 36 h. After a short lag at the diauxic shift, the galactose was totally consumed for 96 h [22].An ethanol concentration of 14.6g/l with Y_{EtOH} = 0.44 was obtained from 25.6 g/l galactose and 7.6 g/l glucose as shown in Fig. 4(b).As results, the

overall mass balance and ethanol yields were described in Fig 1. Therefore, the acclimation of yeasts to high concentration of galactose could make simultaneous utilization of galactose and glucose for the production of ethanol from seaweed *G. amansii*. Acclimated yeasts produced a higher ethanol concentration than non-acclimated yeasts.

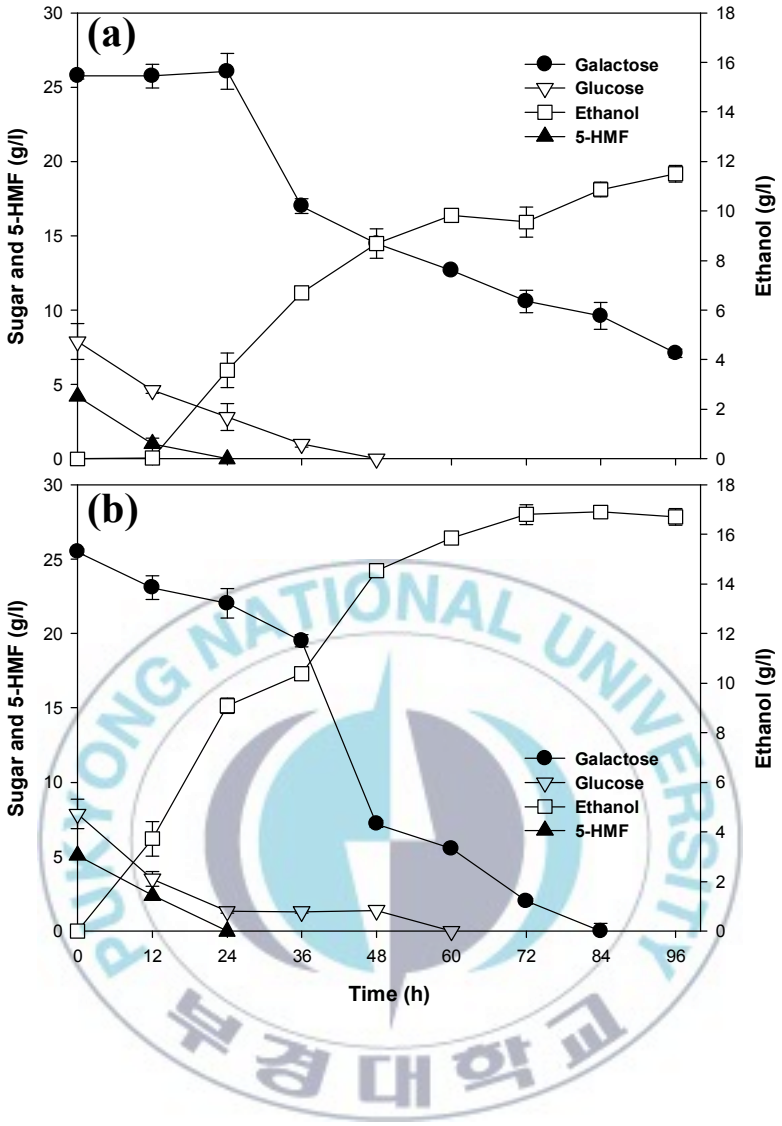

Fig. 3. Ethanol production from hydrolysis of *G. amansii* by SHF with *P. stipitis* KCTC 7228

(a) non-acclimated *P. stipitis* and **(b)** acclimated *P. stipitis* to high concentration of galactose

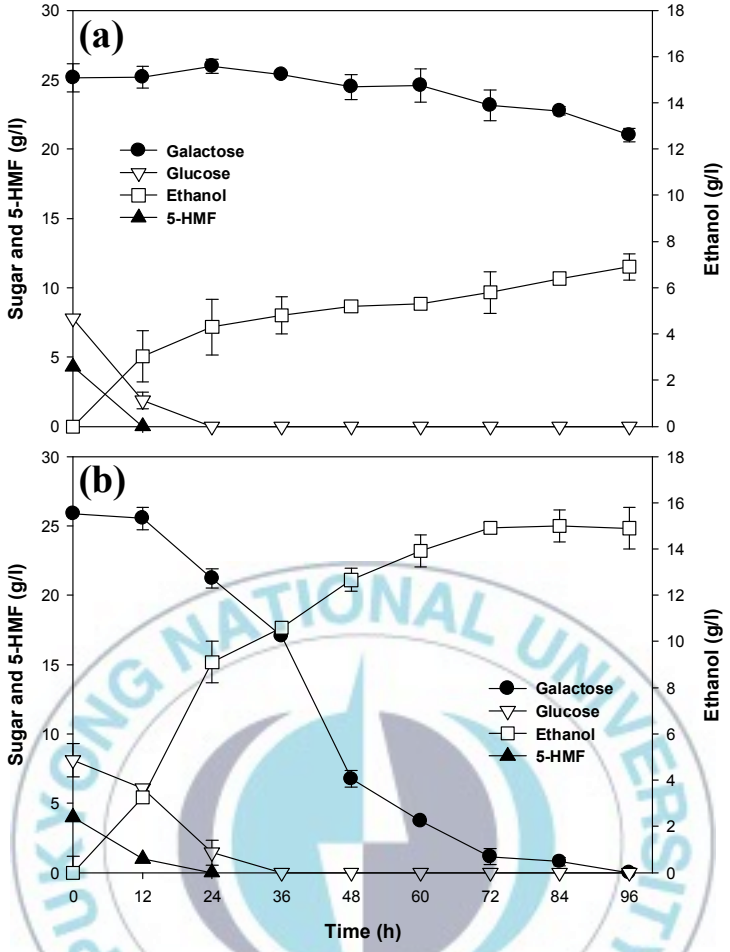

Fig. 4. Ethanol production from hydrolysis of *G. amansii* by SHF with *S. cerevisiae* KCCM 1129

