Thesis for the Degree of Doctor of Philosophy

Biofuel productions from soybean residue and seaweed via separate hydrolysis and fermentation (SHF) process

by

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The Graduate School

Pukyong National University

February 2019

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Advisor: Prof. Sung-Koo Kim

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A dissertation

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Strategy for bioenergy production from various biomass via separate hydrolysis and fermentation (SHF) process

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Abstract

Bioenergy can be produced via fermentation from any biomaterial containing sufficient polysaccharide or equivalent materials that can be degraded into monosaccharides, such as starch or cellulose. Traditionally, bioenergy has been produced from first-generation biomass, such as starch or sugars using sugarcane, wheat, and corn. However, first-generation biomass can also be used as a human food or animal feed, which has caused moral problems and concerns regarding increasing prices. Bioenergy has also been produced from second-generation biomass such as lignocellulosic biomass and agricultural waste products as second-generation biomass, such as the stalks of corn and wheat, straw, grass and wood chips. However, feedstock has low yields and high costs with efficient hydrolysis processes using current technologies. Therefore, the soybean residue and seaweed were used as a new biomass in this study for bioenergy production.

The polysaccharide from the soybean residue was used for bioethanol production via the separate hydrolysis and fermentation (SHF). The study focused on the pretreatment, enzymatic saccharification and fermentation. The pretreatment to obtain monosaccharide was carried out with 20% (w/v) soybean residue slurry and 270 mM H_2SO_4 at 121℃ for 60 min. More monosaccharide was obtained from enzymatic hydrolysis with 16 Units/mL mixture of commercial enzymes CTec 2 and Viscozyme L at 45℃ for 48 h.

Ethanol fermentation with 20% (w/v) soybean residue hydrolysate was performed using wild-type and adapted *Saccharomyces cerevisiae* KCTC 1126 to high concentrations of galactose using a flask and 5 L fermenter. When wild-type of *S. cerevisiae* was used, the ethanol production of 20.77 g/L with ethanol yield of 0.31 was obtained. The ethanol production of 33.89 g/L and 31.64 g/L with ethanol yield of 0.49 and 0.47 were produced using adapted *S. cerevisiae* to the high concentration of galactose in a flask and 5 L fermenter, respectively. As a results, *S. cerevisiae* adapted to galactose increased the ethanol yield comparing to wild-type of *S. cerevisiae.*

Bioethanol was produced using the separate hydrolysis and fermentation (SHF) process with macroalgae polysaccharide from the seaweed, *Gelidium amansii* as a biomass. The study focused on the thermal acid hydrolysis pretreatment, enzymatic saccharification, detoxification and fermentation of red macroalgae, *G. amansii*. The thermal acid hydrolysis was carried out with H_2SO_4, slurry content (8~16%) and treatment time (15~75 min). As results, 12% (w/v) seaweed slurry, 182 mM H_2SO_4 at 121℃ for 45 min were selected as optimal conditions for thermal acid hydrolysis obtaining 6.8g/L glucose and 26.1g/L galactose. A monosaccharide (mainly glucose) was obtained from enzymatic hydrolysis of thermal acid hydrolysate, with 16 Units/mL commercial enzyme (Celluclast 1.5 L) at 45℃ for 36 h. Detoxification were carried out with adsorption method using activated carbon, overliming method using $Ca(OH)_2$, and ion-exchange method using polyethyleneimine. Among those detoxification methods, activated carbon showed the best result for removal of hydroxymethylfurfural. Ethanol fermentation with 12% (w/v) seaweed hydrolysate was performed using wild-type *Saccharomyces cerevisiae* and adapted *S. cerevisiae* to galactose.

Acetone, butanol and ethanol (ABE) were produced following the separate hydrolysis and fermentation (SHF) method using polysaccharides from the

green macroalgae *Enteromorpha intestinalis* as biomass. We focused on the optimization of enzymatic saccharification as pretreatments for the fermentation of *E. intestinalis*. Pretreatment was carried out with 10% (w/v) seaweed slurry and 270 mM H_2SO_4 at 121°C for 60 min. Monosaccharides (mainly glucose) were obtained from enzymatic hydrolysis with a 16 Units/mL mixture of Celluclast 1.5 L and Viscozyme L at 45°C for 36 h. ABE fermentation with 10% (w/v) *E. intestinalis* hydrolysate was performed using the anaerobic bacteria *Clostridium acetobutylicum* with either uncontrolled pH, pH controlled at 6.0, or pH controlled initially at 6.0 and then 4.5 after 4 days, which produced ABE contents of 5.6 g/L with an ABE yield (Y_{ABE}) of 0.24 g/g, 4.8 g/L with an Y_{ABE} of 0.2 g/g, and 8.5 g/L with an Y_{ABE} of 0.36 g/g, respectively.

As a results, The maximum ethanol concentration was 33.89 g/L, with Y_{EtOH} of 0.49 and obtained using SHF with *S. cerevisiae* adapted to the high concentration of galactose when soybean residue was used as a biomass.The activated carbon can be suitable for detoxification of *G. amansii* hydrolysate using for ethanol fermentation which showed the highest efficiency reducing HMF by 89.5% and ethanol concentration of 20.28 g/L with Y_{EtOH} of 0.47 were obtained. ABE fermentation from *E. intestinalis* was carried out with pH controlled at 6.0 and then at 4.5 on day 4, which produced an ABE content of 8.5 g/L with a Y_{ABE} 0.36 g/g.

GENERAL INTRODUCTION

Recently, Many countries around the world are shifting their focus toward renewable sources for energy production because of depleting crude oil reserves [1]. Several countries have already planned for progressive replacement of conventional fossil fuels with alternative fuels especially in the transport sector.

Soybean residue (soy pulp, Okara, Biji) is generated from the processing of soymilk, tofu and fried bean curd. Recently, it has become a typical agricultural waste because the reuse of soybean residue is very difficult, although many trials of its use have been done [2]. It is considered to be hard to digest due to its composition of complicated fibers [3].

Seaweed biomass has become an attractive option as a bioresource for a biofuel. Seaweed as a third-generation biomass can replace first- and second-generation biomass for ethanol production with economic, social, and environmental benefits [4, 5]. Seaweeds are classified into three groups: green, brown and red. They contain various types of glucans, i.e., polysaccharides composed of glucose. The glucans found in green, brown and red seaweeds are cellulose and starch, cellulose and laminarin and cellulose and floridean starch, respectively [6]

Saccharomyces cerevisiae is a facultative yeast that is known for its high fermentative ability, ethanol tolerance, and ethanol yield. Galactose and glucose are monosaccharides obtained from biomass that can be used for ethanol fermentation. However, glucose in the hydrolysate can

repress galactose uptake, which decreases ethanol yield. Yeast adaptation to galactose allows simultaneous utilization of glucose and galactose [7]. *Clostridium acetobutylicum* is a gram-positive, rod shaped obligate anaerobic bacterium that forms spores. In addition, it is one of the few microorganisms that can use a variety of sugars to produce desirable ABE products [8, 9]

The various pretreatment techniques were introduced to enhancing hydrolysis yield [10]. For the economic reasons, the thermal acid hydrolysis is commonly used to hydrolyze seaweed and typically used to enhance cellulose accessibility for subsequent enzymatic saccharification. [11]. However, considerable amount of inhibitors such as hydroxymethylfurfural (HMF), furfural, aliphatic acids and phenolic compounds have been generated in thermal acid hydrolysis, which affects on microbial fermentation [12]. Therefore, the detoxification of hydrolysates is required before ethanol fermentation. Detoxification by activated carbon has been known as a cost effective method with high capacity to absorb compounds without affecting levels of monosaccharides in hydrolysate [13]. Overliming has been considered as a promising detoxification method of lignocellulosic hydrolysate for a long time [14] and the principle of this method is the precipitation of toxic components and the instability of some inhibitors at high pH [15]. Ion-exchange method has been known as one of the most efficient detoxification method for removing inhibitors and improving significantly the yield fermentation [16].

Separate hydrolysis and fermentation (SHF) as a process alternative

in an industrial bioethanol plant has both potential and limitations. The main advantage is the possibility to separately optimize the process steps, especially to be able to run the enzymatic hydrolysis at an optimal temperature. Although, it is important to include all the process steps in the optimization work. The fermentation difficulties together with the end product inhibition are two limitations of the SHF process that have to be improved before SHF is a preferable alternative in a large scale bioethanol plant.

PURPOSE OF THIS STUDY

The aims of this study in Chapter I were to evaluate the optimization of some important variables for thermal acid hydrolysis (concentration of soybean residue, the concentration of acid and treatment time) and to determinethe optimal condition for enzymatic saccharification using various commercial enzymes to obtain more monosaccharide. Another goal was the enhancement of ethanol production by using adaptive evolution of yeast such as galactose adaptation of yeast for better performance of monosaccharide utilization. The evaluation of fermentations using a flask and 5 L fermenter was performed.

In Chapter II, the red seaweed *Gelidium amansii* was used as a substrate for ethanol production through thermal acid hydrolysis and enzymatic saccharification. The detoxification of *G. amansii* was carried out with activated carbon, overliming method using $Ca(OH)_2$ and ion-exchange using polyethylenimine(PEI). Ethanol fermentation of detoxified *G. amansii* hydrolysates were performed using wild-type *Saccharomyces cerevisiae* and *S. cerevisiae* adapted to high concentration of galactose as adaptive evolution.

The objective of this study in chapter III, thermal acid hydrolysis and enzymatic saccharification were employed to produce monosaccharides, and to support fermentation in the production of ABE from the green macroalgae *Enteromorpha intestinalis*. The optimal thermal acid hydrolysis parameters were determined using the

one-factor-at-a-time optimization method. Enzymatic saccharification was performed using the commercial enzymes Celluclast 1.5 L and Viscozyme L. Finally, ABE production was quantified under different fermentation conditions based on pH using *Clostridium acetobutylicum* KCTC 1790.

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CHAPTER I

Bioethanol production from soybean residue via separate hydrolysis and fermentation

1.1. Introduction

Nowadays, ethanol is an alternative transportation fuel which is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by natural energy resources such as petroleum and coal. Traditionally, the major sources of ethanol have been sucrose from sugarcane [1], and glucose from corn starch [2], however, this biomass can also be used as human food and an animal feed, which has caused moral problems and concerns regarding increasing prices. An opportunity, therefore, exists to shift the use of agricultural waste streams to renewable resources [3, 4]. These materials are a promising carbon source for ethanol production because of its wide availability, low cost and little competence with foods.

Soybean is an important grain containing good proteins and oil, and many foods and feedstuffs are made from that. Moreover, a by-product of soybean can be used as a biomass for useful chemical production such as bioethanol from soybean molasses [5], polymalic acid from soybean hulls [6]. Soybean residue (soy pulp, Okara, Biji) is generated from the processing of soymilk, tofu and fried bean curd. About 1.1 kg of fresh soybean residue which contains 76 - 80% moisture is produced from processing 1.0 kg of dry beans to produce soymilk or tofu [7]. Recently, it has become a typical agricultural waste because the reuse of soybean residue is very difficult, although many trials of its use have been done [8]. It is considered to be hard to digest due to its composition of complicated fibers [9]. To obtain

high bioconversion levels of ethanol from this biomass sources requires a thermochemical process as thermal acid hydrolysis, prior to enzymatic hydrolysis of polysaccharide to monosaccharide for the fermentation to ethanol by yeast [10].

Fermentation process for bioethanol production usually uses either by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). The main advantage of SSF is the use of a single reaction vessel and reducing fermentation period, However, the optimal temperatures for the yeast and the enzymes are different, which means that the conditions used in SSF cannot be optimal for both the enzymes and the yeast and might result in lower efficiency and lower production yield. Otherwise, the SHF processing allows optimal conditions for the hydrolysis and fermentation steps. Therefore, many reports show SHF with higher efficiency than SSF process when bioethanol production was carried out using cellulosic biomass [11-13].

The aims of this study were to evaluate the optimization of some important variables for thermal acid hydrolysis (concentration of soybean residue, the concentration of acid and treatment time) and to determine the optimal condition for enzymatic saccharification using various commercial enzymes to obtain more monosaccharide. Another goal was the enhancement of ethanol production by using adaptive evolution of yeast such as galactose adaptation ofyeast for better performance of monosaccharide utilization. The evaluation of fermentations using a flask and 5 L fermenter was performed.

1.2. Material & Method

1.2.1. Raw materials and composition analysis

Soybean residue was obtained from Saebyeok market (Sasang, Busan, Korea). Soybean residue was dried to a constant weight at 60°C, ground using a roller mill, and sieved with a 200-mesh sieve prior to pretreatment. Samples were stored in a dry environment at room temperature in order to avoid rehydration. The composition analysis of soybean residue was conducted by the Feed and Foods Nutrition Research Center at Pukyong National University in Busan, Korea.

1.2.2. Thermal acid hydrolysis

Pretreatment was focused on the effects of the three factors such as slurry contents, H_2SO_4 concentration and treatment time. The pretreatment was carried out using the weight/volume fraction of slurry contents ranging 14 - 24% (w/v) with 180 mM H_2SO_4 at 121°C for 45 min. Then, H_2SO_4 concentration was optimized. The pretreatment was carried out using the optimal condition of slurry content determined previously and H_2SO_4 concentrations ranging 0 - 540 mM at 121°C for 45 min. Thermal hydrolysis time was optimized. The pretreatment was carried out using the optimal slurry content and optimal H_2SO_4 concentration at 121°C for the determination the thermal

hydrolysis time ranging 15 - 90 min.

Soybean residue slurry (100 mL working volume in a 250 mL flask) was heated to 121°C, and soybean residue hydrolysate was neutralized to pH 5.0 using 10M NaOH. The efficiency of thermal acid hydrolysis was calculated using Eq. (1) as follows:

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

where ΔS_p is the increase in monosaccharide (g/L) during the thermal acid hydrolysis, and TC is total carbohydrate content (g/L) of the soybean residue [14]

1.2.3. Selection of enzyme and enzymatic saccharification

The optimal conditions for the enzymatic saccharification of soybean residue were determined after finding the optimal condition for thermal acid hydrolysis using 20% (w/v) slurry concentration. For enzymatic saccharification, pH of acid hydrolysates was adjusted to pH 5 with 10M NaOH. Various enzymes such as Cellic CTec2 (120 filter paper unit (FPU)/mL), Viscozyme L (121 β-glucanase unit (FBG)/ml), Ultraflo max (295 fungal xylanase unit (FXU)/mL, 826 endoglucanase unit (EUG)/mL), Celluclast 1.5 L (854 endo-glucanase unit (EGU)/mL), Viscoferm (262 β-glucanase unit (FBG)/mL), Viscoflow MG (500 β -glucanase unit (FBG)/mL), Spirizyme Fuel (862 amyloglucosidase unit (AGU)/mL), AMG 300L (300 amyloglucosidase unit (AGU)/mL) (all

from Novozymes, Bagsvaerd, Denmark) were added at a level of 16 Units/mL in 100 mL working volume in a 250 mL flask. Then, 3 enzymes with highly efficient saccharification were selected for mixed enzyme experiments to find the optimal condition for the enzymatic saccharification. The saccharification reaction was performed at 50°C on shaking incubator at 150 rpm. Samples of 1 mL were taken periodically and analyzed for the degree of enzymatic saccharification. The concentrations of monosaccharide were analyzed using HPLC. The efficiency of enzymatic saccharification (E_s) was calculated using Eq.(2) as follows:

$$E_s(\%) = \frac{\Delta S_s\ (g/L)}{TF\ (g/L)} \times 100 \qquad \text{Eq. (2)}$$

where ΔS_s is the increase in monosaccharide concentration (g/L) when enzymatic saccharification was carried out. TF is total fiber content (g/L) of the soybean residue.

1.2.4. Fermentation

1.2.4.1. Seed culture and adaptation of yeasts

Kluyveromyces marxianus KCTC 7150, *Saccharomyces cerevisiae* KCTC 1126, *Candida tropicalis* KCTC 7212, *Pichia angophorae* KCTC 17574 were obtained from the Korean Collection for Type Cultures

(KCTC) of Biological Resource Center (Korea) and *Candida lusitaniae* ATCC 42720 was obtained from the American Type Culture Collection (ATCC). These yeasts were grown in YPD medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose as a seed culture. The culture was incubated with agitation at 150 rpm for 24 h at 30°C. The adaptation of yeasts was carried out to improve the uptake of galactose and ethanol production from the mixed monosaccharides in soybean residue hydrolysates. Thus, 10 mL of seed was inoculated to 100 mL of Yeast extract, Peptone and High Galactose (YPHG) medium composed of 10 g/L yeast extract, 20 g/L peptone and 120 g/L galactose, and cultured under the same conditions of seed culture. The cells were centrifuged at 1,390 × g for 10 min to remove the YPHG medium and transferred to a 250 mL Erlenmeyer flask containing 100 mL of 0.2 μm filtered soybean residue hydrolysate. Cultured yeast strains were sampled to determine the dry cell weight using the optical density (OD_{600}) using standard curve of dry cell weight and OD_{600}.

1.2.4.2. Ethanol fermentation

Fermentation was evaluated in 250 mL flasks with a working volume of 100 mL. Following pretreatment, neutralization to pH 5.0 and enzymatic saccharification was carried out. Following nutrients were added to the fermentation medium: 2.5 g/L of NH_4Cl, 5g/L of K_2HPO_4, 0.25g/L of $MgSO_4$ and 2.5 g/L of yeast extract. Fermentation

was performed with *K. marxianus*, *S. cerevisiae*, *C. tropicalis*, *P. angophorae* or *C. lusitaniae* with adaptation to high concentrations of galactose to find the best suitable yeast for the fermentation at 30°C, 150 rpm and for 144 h. After the best suitable yeast was found, ethanol fermentation was carried out at 30°C and 150 rpm by using a 5L fermentor (KF-5; Korea Fermentation Company (KFC), Incheon, Korea) with a 3L working volume. The anaerobic condition was maintained by gas packing with N_2. Samples were taken periodically to measure sugar consumption and ethanol production. The bioethanol yield coefficient was calculated using E_q.(3).

$$Y_{EtOH}\ (g/g) = \frac{[EtOH,\ g/L]_{max}}{[Sugar,\ g/L]_{ini}} \qquad \text{Eq. (3)}$$

where $[EtOH]_{max}$ is highest ethanol concentration achieved during fermentation and $[sugar]_{ini}$ is totalinitial sugar concentration at the start of fermentation. Definition of yield coefficient is generally accepted for the ethanol fermentation. The maximum theoretical ethanol yield

100 g of hexose produce 51.1 g of ethanol and 48.9 g of CO_2. Therefore, 0.51 is the maximum yield coefficient [15, 16] by the total conversion of 2 mole ethanol (M.W.= 46) from the hexose (M.W.= 180) [Y_{EtOH}^{max}=92/180=0.51].