(a) non-acclimated *S. cerevisiae* and **(b)** acclimated *S. cerevisiae* to high concentration of galactose

Ⅳ. CONCLUSION

In producing ethanol from seaweed *G. amansii*, acclimation of yeasts to high concentration of galactose is an important factor in increasing the ethanol yield from *G. amansii*. Through the thermal acid hydrolysis, 25.6 g/l of galactose was obtained with a saccharification yield of 59.6 % from the total agar of *G. amansii*, and a glucose concentration of 7.6 g/l was obtained by enzymatic saccharification using Celluclast 1.5 L (Novozyme) and Viscozyme L (Novozyme). In the case of non-acclimated yeasts to high concentration of galactose at the SHF process with a working volume of 250 ml, an ethanol concentration of 12.1 g/l was obtained using *P. stipitis* and 9.1 g/l was produced by *S. cerevisiae*, respectively. In the mixed sugars with galactose and glucose, galactose consumption was repressed due to glucose preference of yeasts. The glucose preference was overcome through the acclimation of yeasts to high concentration of galactose. Using acclimated *P.stipitis* and *S. cerevisiae* to high concentration of galactose, ethanol concentrations of 16.6 g/l and 16.3 g/l were produced, respectively. The acclimation of yeasts to high concentration of galactose could make simultaneous utilization of galactose and glucose. Acclimated yeasts produced a higher ethanol concentration than non-acclimated yeasts.

Ⅴ. ACKNOWLEDGMENTS

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Ⅵ. REFERENCES

1. Alfani F, Gallifuoco A, Saporosi A, Spera A,CantarellaM. 2000. Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. *J. Ind. Microbiol. Biotechnol.***25:** 184-192

2. BaiFW, Anderson WA,Moo-Young M.2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.***26**: 89-105.

3. Bro C, Knudsen S, Regenberg B, Olsson L, Nielsen J. 2005. Improvement of galactose uptake in *Saccharomyces cerevisiae* through overexpression of phosphoglucomutase: Example of transcript analysis as a tool in inverse metabolic engineering. *Appl. Environ. Microbiol.***71:** 6465-6472

4. DelgenesJ, Moletta R, Navarro J. 1988. Fermentation ofD-xylose, D-glucose and L-arabinose mixture by *Pichia stipitis* Y7124: Sugar tolerance. *Appl. Microbiol. Biotechnol.***29:** 155-161.

5. ErnandesJR, William JW,Stewart GG. 1992. Simultaneous utilization of galactose and

glucose by *Saccharomyces* spp. *Biotechnol. Adv.***6:** 233-238.

6. JangJS, Cho Y, Jeong GT,Kim SK. 2012. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*. *BioprosessBiosyst. Eng.***35:**11-18.

7. JeongTS,Kim YS, and Oh KK. 2011. Two-stage acid saccharification of fractionated *Gelidium amansii* minimizing the sugar decomposition. *Bioresour. Technol.***102:**10529-10534.

8. Jol CN, Neiss TG, Penninkhof B, Rudolph B, De Ruiter GA. 1999. A novel high-performance anion-exchange chromatographic method for the analysis of carrageenans and agars containing 3,6-anhydrogalactose. *Anal. Biochem.* **268:** 213-222.

9. Kamireddy SR, Li J, Tucker M, Degenestein J, Ji Y. 2013. Effects and mechanism of metal chloride salts on pretreatment and enzymatic digestibility of corm stover.*Ind. Eng. Chem. Res.* **52**: 1775-1782.

10. Kang HK, Kim NM, Kim GJ, Seo ES, Ryu HJ, Yun SI, Choi HC, Day DF, Kim J, Cho

DL,Kim D. 2011. Enhanced saccharification of rice straw using hypochlorite-hydrogen peroxide. *Biotechnol. Bioprocess Eng.***16:** 273-281.

11. KimC, Ryu HJ, Kim SH, Yoon JJ, Kim HS,Kim YJ. 2010. Acidity tunable ionic liquids as catalysts for conversion of agar into mixed sugars. *Bull. Korean Chem. Soc.***31:**511-514.

12. KimH, Ra CH, and Kim SK. 2013. Ethanol production from seaweed (*Undariapinnatifida*) using yeast acclimated to specific sugars. *Biotechnol. Bioprocess Eng.***18:** 533-537.

13. Kim NJ, Li HJ, Chang HN, Lee PC. 2011. Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. *Bioresour. Technol.* **102:** 7466-7469.

14. KimSR, Ha SJ, Wei N, Oh EJ, Jin YS. 2012. Simultaneousco-fermentation of mixed sugars: A promising strategy.*Trends Biotechnol.***30:** 274-282.

15. Kubicek CP. 1982. β-glucosidase excretion by *Trichodermapseudokoningii* correlation with cell wall bound β-1,3-glucanase activities. *Arch. Microbiol*. **132**: 349-354.

16. Limayem A, Ricke SC. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects.*Prog.Energ. Combust.* **38:** 449-467.

17. Liu R, Li J, Shen F. 2008. Refining bioethanol from stalk juice of sweet sorghum by immobilized yeast fermentation. *Renew. Energy.* **33:** 1130-1135

18. Mandels M, Aldreotti R, Roche C. 1976. Measurement of saccharifyingcellulase. *Biotechnol. Bioeng. Symp.***6**: 21-23.

19. ParkJH, Hong JY, Jang HC, Oh SG, Kim SH, Yoon JJ,Kim YJ. 2012. Use of *Gelidium amansii* as a promising resource for bioethanol: A practical approach for continuous dilute-acid hydrolysis and fermentation. *Bioresour. Technol.***108:**83-88.

20. Ra CH, Jeong GT, Shin MK, Kim SK. 2013.Biotransformation of 5-hydroxymethylfurfural (HMF) by *Scheffersomyces stipitis* during ethanol fermentation of hydrolysate of the seaweed *Gelidium amansii.Bioresour. Technol.* **140:** 421-425.

21. Saha BC, Iten LB, Cotta MA, Wu YV. 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem.***40:** 3693-3700

22. Stahl G, Nakamura SNB, Ariga O, Nakasaki K. 2004. Translational accuracy during exponential, postdiauxic, and stationary growth phases in *Saccharomyces cerevisiae*. *Eukaryotic cell*. **3:** 331-338

23. Yanagisawa M, Nakamura K, Ariga O,Nakasaki K. 2011. Production of high concentrations of bioethanol from seaweeds that contain easily hydrolysablepolysaccharides. *Process*

Biochem. **46:** 2111-2116

24. Zheng Y, Pan Z, Zhang R, Wang D. 2009. Enzymatic saccharification of dilute acid pretreated saline crops for fermentable sugar production.*Appl. Energy*. **86**:2459-2465

Chapter2. Enhanced bioethanol production from seaweed, Gelidium amansii, using acclimated yeasts to high salt concentration

Ⅰ. INTRODUCTION

Seaweed biomasshas been regarded as an alternative to fossil fuel. As a renewable and eco-friendly biomass [2, 22],seaweed is a third-generation biomass that can be used for bioenergy production.Seaweed grows quickly, is lignin-free however it is not used as a primary food crop [2, 6]. Especially, *Gelidium amansii*,red seaweed, hasthe advantage of high carbohydrate contents.*G. amansii* is composed of cellulose and agar (galactan). Agar is composed of galactose and 3,6-anhydrogalactose (AHG) [7].In ethanol production using *G. amansii*, thermal acid hydrolysis and enzymatic hydrolysis have been usedforthe saccharification.The agar is hydrolyzed to galactose and 3,6-AHG by thermal acid hydrolysis. Thermal acid hydrolysis is a simple processthat has a short reaction time. Enzymatic saccharification has been used for the saccharification of cellulose in order to overcomethe low yield of glucose production [9, 10, 11]. Therefore, galactose and glucose are monosaccharides for ethanol fermentation from*G. amansii*. Separated hydrolysis and fermentation (SHF) is a separated process of saccharification and fermentation. The main advantage of SHF is to separately optimize the process steps. Especially, enzymatic saccharification and fermentation need different optimal pH and temperature. Therefore, the SHF process is more efficient for high ethanol

yield than simultaneous saccharification and fermentation (SSF) process when *G. amansii* is used [1, 7, 13].

In order to produce ethanol using seaweed which has problem of high salinity, this study conducted a thermal acid hydrolysis and enzymatic saccharification of *G.amansii*. Therefore, fermentations were carried out using *Pichia stipitis* and *Saccharomyces cerevisiae.* The yeasts were acclimated to produce the high concentration of ethanolby the prevention of repression on salinity.

Ⅱ. MATERIALS AND METHODS

1. Raw materials and composition analysis

Gelidium amansii was obtained from the Gijang fisheries market in Busan, Korea. A composition analysis of *G. amansii* was conducted at the Feed & Foods Nutrition Research Center ofPukyongNational University in Busan, Korea.

2. Thermal acid hydrolysis

Milled *Gelidium amansii* was added to182 mM H_2SO_4 to make solid/liquid (S/L)contents of 16 % (w/v). Then,a thermal acid hydrolysis was carried out in the autoclave at 121℃ for 60 min [6, 21]. The efficiency of thermal acid hydrolysis was calculated as follow:

$$E_{TAH} = \frac{\Delta S_{gal}}{[Gal]_{max}} \times 100$$

in which E_{TAH} is efficiency of thermal acid hydrolysis (%), ΔS_{gal} is galactose increase (g/l) during thermal acid hydrolysis and $[Gal]_{max}$ is the theoretical maximum galactose concentration in pretreated *G. amansii* [20].