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad \text{Eq. (4)}$$

1.2.5. Analytical methods

Cell growth was determined based on the optical density at 600 nm (OD_{600}) using ultra violet-visible spectrophotometer (Amersham Biosciences Ultrospec 6300Pro, Biochrom, Cambridge, England). Optical density values was converted to the dry cell weight (dcw) using a standard curve of dry cell weight and OD_{600nm}. The pH was measured by a pH-meter (Meltler-Toledo AG, CH-8603, Schwerzenbach, Switzerland). The glucose, galactose, acetic acid and ethanol concentrations were determined using HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID). A Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) was used with filtered and degassed 5 mM H_2SO_4 as an eluent at a flowrate of 0.6 mL/min and a temperature of 65°C. Before analysis, aqueous samples were centrifuged at 14,240×g for 10 min and the supernatant was filtered by using a 0.2 μm syringe filter.

1.2.6. Statistical analysis

Each experiment was carried out in triplicate. The statistical significance of differences in pretreatment, saccharification and monosaccharide contents were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range test ($P<0.05$) using SPSS version 23 (SPSS, Cary, NC, USA).

1.3. Results and Discussion

1.3.1. Composition of soybean residue

The composition of soybean residue was analyzed by the AOAC method [17] and 34.43% carbohydrate, 30.69% crude protein, 16.42% crude lipids, 22.9% crude ash and 13.43% fiber were contained in soybean residue as shown in Table. 1. The total carbohydrate content of the soybean residue used in this study was 47.86% including fiber lower than other residues from soybean (Table 1). The carbohydrate in raw soybean residue contained mainly of glucose, galactose, arabinose and xylose [18].

Table 1.1 Compositional analysis of soybean residue

By-products	Protein (%)	Fat (%)	Fiber (%)	Ash (%)	Carbohydrate (%)	References
Soybean hull	14.38	3.97	-	4.94	76.71	[6]
Soybean molasses	9.44	21.20	5.70	6.36	57.30	[5]
Soybean residue	27.40	9.50	13.60	4.00	45.50	[18]
Soybean residue	30.69	16.42	13.43	5.03	34.43	This study

1.3.2. Effect of optimal slurry content

The effects of the three factors such as slurry contents, H_2SO_4 concentration and treatment time were evaluated for monosaccharide production by thermal acid hydrolysis. The first factor, the slurry content was varied in the range 14 - 24% (w/v) and 182 mM H_2SO_4 at 121°C for 45 min as thermal acid hydrolysis and enzymatic saccharification to determine the optimal slurry contents as shown in Fig.1. However, the sugar concentration did not show the difference as the slurry content in creased over 20% of soybean residue. Thus, 16 Units/mL of Viscozyme was added for enzymatic saccharification [19]. As a result, the sugar concentration increased as the slurry content increased, and the monosaccharide concentrations with slurry contents of 14%, 16%, 18%, 20% (w/v) were 47.03 g/L, 50.58 g/L, 55.71 g/L, 60.58 g/L, respectively. However, increasing the slurry contents over 20% during pretreatment and enzymatic saccharification did not produce more monosaccharides comparing to that of 20% slurry contents (Fig. 1). Therefore, 20% slurry content was selected as an optimal slurry content for thermal acid hydrolysis.

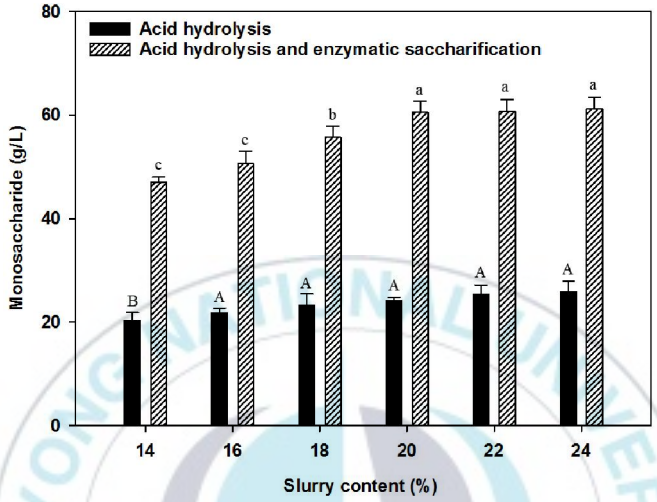

Fig. 1.1 Optimization of thermal acid hydrolysis of soybean residue with various slurry contents. Different letters indicate that it is significantly different with each concentration of seaweed ($P < 0.05$, Duncan's test).

1.3.3. Optimization of thermal acid hydrolysis

The first factor, the H_2SO_4 concentration was varied in the range of 0 - 540 mM and 20% (w/v) slurry content at 121°C for 45min thermal acid treatment. As shown in Fig. 2a, monosaccharide concentration increased with increasing H_2SO_4 concentration from 7.03 g/L of 0 mM to 36.21 g/L of 540 mM. The previous study reported that the release of high amounts of monosaccharide was obtained by high acid concentrations [20]; however, high H_2SO_4 concentrations in the range360 - 540 mM H_2SO_4 resulted in a similar value of E_p (37.3-37.8%) compared with that of 270 mM H_2SO_4 (37.1%). Therefore, 270 mM H_2SO_4 was selected as the optimal acid concentration, giving 35.54 g/L of monosaccharide.

The second factor, the thermal treatment time was varied in the range 15-90 min, with a slurry content of 20% (w/v) and H_2SO_4 concentration of 270 mM at 121°C. Fig. 2b shows that monosaccharide concentration and efficiency of pretreatment increased from 22.33 g/L, 23.3% to 42.28 g/L, 44.1% when treatment time increased from 15 to 60min, respectively. However, when treatment time over 60minwas used, the monosaccharide and efficiency of pretreatment did not increase. Therefore, 60 min was selected as the optimal hydrolysis time, giving 42.28 g/L of monosaccharide.

From these results, the optimal condition for thermal acid pretreatment was selected as follows: 20% (w/v) slurry and 270 mM H_2SO_4 at 121°C for 60 min. Thermal acid hydrolysis with optimal

conditions produced 42.28 g/L monosaccharide. The previous study reported that thermal acid hydrolysis is reported as one of the mostly used and oldest methods among all types of chemical pretreatments of biomass for reducing in size [21]. Therefore, the pretreatment of biomass is crucial before enzymatic hydrolysis.

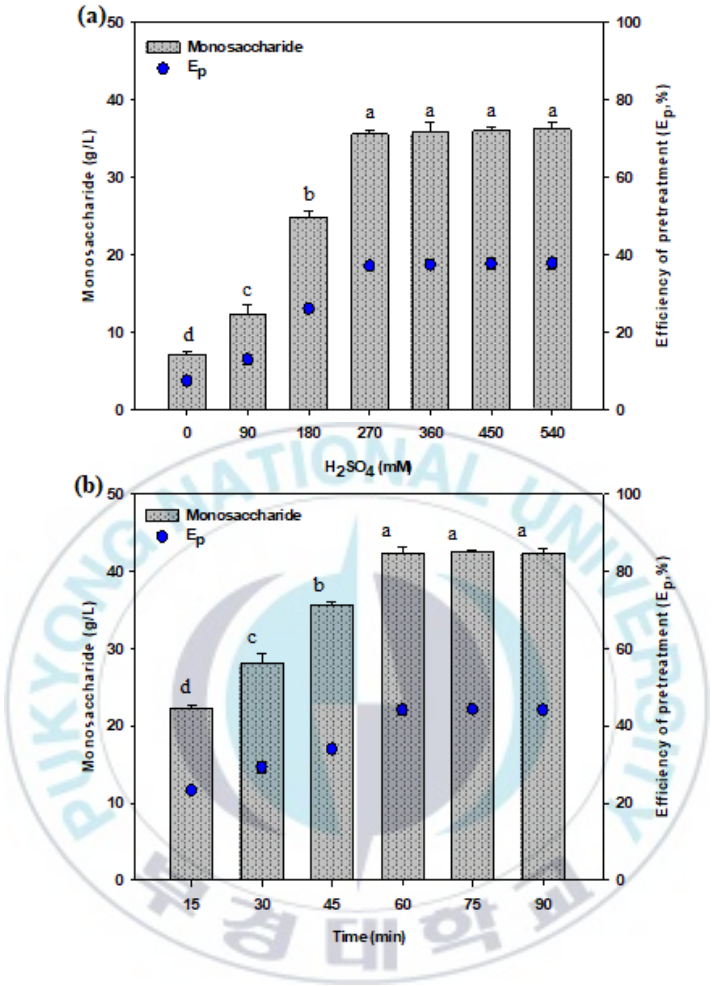

Fig. 1.2. Effects of thermal acid hydrolysis of (a) H_2SO_4 concentration (slurrycontent: 20%, 45 min, 121℃) and (b) Thermal hydrolysis time (slurry content: 20%, H_2SO_4: 270mM, 121℃). Different letters indicate that it is significantly different with each acid concentration and time treatment($P < 0.05$, Duncan's test).