3. Enzymatic saccharification

The enzymatic saccharification of acid hydrolysate was performed by adding 8.4 EGU/ml ofCelluclast 1.5 L (Novozyme) and1.2 FBG/ml of Viscozyme L (Novozyme) with 2 % (w/v) sodium azide to inhibit microbial growth during the enzymatic hydrolysis after adjusting pH 4.5 with 10N NaOH [9, 24]. Viscozyme L has endo-beta-glucanase that hydrolyzes (1,3)- or (1,4)-likages in beta-D-glucans with side activities of xylanase, cellulase and hemicellulose. Celluclast 1.5L has cellulase that hydrolyzes (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans.

The reaction was carried out in water bath at 45℃, 30 rpm for 60 h [13].The efficiency of enzymatic saccharification was calculated as follow:

$$E_{ES}(\%) = \frac{\Delta S_{glu}}{[Glu]_{max}} \times 100$$

in which E_{ES} is efficiency of enzymatic saccharification (%), $\Delta S_{glu}$is glucose increase (g/l) during enzymatic saccharification and $[Glu]_{max}$ is thetheoreticalmaximum glucose concentration from fiber of *G. amansii* [20].

4. Detoxification

After thermal acid hydrolysis and enzymatic saccharification, active charcoal (Samchun., Inc.) was

used for remove HMF was added with active charcoal of 5% and stirred for 1 hr. The treated hydrolysate was then used for the fermentation studies.

5. Seed culture and fermentation medium

Stocked *Candida tropicalis* KCTC 7228 and *Saccharomyces cerevisiae* KCCM 1129 were cultured in YPG agar plate composed of 10 g/l yeast extract, 20 g/l peptone, 20 g/l galactose,15 g/l agar for 24 h [19]. Each colony of yeasts was inoculated with 15 ml YPG (Yeast extract, Peptone and Galactose) medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l galactose. The mixture was cultured at 30℃, 30 rpm for 24 h. Five ml ofcultures were transferred to 50 ml to YPG medium and culturedunder the same condition. The acclimation of *C.tropicalis* and *S. cerevisiae* to high concentration of galactose was carried out. Five ml of yeasts were inoculated 50 ml YPG (Yeast extract, Peptone, galactose and High concentration of NaCl) medium composed of 10 g/l yeast extract, 20 g/l peptone, 20 g/l galactose and 70 g/l NaCl and cultured for 18 h [19].The each cell of 25 ml was centrifuged at 3000 rpm for 5 min to remove the YPG medium and cell pellets were inoculated, respectively.

6. Separated hydrolysis and fermentation (SHF)

The fermentation was carried out in 500 ml flasks with a working volume of 250 ml [17]. Afterenzymatic saccharification and final neutralization to pH 6.4 were carried out. Fermentations were carried out with acclimated and non-acclimated yeasts, *C. tropicalis*and *S. cerevisiae.* The fermentation was carried out at 30℃, 30 rpm for 168 h.The efficiency of ethanol yield was calculated as follow:

$$Y_{EtOH} = \frac{[EtOH]}{[Sugar]_{ini}}$$

in which Y_{EtOH} is ethanol yield (g/g), [EtOH] is the ethanol concentration achieved during fermentation (g/l). $[Sugar]_{ini}$ is total initial fermentable sugar (galactose+glucose) concentration at onset fermentation (g/l). $Y_{EtOH}$of 0.51 is the theoretical maximumethanol yield [19].

7.Analytical methods

The cell concentrations were determined by the optical density (OD) measurement of the cells using a UV-Vis spectrophotometer and converted to dry cell weight. The concentrations of glucose, galactose, ethanol and 5-HMF were measured by HPLC (Agilent 1100 Series, Agilent. Inc., USA) equipped with Agilent G1362A refractive index detector. A BioradAminex HPX-87H column and Supelguard C610H column were used with filtered and degassed 5 mM H_2SO_4 as eluent at the flow

rate of 0.6 ml/min and a column temperature of 65℃. The activities of cellulaseand β-glucosidase were determined according to the procedure described in Mandels et al. and Kubicek et al. [15, 18].

Ⅲ. RESULTS AND DISCUSSION

1. Composition of *G. amansii*

G. amansii isred algae and has the highest carbohydrate contents among seaweeds[11]. The analytical results indicated that the total carbohydrate content was 74.4 % (w/w). Agar and fiber in the total carbohydrate were 62.8 % (w/w) and 11.6 % (w/w), respectively. The ratio of galactose to 3,6-anhydrogalactose was reported as 1:1.13 (0.47:0.53) [8]. Therefore, the maximum galactose content was calculated as 33 %; (0.628g agar/g *G.amansii*) x (0.47g galactose unit/g agar) x (180g galactose/162g galactose unit) x 100. The maximum glucose content was calculated as 13 %; (0.116g cellulose/g *G.amansii*) x (180g glucose/162g unit of cellulose) x 100.The total initial fermentable sugar contentwas calculated as 46 % of *G. amansii*. Therefore, the maximum galactose concentration can reach 52.8 g/l; (160g *G.amansii* /l liquid) x (0.33 g galactose/g *G.amansii*)and glucose concentration can reach 20.8 g/l; (160g *G.amansii* /l liquid) x (0.13 g glucose/g *G.amansii*)from 160 g/l of slurry [19]. Also, the components in *G. amansii* were comprised of 18.1 % (w/w) crude protein, 0.2 % (w/w) crude lipid and 7.3 % (w/w) crude ash as shown in Fig. 1.