1.3.4. Selection of enzyme for enzymatic saccharification

Enzymatic saccharification is applied for the hydrolysis of the cellulosic fiber to form monosaccharides to facilitate the ethanol fermentation using yeasts [22]. An initial monosaccharide concentration of 42.28 g/L was obtained after thermal acid hydrolysis. The effect of enzymatic saccharification on the release of glucose from 20% soybean residue hydrolysate after thermal acid hydrolysis was evaluated using single and mixed enzymes treatments of Cellic CTec2, Viscozyme L, Ultraflo max, Celluclast 1.5 L, Viscoferm, Viscoflow MG, Spirizyme Fuel, AMG 300L as shown in Fig. 3. The increase in reaction time over 36 h had no more significant effect on enzymatic saccharification as shown in Fig. 3a, therefore the optimum enzyme reaction time was selected as 36 h. Table 2 shows the effects of single enzyme saccharifications on glucose release. When Cellic CTec2, Viscozyme L and Ultraflo max were used, E_s showed of 60.46 g/L monosaccharide with E_s of 67.6%, 59.65 g/L monosaccharide with E_s of 64.6% and 59.07 g/L monosaccharide with E_s of 62.4%, respectively. These enzymes exhibited a high activity for hydrolyzing soybean residue. On the other hand, Celluclast 1.5 L, Viscoferm, Viscoflow MG, Spirizyme Fuel and AMG 300L showed a lower activity for hydrolyzing soybean residue with E_s of 46.1%, 43.2%, 42.1%, 28.9% and 16.5%, respectively. Ahn *et al.* [23] reported that the mixture of enzymes showed higher degradation activity than single enzyme treatment. Therefore, Cellic CTec2, Viscozyme L and Ultraflo max were selected for mixed

enzymes experiment. The mixture of three enzymes (Viscozyme L and Ultraflo max and Cellic CTec2) and two enzymes (Viscozyme L and Cellic CTec2, Viscozyme L and Ultraflo max, Ultraflo max and Cellic CTec2) were used to increase E_s. As results, the maximum E_s was obtained 92.7% with 67.20 g/L monosaccharide from 20% soybean residue hydrolysate (336 g monosaccharide per kg raw soybean residue) using mixture of Viscozyme L and Cellic CTec2 as shown in Fig.3b and followed by mixture of Viscozyme L and Ultraflo max, mixture of three enzymes and mixture of Ultraflo max and Cellic CTec2 with E_s of 78.5%, 76.2% and 69.8%, respectively. The same biomass was used in the previous study. However thermal acid hydrolysis was not carried out before enzymatic saccharification, therefore, 293 g monosaccharide per kg of raw soybean residue was obtained for less monosaccharide than that of this study [18].

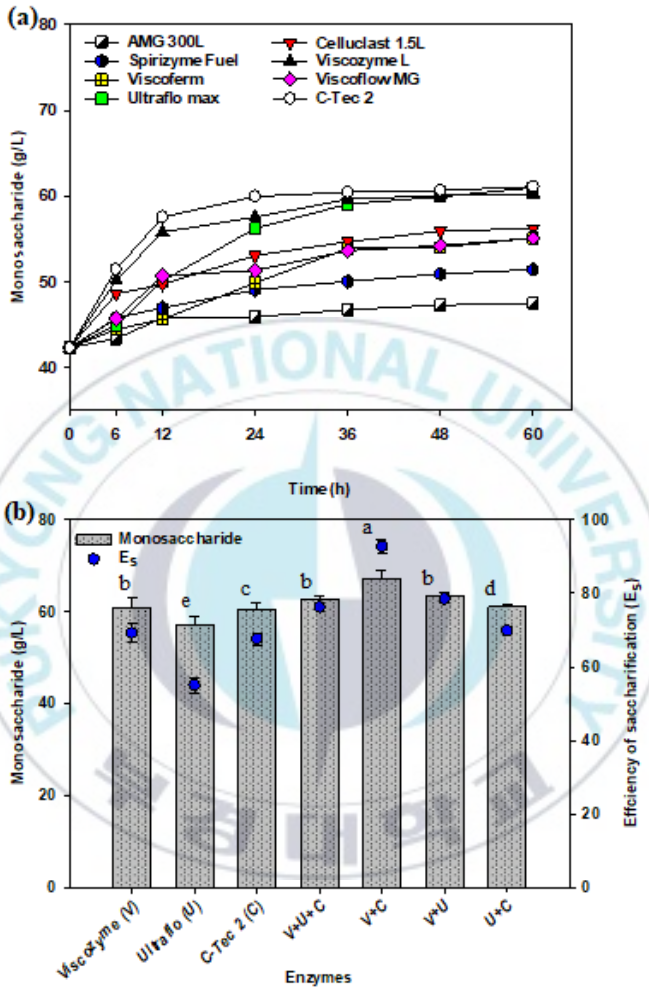

Fig. 1.3. Saccharification of soybean residue by using various commercial enzymes (a) Single enzyme (b) Mixed enzymes. Different letters indicate that it is significantly different with each enzyme ($P < 0.05$, Duncan's test).

Table 1.2. Monosaccharide concentrations produced by enzymatic saccharification (all samples were treated by optimal thermal acid hydrolysis)

Enzymes	Enzyme activity	Monosaccharides (g/L)	E_s(%)
Cellic CTec2	Cellulase, xylanase	60.46	67.6
Viscozyme L	Cellulase, arabanase, beta-glucanase, hemicellulase and xylanase	59.65	64.6
Ultraflo max	Xylanae (endo-1,4-), beta-glucanase (endo- 1,3(4)-)	59.07	62.4
Celluclast 1.5 L	Cellulase	54.68	46.1
Viscoferm	Beta-glucanase (endo-1,3(4)-), cellulase, xylanase (endo-1,4-)	53.91	43.2
Viscofow MG	Beta-glucanase (endo-1,3(4)-), cellulase, alpha-amylase, xylanase	53.62	42.1
Spirizyme Fuel	Amylase and glucoamylase	50.06	28.9
AMG 300L	Glucoamylase	46.74	16.5

1.3.5. Selection of yeast

After thermal acid hydrolysis and enzymatic saccharification, 67.2 g/L monosaccharide (27.90 g/L glucose and 39.29 g/L galactose) was obtained. Yeast preferentially utilizes glucose via the Embden-Meyerhof glycolysis pathway, and the uptake of galactose by yeast requires the expression of enzymes in the Leloir pathway. These enzymes expression is induced by yeast growth in galactose and repressed when glucose is existed to the medium [25,25]. Therefore, the selection of yeastwas performed with *K. marxianus*, *S. cerevisiae*, *C. tropicalis*, *P. angophorae* or *C. lusitaniae* with adaptation to high concentrations of galactose to find the suitable yeast for the fermentation at 30°C, 150 rpm and for 144 h. As shown in Fig. 4, the highest ethanol production was obtained using *S. cerevisiae* with 33.89 g/L ethanol at 72 h and followed by *C. lusitaniae*, *C. tropicalis*, *K. marxianus* and *P. angophorae*with 27.68 g/L, 27.21 g/L, 23.17 g/L and 21.95 g/L, respectively. Mishra *et al*. also reported that best results were obtained with *S. cerevisae* when ethanol production from various agro residues [4]. Therefore *S. cerevisiae* was selected as the suitable yeast for ethanol production from soybean residue.

1.3.6. fermentation with wild-type *S. cerevisiae* and *S. cerevisiae* adapted to galactose

Fig. 5a shows the results of fermentation using a wild-type of *S.*

cerevisiae. The glucose and galalactose concentrations at the start of fermentation were 27.26 g/Land 39.05 g/L, respectively. Because glucose is the preferred substrate to galactose, glucose was consumed during initial 48 h, and then galactose was consumed until 144 h. However, the galactose was not totally consumed at 144 h, and 21.06 g/L of galactose remained. The ethanol concentration after 144 h of fermentation with wild-type *S. cerevisiae*was 20.77 g/L, with Y_{EtOH} = 0.31. Fig. 5b shows the results of fermentation with *S. cerevisiae* adapted to galactose. Initial galactose concentration was 28.04 g/L, and the initialglucose concentration was 40.16 g/L. The glucose and galactose were consumed simultaneously because the adaptation of high galactose concentration could reduce the glucose repression [25]. The glucose was consumed after 24 h, and 3.06 g/L of galactose remained after 72 h. The final ethanol concentration was 33.89 g/L, with Y_{EtOH} = 0.49. Previous study, Letti *et al* [5] reported that 26.0 g/L of ethanol was produced using 200 g/L soybean molasses with *Zymomonas mobilis*. The similar result of the previous study also reported that ethanol yields of 0.34 and 0.46 were obtained using a mixture of glucose and galactose which come from the biomass of red seaweed *Gracilaria verrucosa* when wild-type and adapted *S. cerevisiae* were used, respectively [25]. Meinita *et al.* also reported when wild-type of *S. cerevisiae* was used, the ethanol yield coefficient was 0.21 with galactose and glucose from *Kappaphycus alvarezii* hydrolyzate for ethanol fermentation [26]. Therefore, the adaptation of *S. cerevisiae* to high concentrations of galactose is important to

increase the ethanol yield of ethanol from soybean residue.

Fig. 5c shows the results of fermentation with *S. cerevisiae* adapted to galactose using 5 L fermenter under anaerobic condition. Initial glucose and galactose concentrations were 28.45 g/L and 39.08g/L. The glucose and galactose were consumed simultaneously. The glucose was completely consumed in 24 h, however, slow galactose consumption was observed until 120 hshowing delayed fermentation due to the changes in fermentation scale. After the fermentation was finished, 31.64 g/L ethanol concentration was obtained with Y_{EtOH} of 0.47. The similar result was reported by Lin *et al.* [27] when bioethanol production at the pilot-scale using rice straw by *Pichia stipites*, a slight decrease in ethanol yield was found in the 100 L volume fermentation comparing to flask fermentation. In addition, Khambhaty *et al.* [28] also reported that when scale-up fermentation of *Kappaphycus alvarezii* hydrolysate from 100 mL to 10 L, the fermentation time increased from 48 h to 120 h.