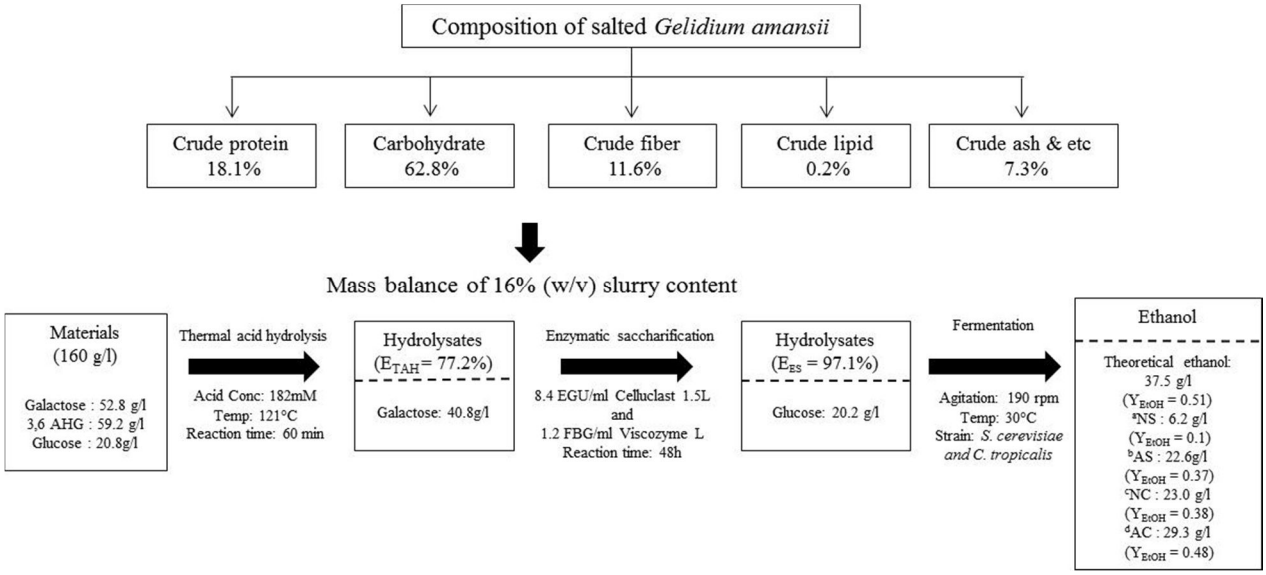

Fig. 1. Compositional analysis data and mass balance flow chart of bioethanol production process from *Gelidium amansii*

(E_{TAH} = Efficiency of thermal acid hydrolysis: $E_{TAH} = \frac{\Delta S_{gal}}{[Gal]_{max}} \times 100$

; E_{ES} = Efficiency of enzymatic saccharification: $E_{ES}(\%) = \frac{\Delta S_{glu}}{[Glu]_{max}} \times 100$

; Y_{EtOH} = The efficiency of ethanol yield: $Y_{EtOH} = \frac{[EtOH]}{[Sugar]_{ini}}$

; [a]NC = Non-acclimated *C. tropicalis*; [b]AC = Acclimated *C. tropicalis*; [c]NS = Non-acclimated *S. cerevisiae*; [d]AS = Acclimated *S. cerevisiae*)

2. Thermal acid hydrolysis

The agar can be hydrolyzed by the acid however, cannot be hydrolyzed by the enzyme [7]. Sulfuric acid of 182 mM was added to16 % (w/v) slurry of *G. amansii* and treated at 121℃ for 60 min. As a result, the galactose concentration of 40.8g/l was obtained and the E_{TAH} was 77.2 % as shown in Fig.1. Because the physical morphology of the agar is softer than that of cellulose, the optimal reaction conditions for thermal acid hydrolysis would be milder than that of cellulose [15]. Therefore, the glucose was not found in the thermal acid hydrolysis.When thermal acid hydrolysis was used, the 5-hydroxy-methyl furfural (5-HMF) was generated from the degradation of 3,6-anhydrogalactose due to its acid-labile character [11].In this study, 4.8 g/l of 5-HMF as inhibitors in fermentation process was found due to the process of detoxification. According to a previous study, the 5-HMF concentration above 5 g/l shows the crucial role of inhibiting fermentation process when using red seaweed hydrolysates [11, 20]. Therefore 5-HMF concentration of thermal acid hydrolysis did not affect the fermentation process.

3. Enzymatic saccharification

For the hydrolysis of fiber, Celluclast 1.5 L, Viscozyme L and mixed enzymes of Celluclast 1.5 L and Viscozyme L were used.After a thermal acid hydrolysis, enzymatic saccharification was carried out at pH 4.5, 130 rpm, 45℃ for 60 h. The final glucoseconcentration of 20.2g/l with E_{ES} of 97.2 % was obtained.

4. Separated hydrolysis and fermentations (SHF)

SHF was carried out by the addition of thegalactose acclimated or non-acclimated *C. tropicalis*(Fig. 2)or*S. cerevisiae*(Fig. 3). Fermentation with non-acclimated *P. stipitis* to high concentration of galactose was shown in Fig. 2 (a). Glucose was consumed first as the fermentation started because glucose was the preferred substrate to galactose. Glucose was consumed in 48 h, and then galactose was consumed for 24 h. However, galactose was not totally consumed until 168 h as shown in Fig. 2 (a). The ethanol concentration after 168 h of fermentation with non-acclimated *C. tropicalis* was 23.0 g/l with Y_{EtOH} = 0.38 as shown in Fig. 2 (a).