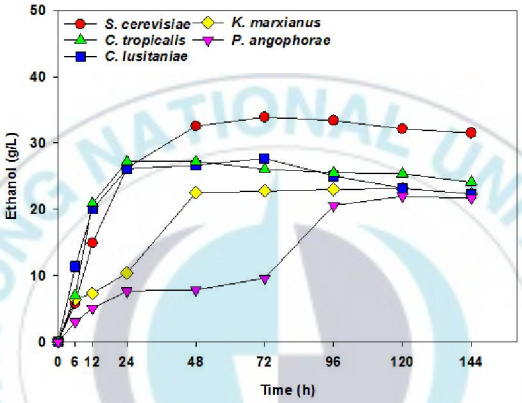

Fig. 1.4. Bioethanol production from hydrolysate of soybean residue by SHF with various yeast.

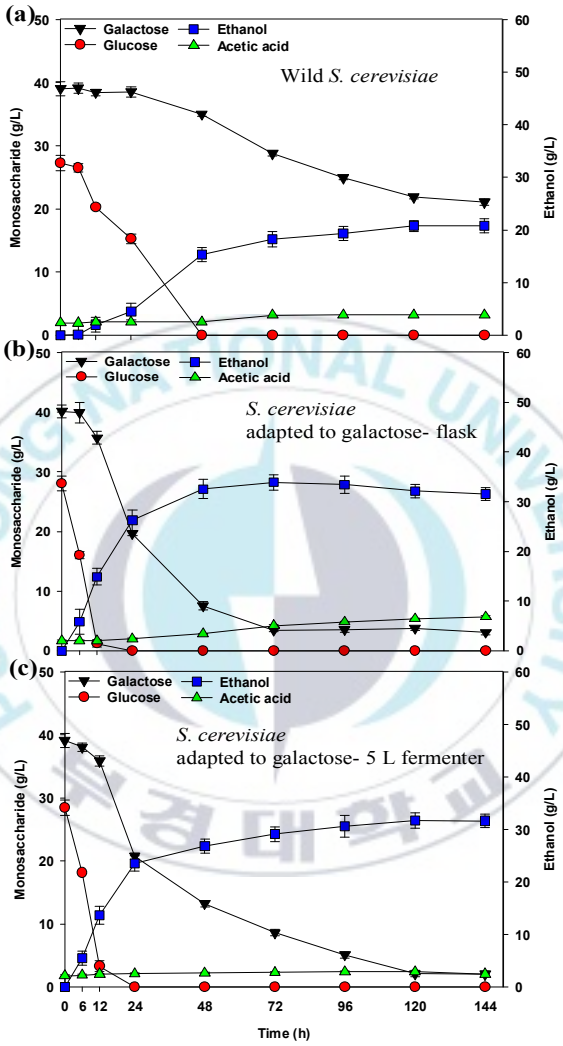

Fig. 1.5. Bioethanol production from the hydrolysate of soybean residue by SHF with (a) wild *S. cerevisiae* in flask culture, (b) adapted *S. cerevisiae* to galactose in flask culture and, (c) adapted *S. cerevisiae* to galactose in 5 L fermenter.

1.4. Conclusion

The optimal pretreatment conditions of soybean residue were 270 mM H_2SO_4, with a 20% (w/v) slurry at 121℃ for 60 min, and the optimal conditions for saccharification were 16 Units/mL mixture of Viscozyme L and Cellic CTec2 at 45℃ for 48 h. Adapted *S. cerevisiae* to high concentrations of galactose showed significantly higher ethanol production compared to that of the wild-type strain. The maximum ethanol concentration was 33.89 g/L, with Y_{EtOH} of 0.49 and obtained using SHF with *S. cerevisiae* adapted to the high concentration of galactose. Ethanol concentration was 31.13 g/L with Y_{EtOH} of 0.47 when 5 L fermentor was used for the ethanol production.

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CHAPTER II

Bioethanol production from red seaweed, *Gelidium amansii* via detoxification (HMF removal) and SHF

2.1. Introduction

Seaweed which is among the most abundant raw materials on Earth, comprises green, red, and brown types. Of these, red seaweed is known as a potential substrate for the production of value-added products such as agar [1] and K-carrageenan [2], which are used to produce pharmaceuticals, cosmetics, and culture media for laboratory microorganisms and other purposes. Major carbohydrates of red seaweed are carrageenan, agar, and glucans such as floridean starch and cellulose that provides monosaccharides for ethanol fermentation following hydrolysis [3].

Hydrolysis yield can be enhanced by various pretreatment techniques [4]. For economic reasons, thermal acid hydrolysis is usually used to hydrolyze seaweed and enhance cellulose accessibility for subsequent enzymatic saccharification. [5]. However, a considerable number of inhibitors, such as phenolic compounds, furfural, aliphatic acids, and hydroxymethylfurfural (HMF) are generated during thermal acid hydrolysis; these in turn affect microbial fermentation [6]. Therefore, hydrolysate detoxification is required prior to ethanol fermentation. Detoxification by activated carbon is a cost effective method with high capacity to absorb inhibitors without affecting hydrolysate monosaccharides levels [7]. Overliming is a promising lignocellulosic hydrolysate detoxification method [8], in which toxic components are precipitated and inhibitors destabilized at high pH [9]. The ion exchange method is one of the most efficient detoxification methods

for removing inhibitors, and significantly improves fermentation yield [10].

In this study, we used *Gelidium amansii* as a substrate for ethanol production through thermal acid hydrolysis pretreatment and enzymatic saccharification. Detoxification was performed using the adsorption method with activated carbon, the overliming method with $Ca(OH)_2$, and the ion-exchange method with polyethyleneimine (PEI). Ethanol fermentation of detoxified *G. amansii* hydrolysates was carried out using wild-type (WT) *Saccharomyces cerevisiae* and *S. cerevisiae* adapted to high concentrations of galactose through adaptive evolution.

2.2. Material & Method

2.2.1. Raw materials and composition analysis

Gelidium amansii was obtained from the Gijang fisheries market in Busan, Korea. *G. amansii* was dried using sunlight, ground using a roller mill, and sieved with a 200-mesh sieve before pretreatment. The composition analysis of *G. amansii* was performed according to AOAC method [11] by the Feed and Foods Nutrition Research Center at Pukyong National University in Busan, Korea.

2.2.2. Thermal acid hydrolysis pretreatment

The optimization of thermal acid hydrolysis pretreatment of *G. amansii* was carried out using the one factor at a time method. The thermal acid hydrolysis conditions were optimized in terms of seaweed slurry content (8 - 16%, w/v), H_2SO_4 concentration (90 - 450mM), and thermal acid hydrolysis time (15 - 75 min). The experiment was performed at 121°C. *G. amansii* hydrolysate was adjusted to pH 5 using 10 N NaOH. After thermal acid hydrolysis pretreatment, high-performance liquid chromatography (HPLC) (1100 Series; Agilent Technologies, Santa Clara, CA, USA) was used for determination of the sugar concentrations of each sample. The efficiency was calculated using Eq. (1), as follows:

$$E_p(\%) = \frac{\Delta S_{mono}}{TC} \times 100 \qquad \text{Eq. (1)}$$

where E_p is the thermal acid hydrolysis pretreatment efficiency (%), ΔS_{mono} is the increase in glucose and galactose (g/L) during experiment, and TC is the concentration of total carbohydrate (g/L) of *G. amansii.* Optimization of enzymatic saccharification of *G. amansii* were evaluated. Celluclast 1.5 L (854 endoglucanase units/mL; Novozymes, Bagsværd, Denmark) was used to hydrolyze the fiber. Celluclast 1.5 L (16 U/mL) was added to 120 g/L of *G. amansii* slurry following thermal acid hydrolysis pretreatment at pH 5.0, 45°C, and 150 rpm for 0 - 48 h. Celluclast 1.5 L contains cellulase, which hydrolyzes the β(1, 4)-D-glucosidic linkages of cellulose and other β-D-glucans. We determined β-glucosidase and cellulase activities following the methods of Mandels *et al.* [12] and Kubicek *et al.* [13]. The efficiency was calculated using Eq. (2), as follows:

$$E_{ps}(\%) = \frac{\Delta S_{mono}}{TC} \times 100 \qquad \text{Eq. (2)}$$

where E_{ps} is the efficiency of the thermal acid pretreatment and enzymatic saccharification (%), ΔS_{mono} is the increase in monosaccharides(g/L) during thermal acid hydrolys is and enzymatic saccharification, and TC is the total carbohydrate concentration (g/L) of *G. amansii*.

2.2.3. Removal of HMF using various methods

Activated carbon (Duksan Pure Chemical Co., Ltd., Ansan, Korea) was used to remove HMF from *G. amansii* hydrolysates following enzymatic saccharification. The adsorption surface areas of the active carbon used was 1,400 - 1,600 m^2/g. These experiments were conducted using a 250-mL flasks with working volume of 100 mL hydrolysate containing 0, 1, 2, 3, 4, or 5% activated carbon. The experiments were carried out in a shaking water bath at 100 rpm and 50°C for adsorption times of 0, 1, 2, 3, 4, 5, and 10 min. The supernatant was recovered by centrifugation (14,240 × g, 10 min) and used to determine sugar and HMF content.

The overliming method was used to remove HMF by adding $Ca(OH)_2$ (Duksan Pure Chemical Co., Ltd., Ansan, Korea) at pH 11.0 and incubating for 0, 15, 30, 45, and 60 min at 30°C. After incubation, the mixture was centrifuged at 14,240 × g for 10 min and the supernatant was then neutralized to pH 5.0 using a 5 M H_2SO_4 solution, and used to determine sugar and HMF content.

The ion exchange method was then used to remove HMF by adding branched polyethylenimine with a molecular weight (MW) of 10,000 (PEI; Polysciences, Inc., Warrington, PA, USA). PEI is highly branched liquid water with high cationic charge density, containing amine groups of primary, secondary, and tertiary at a ratio of approximately 25:50:25. PEI was added to the hydrolysates at ratios of 1:2, 1:1, 2:1, and 3:1 (g PEI:g HMF) for 30 min to determine the optimal ratio.

Treatment times were varied as 0, 15, 30, and 45 min. The resulting mixture was stirred at room temperature (~22°C). Following the reaction, the mixture was centrifuged at 14,240 × g for 10 min and the pH of the supernatant was then neutralized to 5.0 with 5 M H_2SO_4 and used to determine sugar and HMF content.

2.2.4. Seed culture and adaptation of yeasts to high concentration of galactose

S. cerevisiae KCCM 1129 was purchased from the Korean Culture Center of Microorganisms (KCCM). Stocked *S. cerevisiae* was cultured on a YPG agar plate containing 15 g/L agar, 10 g/L yeast extract, 20 g/L peptone and 20 g/L galactose for 24 h [14]. One yeast colony was inoculated with 30 mL YPG medium and cultured at 30°C and 120 rpm for 24 h. We transferred 10 mL from the cultures at 5.3 g dcw/L to 100 mL of YPG medium, cultured under the same conditions. Adaptation of the seed culture to high galactose concentration was performed using 10 mL yeast that had been inoculated in 100 mL yeast extract, peptone, and high-concentration galactose (YPHG, 10 g/L yeast extract, 20 g/L peptone, and 80 g/L galactose) and cultured until the dry cell weight (dcw) of *S. cerevisiae* reached 8.2 g dcw/L [15].

2.2.5. Ethanol fermentation

Fermentation was carried out with a working volume of 100 mL in 250-mL flasks at 30°C and 150 rpm for 144 h. The following nutrients were added to the fermentation medium: 2.5 g/L NH_4Cl, 5 g/L K_2HPO_4, 0.25 g/L $MgSO_4$, and 2.5 g/L yeast extract. Fermentation was performed using WT *S. cerevisiae* and *S. cerevisiae* adapted to high concentration galactose. The ethanol yield coefficient was calculated using Eq. (3), as follows:

$$Y_{EtOH}\ (g/g) = \frac{[EtOH]_{max}}{[Sugar]_{ini}} \qquad \text{Eq. (3)}$$

where $[EtOH]_{max}$ is the highest bioethanol concentration obtained during experiment (g/L) and $[Sugar]_{ini}$ is the total initial glucose and galactose concentration of fermentation (g/L). This definition of the yield coefficient is generally used for fermentation. The maximum yield coefficient was 0.51 [5] for a total conversion of hexose (M.W.: 180) to 2 mol ethanol (M.W.: 46).

2.2.6. Analytical Methods

Cell growth was determined based on the optical density at 600 nm (OD_{600}) and converted to the dry cell weight (dcw) using as tandard curve. The glucose, galactose, 5-hydroxymethylfurfural (HMF) and

ethanol concentration were determined using HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a RID. A Bio-Rad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) was used with filtered and degassed 5 mM H_2SO_4asaneluentatthe flow rate of 0.6 mL/min and the temperature of 65℃.The fermentation samples were centrifuged for 10 min at 14,240× *g* and the supernatant was filtered using 0.2μm filter paper prior to analysis.

2.2.7. Statistical analysis

All experiments were performed in triplicate. The statistical significance of the differences among the pretreatment, saccharification, and monosaccharide contents were evaluated using one-way analysis of variance and Duncan's multiple range test ($P < 0.05$) in SPSS software (ver. 23; SPSS Inc., Chicago, IL, USA).

2.3. Results and Discussion

2.3.1. Composition of *G. amansii*

G. amansii is a red alga known for high carbohydrate content as one of the most abundantly available seaweed species. The major carbohydrates comprise a neutral polymer (agarose) and a sulfate polysaccharide (agaropectin) [5] and cellulose consisting glucose [16]. The composition of *G. amansii* was analyzed by the AOAC method and found to contain 62.8% carbohydrate, 18.1% crude protein, 0.2% crude lipids, 7.3% crude ash and 11.6% cellulose. The total carbohydrate content of the *G. amansii* used in this study was 74.4% including cellulose.

2.3.2. Thermal Acid Hydrolysis

Thermal acid hydrolysis pretreatment was conducted for sugars produced from *G. amansii*. Concentrations of *G. amansii* slurry and H_2SO_4, as well as treatment time, were determined to obtain the optimal conditions for thermal acid hydrolysis.

Optimal slurry content (8 - 16%, w/v) was obtained by thermal acid hydrolysis at 182 mM H_2SO_4 and 121°C for 60 min. Figure 1a shows the increase in sugar as slurry content increased. The sugar concentration at slurry content from 8 to 16% (w/v) was 22.24 to 35.92 g/L. However, as slurry content increased above 12% during

thermal acid hydrolysis, E_p decreased from 37.2 to 30.3%. Levels of HMF concentration increased from 4.5 to 8.4 g/L as slurry content increased from 8 to 16 % (w/v). A previous study also reported that HMF increased with increasing slurry content during thermal acid hydrolysis pretreatment of red seaweed [17]. Therefore, 12% (w/v) slurry content with E_p=37.2% was selected for thermal acid hydrolysis pretreatment.

H_2SO_4 concentration was varied from 90 to 450 mM at 12% (w/v) slurry content, at 121°C for 60 min. Sugar concentration increased from 22.69 to 33.07 as the H_2SO_4 concentration increased from 90 to 180 mM as shown in Fig. 1b. Redding *et al.* [18] reported that high amounts of sugar were released at high acid concentrations; however, the range 180 - 450 mM of H_2SO_4 concentrations resulted in the same E_p value. Increasing the H_2SO_4 concentration during thermal acid hydrolysis has been reported to result in a decrease in HMF due to subsequent conversion of HMF into other inhibitors [19]. These results are consistent with sugar decomposition to HMF and subsequent decomposition to other acids. However, salt was generated from the acid - base reaction during pH neutralization before fermentation, which inhibits cell growth and fermentation by *S. cerevisiae*, requiring more NaOH to control the pH of the hydrolysates at high concentrations of H_2SO_4 [20]. Therefore, 180 mM H_2SO_4 was selected as the optimal acid concentration at E_p=37.2%.

Thermal acid hydrolysis pretreatment time was varied in the range of 15 - 75 min at a slurry content of 12% (w/v) and H_2SO_4

concentration of 180 mM at 121°C. Figure 1c shows that the sugar concentration increased from 22.36 to 32.89 g/L as hydrolysis time increased from 15 to 45 min. Treatment times in the range of 45 - 75 min resulted in similar sugar and HMF concentrations as hydrolysis time increased. A previous study reported that ethanol production was decreased with the hydrolysis pretreatment times over 60 min due to high HMF concentration [21]. Therefore, 45 min was considered the optimal time for thermal acid hydrolysis pretreatment.

From these results, the optimal conditions for thermal acid hydrolysis pretreatment were as follows: 12% (w/v) slurry content and 180 mM H_2SO_4 at 121°C for 45 min. This optimal conditions produced 7.36 g/L HMF.

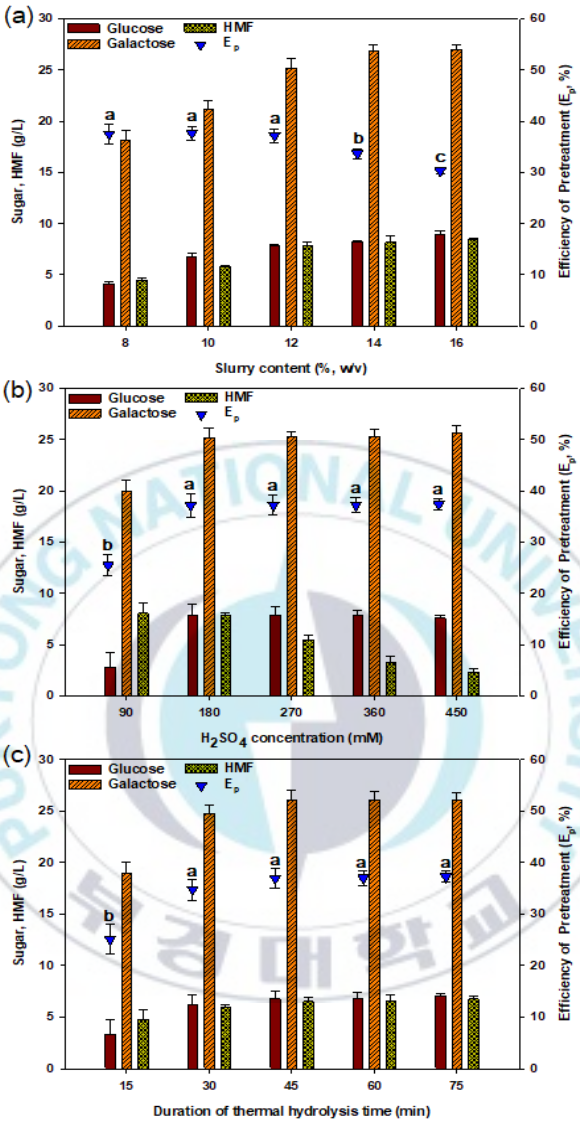

Fig. 2.1. Results of thermal acid hydrolysis based on (a) slurry content, (b) H_2SO_4 concentration, and (c) thermal hydrolysis time. Different letters indicate significant differences in slurry content, acid concentration and treatment time ($P < 0.05$, Duncan's test).