A galactose concentration of 40 g/l and a glucose concentration of 20 g/l were consumed by high concentration of galactose acclimated *C. tropicalis*as shown in Fig 2 (b). The galactose was consumed until 168h, and the final ethanol concentration of 29.3 g/l with Y_{EtOH} = 0.48 was produced as shown in Fig. 2 (b).

The fermentation with non-acclimated *S. cerevisiae* to high concentration of galactose produced ethanol concentration of 6.2 g/l with Y_{EtOH} = 0.1 for 168 h as shown in Fig. 3 (a). As the result of saccharification, 40.8 g/l galactose was hydrolyzed by thermal acid hydrolysis and 20.2 g/l glucose was obtained by enzymatic hydrolysis.*S. cerevisiae*preferred glucose to galactose. The glucose and galactose was not consumed in 168 h, Therefore, the ethanol production from high concentration of slurry contents was inhibited due to the salinity. The fermentation with acclimated *S. cerevisiae*utilized 40.8 g/l galactose and 20 g/l glucose as shown in Fig. 3 (b). An ethanol concentration of 29.3 g/l with Y_{EtOH} = 0.48 was obtained as shown in Fig. 3 (b). As results, the overall mass balance and ethanol yields were described in Fig 1. Therefore, the acclimation of yeasts to high concentration of high salt concentration could make utilization of galactose and glucose for the production of ethanol from seaweed *G. amansii*. Acclimated yeasts produced a higher ethanol concentration than non-acclimated yeasts.

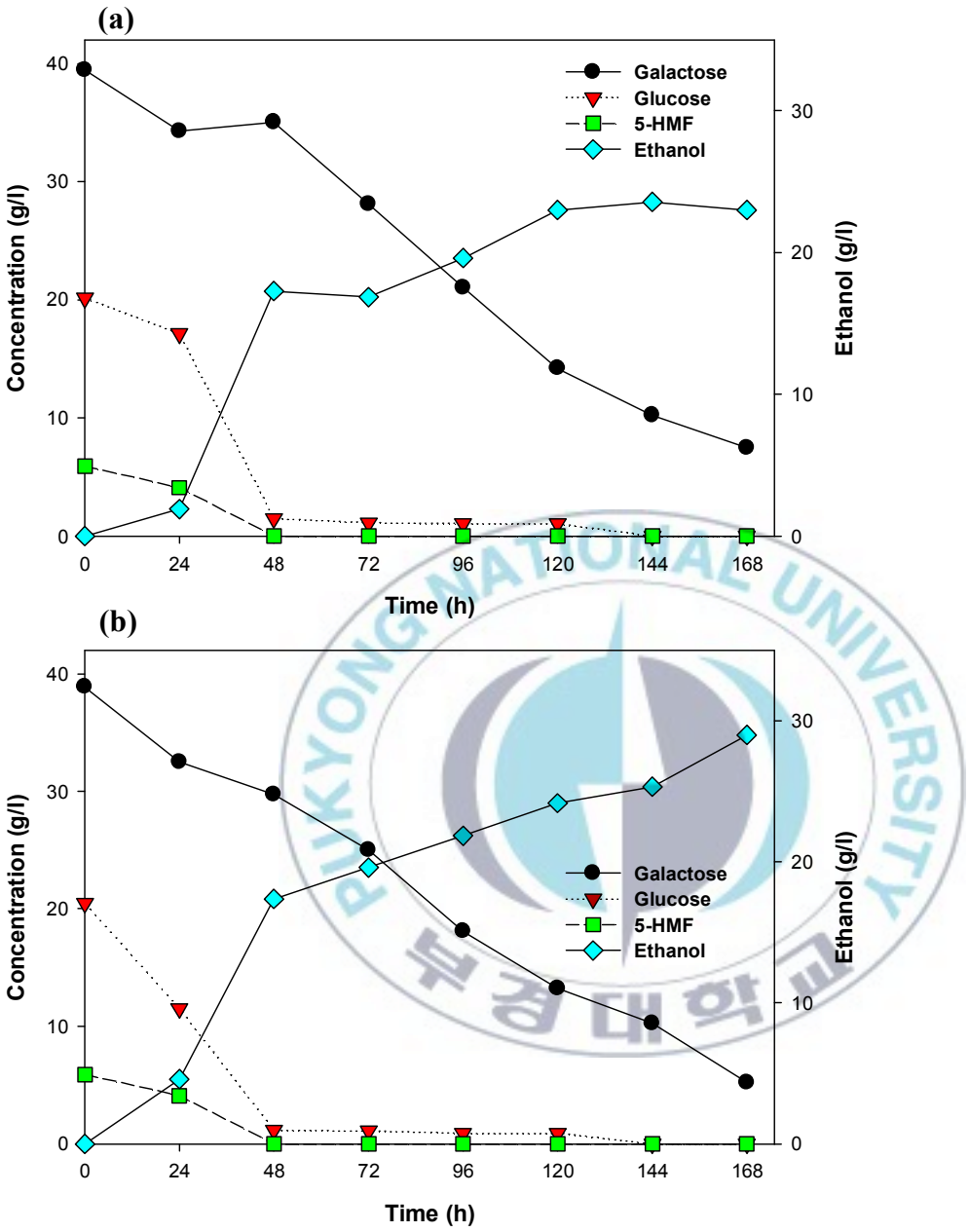

Fig. 2. Ethanol production from hydrolysis of *G. amansii* by SHF with *C. tropicalis*