2.3.3. Enzymatic saccharification

Enzymatic saccharification is an ideal approach for degrading cellulose into reducing sugars, because mild reaction conditions can be used to facilitate ethanol fermentation by yeasts [22]. After thermal acid hydrolysis, the glucose concentration was 6.78 g/L. The effects of Celluclast 1.5 L on glucose release with 12% (w/v) *G. amansii* hydrolysate are shown in Fig. 2. For cellulose hydrolysis, 16 U/mL Celluclast 1.5 L was used at pH 5.0, 45℃, and 150 rpm with 12% (w/v) *G. amansii* slurry content, as saccharification time was varied from 0 to 48 h. The maximum glucose concentration was 17.26 g/L, obtained at 36 h and a saccharification efficiency of 50.8%; galactose was maintained during enzymatic saccharification. Redding *et al.* [18] reported high production of reducing sugars from enzymatic saccharification of the red seaweed *K. alvarezii* with 16 U/mL Celluclast 1.5 L.

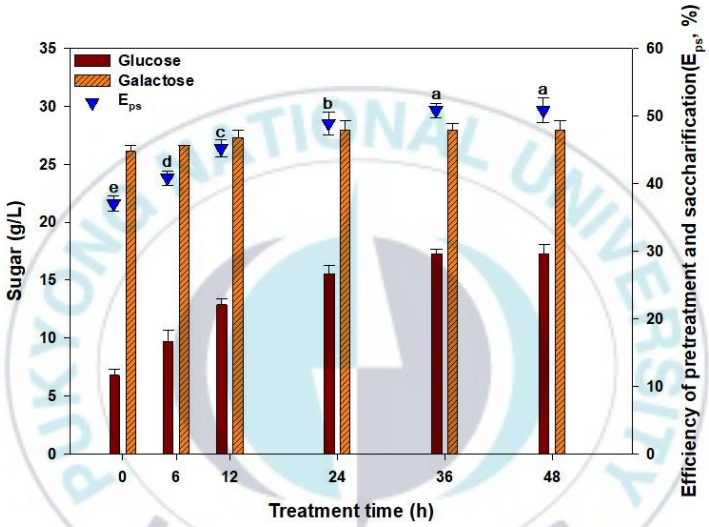

Fig. 2.2. Saccharification of *G. amansii* using 16 Units/mL of Celluclast 1.5 L. Different letters indicate significant differences in saccharification time ($P < 0.05$, Duncan's test).

2.3.4. Detoxification of hydrolysates using various methods

HMF was generated during thermal acid hydrolysis, necessitating a detoxification step to eliminate inhibitors of fermentation efficiency. The adsorption of HMF onto activated carbon was conducted using various activated carbon content levels and adsorption times. A previous study reported that HMF was preferentially adsorbed to activated carbon [23]. Therefore, we developed a strategy to improve the adsorption process efficiency, to remove HMF and maintain high sugar concentrations. As shown in Fig. 3, HMF decreased with the addition of activated carbon and treatment time. HMF decreased by 89.5% from 7.25 g/L to 0.76 g/L at 4 min when 4% and 5% of activated carbon were applied. This increase in activated carbon content resulted in an increase in sugar removal efficiency [24]. Therefore, 4% activated carbon was selected as an optimal condition for HMF removal.

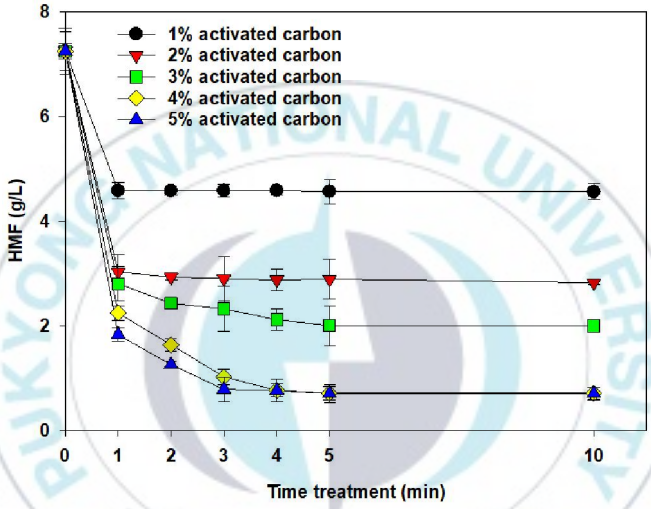

Fig. 2.3. Detoxification of *G. amansii* hydrolysate using activated carbon

Overliming is a widely used HMF removal method [25]. Increasing the pH of hydrolysate to 11.0 with $Ca(OH)_2$ followed by readjustment to pH 5.0 with 10N H_2SO_4, showed efficient detoxification of *G. amansii* hydrolysate. The overliming treatment time was optimized from 0 to 60 min, as shown in Fig. 4. HMF decreased as treatment time increased; a significant decrease (67.4%) in HMF from 7.25 to 2.36 g/L was observed after overliming *G. amansii* hydrolysate for 30 min. However, a marginal decrease in sugar from 45.2 to 42.9 g/L was also observed. Chandel *et al*. [26] reported a 41.75% reduction in furfurals and 7.61% reduction in reducing sugars from *S. spontaneum* hydrolysate after overliming.

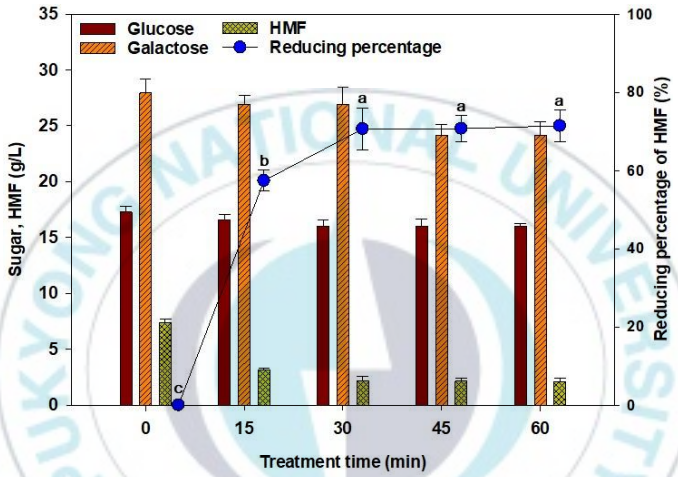

Fig. 2.4. Detoxification of *G. amansii* hydrolysate using the overliming method. Different letters indicate significant differences in treatment time ($P < 0.05$, Duncan's test).

The optimization of HMF removal from *G. amansii* hydrolysate using PEI at various treatment times is shown in Fig. 5. A ratio of 1:1 (g PEI:g HMF) was optimal for ion exchange and the optimal treatment time was 15 min. At these conditions, HMF decreased by 76.2% from 7.25 to 1.72 g/L, and sugar decreased from 45.2 to 41.4 g/L, using the ion exchange method. A previous study also reported that ion exchange led to a significant loss of fermentable sugars [27]

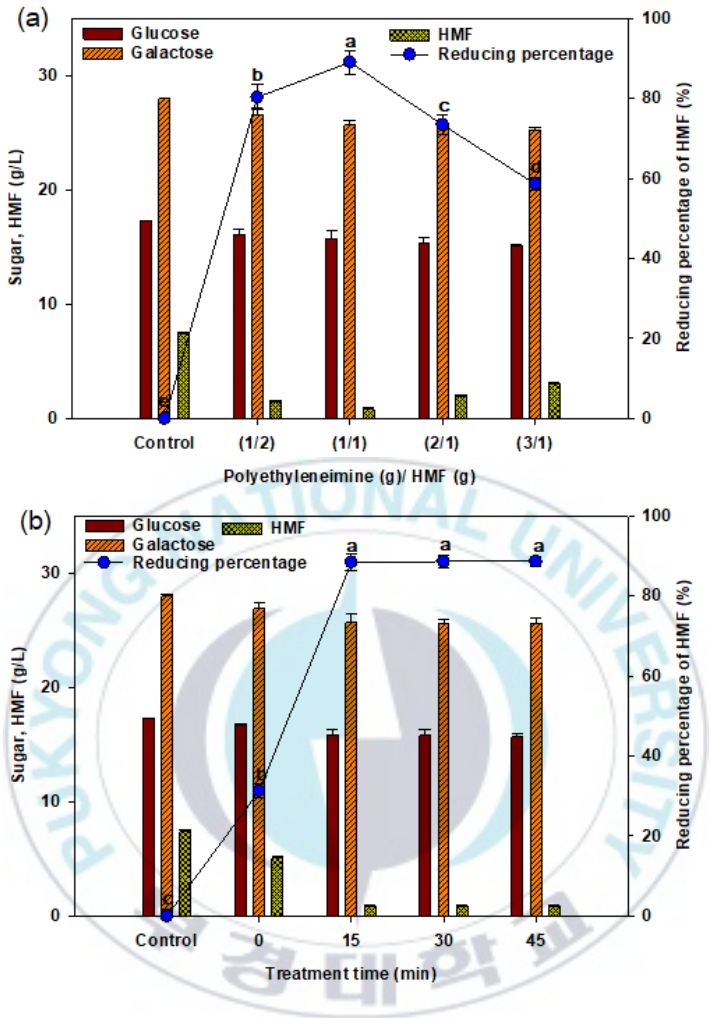

Fig. 2.5. Detoxification of *G. amansii* hydrolysate using the ion exchange method. **a** various ratios of polyethyleneimine (PEI): hydroxymethylfurfural (HMF). **b** detoxification treatment times. Different letters indicate significant differences in ratio and treatment time ($P < 0.005$, Duncan's test).