(a) non-acclimated *C. tropicalis*and **(b)** acclimated *C. tropicalis*to high salt concentration

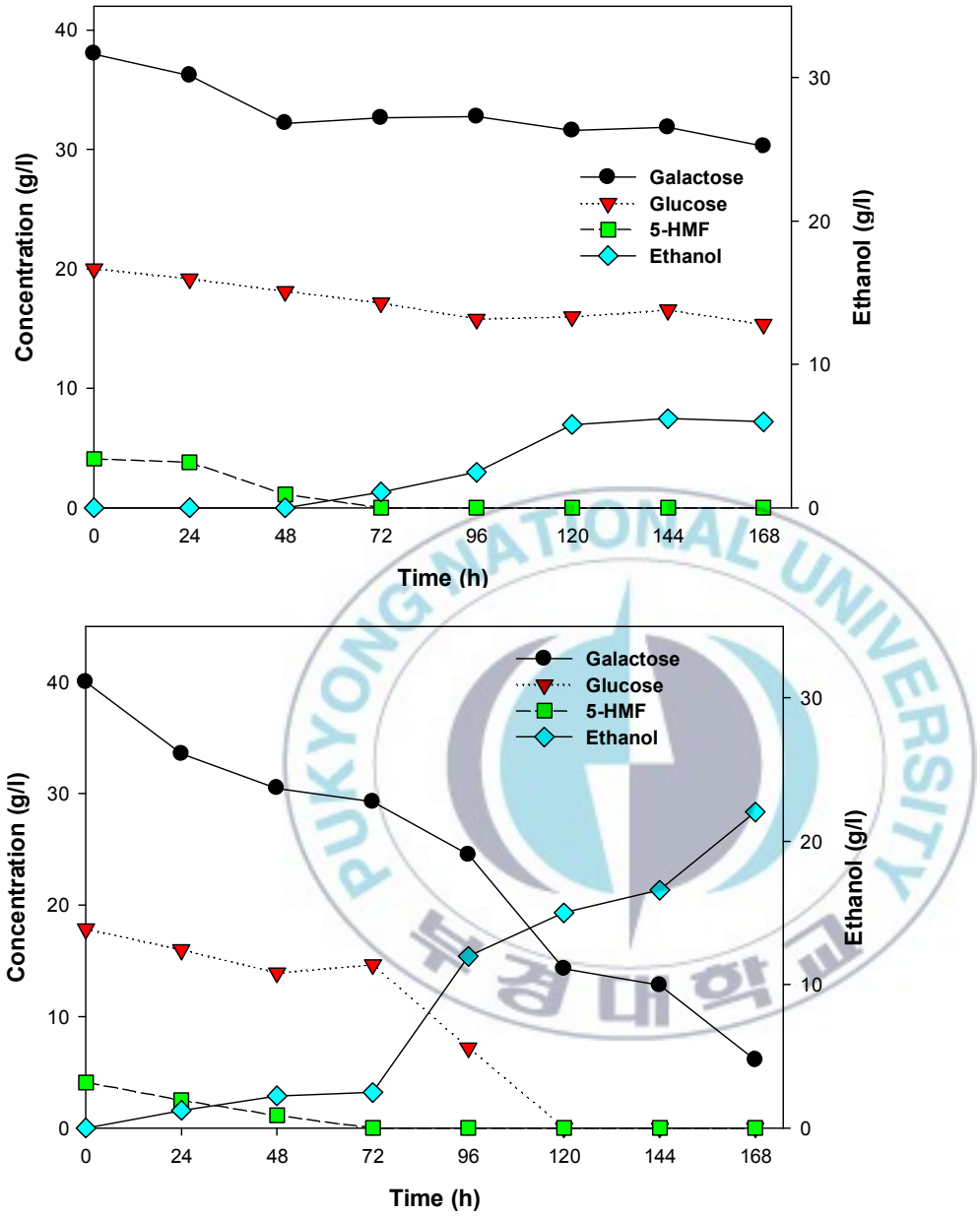

Fig. 3. Ethanol production from hydrolysis of *G. amansii* by SHF with *S. cerevisiae* KCCM 1129

(a) non-acclimated *S. cerevisiae* and **(b)** acclimated *S. cerevisiae* to high salts concentration

Ⅳ. CONCLUSION

In producing ethanol from seaweed *G. amansii*, acclimation of yeasts to high concentration of galactose is an important factor in increasing the ethanol yield from *G. amansii*. Through the thermal acid hydrolysis, 40.8 g/l of galactose was obtained with a saccharification yield of 77.2 % from the total agar of *G. amansii*, and a glucose concentration of 20.2 g/l was obtained by enzymatic saccharification using Celluclast 1.5 L (Novozyme) and Viscozyme L (Novozyme). In the case of non-acclimated yeasts to high concentration of galactose at the SHF process with a working volume of 250 ml, an ethanol concentration of 23.0 g/l was obtained using *C. tropicalis* and 6.2 g/l was produced by *S. cerevisiae*, respectively. When seaweed was used for bioethanol production, fermentation was repressed due to salinity. The problem was overcome through the acclimation of yeasts to high salts concentration. Using acclimated *C. tropicalis*and *S. cerevisiae* to high concentration of galactose, ethanol concentrations of 22.6 g/l and 29.3 g/l were produced, respectively. Acclimated yeasts produced a higher ethanol concentration than non-acclimated yeasts.

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Ⅵ. REFERENCES

1. Alfani F, Gallifuoco A, Saporosi A, Spera A, Cantarella M. 2000. Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. *J. Ind. Microbiol. Biotechnol.***25:** 184-192

2. BaiFW, Anderson WA, Moo-Young M.2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.***26**: 89-105.

3. Bro C, Knudsen S, Regenberg B, Olsson L, Nielsen J. 2005. Improvement of galactose uptake in *Saccharomyces cerevisiae* through overexpression of phosphoglucomutase: Example of transcript analysis as a tool in inverse metabolic engineering. *Appl. Environ. Microbiol.***71:** 6465-6472

4. DelgenesJ, Moletta R, Navarro J. 1988. Fermentation ofD-xylose, D-glucose and L-arabinose mixture by *Pichia stipitis* Y7124: Sugar tolerance. *Appl. Microbiol. Biotechnol.***29:** 155-161.

5. ErnandesJR, William JW, Stewart GG. 1992. Simultaneous utilization of galactose and

glucose by *Saccharomyces* spp. *Biotechnol. Adv.***6:** 233-238.

6. JangJS, Cho Y, Jeong GT, Kim SK. 2012. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*. *BioprosessBiosyst. Eng.***35:**11-18.

7. JeongTS, Kim YS, and Oh KK. 2011. Two-stage acid saccharification of fractionated *Gelidium amansii* minimizing the sugar decomposition. *Bioresour. Technol.***102:**10529-10534.

8. Jol CN, Neiss TG, Penninkhof B, Rudolph B, De Ruiter GA. 1999. A novel high-performance anion-exchange chromatographic method for the analysis of carrageenans and agars containing 3,6-anhydrogalactose. *Anal. Biochem.* **268:** 213-222.

9. Kamireddy SR, Li J, Tucker M, Degenestein J, Ji Y. 2013. Effects and mechanism of metal chloride salts on pretreatment and enzymatic digestibility of corm stover.*Ind. Eng. Chem. Res.* **52**: 1775-1782.

10. Kang HK, Kim NM, Kim GJ, Seo ES, Ryu HJ, Yun SI, Choi HC, Day DF, Kim J, Cho DL,

Kim D. 2011. Enhanced saccharification of rice straw using hypochlorite-hydrogen peroxide. *Biotechnol. Bioprocess Eng.***16:** 273-281.

11. KimC, Ryu HJ, Kim SH, Yoon JJ, Kim HS, Kim YJ. 2010. Acidity tunable ionic liquids as catalysts for conversion of agar into mixed sugars. *Bull. Korean Chem. Soc.***31:**511-514.

12. KimH, Ra CH, and Kim SK. 2013. Ethanol production from seaweed (*Undariapinnatifida*) using yeast acclimated to specific sugars. *Biotechnol. Bioprocess Eng.***18:** 533-537.

13. Kim NJ, Li HJ, Chang HN, Lee PC. 2011. Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. *Bioresour. Technol.* **102:** 7466-7469.

14. KimSR, Ha SJ, Wei N, Oh EJ, Jin YS. 2012. Simultaneousco-fermentation of mixed sugars: A promising strategy.*Trends Biotechnol.***30:** 274-282.

15. Kubicek CP. 1982. β-glucosidase excretion by *Trichodermapseudokoningii* correlation with cell wall bound β-1,3-glucanase activities. *Arch. Microbiol*. **132**: 349-354.

16. Limayem A, Ricke SC. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects.*Prog.Energ. Combust.* **38:** 449-467.

17. Liu R, Li J, Shen F. 2008. Refining bioethanol from stalk juice of sweet sorghum by immobilized yeast fermentation. *Renew. Energy.* **33:** 1130-1135

18. Mandels M, Aldreotti R, Roche C. 1976. Measurement of saccharifyingcellulase. *Biotechnol. Bioeng. Symp.***6**: 21-23.

19. ParkJH, Hong JY, Jang HC, Oh SG, Kim SH, Yoon JJ, Kim YJ. 2012. Use of *Gelidium amansii* as a promising resource for bioethanol: A practical approach for continuous dilute-acid hydrolysis and fermentation. *Bioresour. Technol.***108:**83-88.

20. Ra CH, Jeong GT, Shin MK, Kim SK. 2013.Biotransformation of 5-hydroxymethylfurfural (HMF) by *Scheffersomyces stipitis* during ethanol fermentation of hydrolysate of the seaweed *Gelidium amansii.Bioresour. Technol.* **140:** 421-425.

21. Saha BC, Iten LB, Cotta MA, Wu YV. 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem.***40:** 3693-3700

22. Stahl G, Nakamura SNB, Ariga O, Nakasaki K. 2004. Translational accuracy during exponential, postdiauxic, and stationary growth phases in *Saccharomyces cerevisiae*. *Eukaryotic cell*. **3:** 331-338

23. Yanagisawa M, Nakamura K, Ariga O, Nakasaki K. 2011. Production of high concentrations of bioethanol from seaweeds that contain easily hydrolysablepolysaccharides.

Process Biochem. **46:** 2111-2116

24. Zheng Y, Pan Z, Zhang R, Wang D. 2009. Enzymatic saccharification of dilute acid pretreated saline crops for fermentable sugar production.*Appl. Energy*. **86**:2459-2465