2.3.5. Bioethanol fermentation

A hydrolysate of 12% (w/v) *G. amansii* from thermal acid hydrolysis pretreatment and enzymatic saccharification was used for ethanol fermentation. Bioethanol fermentation was performed by inoculating WT *S. cerevisiae* and *S. cerevisiae* adapted to high-concentration galactose using various detoxification methods through separate hydrolysis and fermentation (SHF) (Fig. 6).

The effects of pretreatment conditions on bioethanol production were assessed without detoxification using WT *S. cerevisiae* and *S. cerevisiae* adapted to galactose as shown in Fig. 6a, b. Detoxification using activated carbon treatment with WT *S. cerevisiae* are shown in Fig. 6c, and those using activated carbon treatment with *S. cerevisiae* adapted to galactose are shown in Fig. 6d. Overliming treatment with WT *S. cerevisiae* is shown in Fig. 6e and that with *S. cerevisiae* adapted to galactose is shown in Fig. 6f. Ion exchange treatment with WT *S. cerevisiae* is shown in Fig. 6g and that with *S. cerevisiae* adapted to galactose is shown in Fig. 6h.

The WT yeast without detoxification resulted in glucose being consumed only after 12 h due to inhibition by HMF as shown in Fig. 6a. However, when HMF decreased below 5 g/L through HMF degradation during yeast fermentation [14], glucose was consumed over 60 h. After glucose was totally consumed, galactose was consumed; however, 10.11 g/L galactose remained at 144 h of fermentation [28]. An ethanol concentration of 16.62 g/L was obtained with a Y_{EtOH} of 0.37 and productivity of 0.139 g/L/h after fermentation. *S. cerevisiae*

adapted to galactose without detoxification led to the consumption of glucose at HMF below 5 g/L; however, galactose was simultaneously consumed due to yeast adaptation to galactose as shown in Fig. 6b. Glucose was totally consumed at 48 h and galactose remained at 1.5 g/L until 144 h. An ethanol concentration of 19.05 g/L was obtained at a Y_{EtOH} of 0.42 and productivity of 0.159 g/L/h during fermentation.

Detoxification of activated carbon treatment with WT *S. cerevisiae* for the production of ethanol is shown in Fig. 6c. After detoxification, only 0.74 g/L of HMF remained; therefore, glucose was consumed immediately at the start of fermentation, and totally consumed at 18 h; galactose was continuously consumed until 120 h. An ethanol concentration of 18.91 g/L was obtained at a Y_{EtOH} of 0.44 and productivity of 0.158 g/L/h during fermentation. Detoxification by activated carbon treatment using *S. cerevisiae* adapted to galactose is shown in Fig. 6d. Galactose and glucose were simultaneously consumed by *S. cerevisiae* adapted to galactose, and were completely consumed in 24 h and 76 h, respectively. An ethanol concentration of 20.28 g/L was obtained at a Y_{EtOH} of 0.47 and productivity of 0.281 g/L/h during fermentation.

Fermentation was performed by overliming treatment with WT *S. cerevisiae* as shown in Fig. 6e. After detoxification, 2.36 g/L HMF remained, and glucose was consumed during 18 h. After glucose was totally consumed, galactose was consumed; however, 8.1 g/L galactose remained at 144 h of fermentation. After fermentation, an ethanol concentration of 15.88 g/L was obtained at a Y_{EtOH} of 0.37 and

productivity of 0.265 g/L/h. When overliming treatment with *S. cerevisiae* adapted to galactose was used for fermentation as shown in Fig. 6f, galactose and glucose were simultaneously and totally consumed in 18 h and 96 h, respectively. An ethanol concentration of 19.66 g/L was obtained at a Y_{EtOH} of 0.46 and productivity of 0.273 g/L/h during fermentation.

When hydrolysates detoxified using ion exchange treatment were used for fermentation, the time to glucose and galactose consumption was reduced in a manner similar to the other detoxification methods. WT *S. cerevisiae* was used to totally consume glucose and galactose in 36 h and 120 h, respectively as shown in Fig. 6g. The fermentation time was reduced from 120 h to 72 h when *S. cerevisiae* adapted to galactose was used as shown in Fig. 6h. After fermentation, ethanol concentrations of 17.02 and 18.90 g/L were obtained at Y_{EtOH} values of 0.41 and 0.46 and productivity of 0.142 and 0.263 g/L/h, using WT *S. cerevisiae* and *S. cerevisiae* adapted to galactose, respectively.

Table 1 presents the overall ethanol production through fermentation of *G. amansii* via SHF. The activated carbon method showed the highest efficiency, reducing HMF by 89.5%, at an ethanol concentration of 20.28 g/L and Y_{EtOH} of 0.47. These results indicate that the activated carbon method is suitable for detoxification of *G. amansii* hydrolysate in ethanol fermentation.

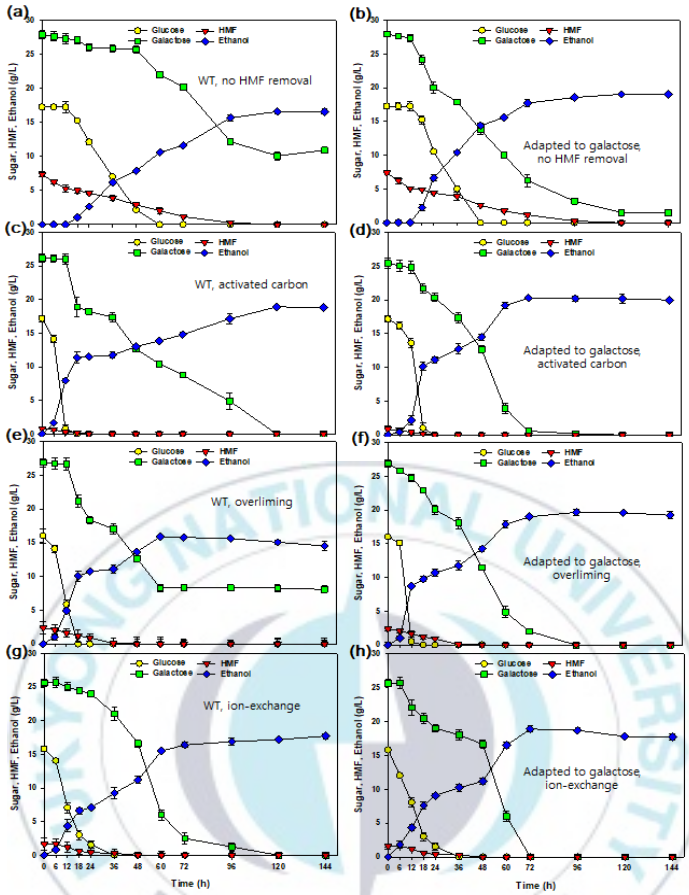

Fig. 2.6. Ethanol fermentation from *G. amansii* using *S. cerevisiae* with (a) no detoxification with wild-type *S. cerevisiae,* (b) no detoxification with *S. cerevisiae* adapted to galactose, (c) activated carbon treatment with wild-type *S. cerevisiae,* (d) activated carbon treatment with *S. cerevisiae* adapted to galactose, (e) Overliming treatment with wild-type *S. cerevisiae,* (f) Overliming treatment of *S. cerevisiae* adapted to galactose, (g) Ion-exchange treatment with wild-type *S. cerevisiae,* (h) Ion-exchange treatment with *S. cerevisiae* adapted to galactose. * WT: Wild-type *S. cerevisiae*

Table 2.1. Fermentation profile of *G. amansii* hydrolysate detoxified with different methods

	Initial sugar (g/L)	Final sugar (g/L)	HMF (g/L)	Ethanol (g/L)	Yield (g/g)	Fermentation time (h)	Productivity (g/L/h)
No detoxification with WT *S. cerevisiae* (control)	45.2	10.1	7.38	16.62	0.37	120	0.139
No detoxification with *S. cerevisiae* adapted to galactose	45.2	2.5	7.38	19.05	0.42	120	0.159
Activated carbon treatment with wild-type *S. cerevisiae*	43.3	0	0.74	18.91	0.44	120	0.158
Activated carbon treatment with *S. cerevisiae* adapted to galactose	43.3	0	0.74	20.28	0.47	72	0.281
Overliming treatment with WT *S. cerevisiae*	42.9	8.1	2.36	15.88	0.37	60	0.265
Overliming treatment with *S. cerevisiae* adapted to galactose	42.9	0	2.36	19.66	0.46	72	0.273
Ion exchange treatment with wild-type *S. cerevisiae* yeast	41.4	0	1.72	17.02	0.41	120	0.142
Ion exchange treatment with *S. cerevisiae* adapted to galactose	41.4	0	1.72	18.90	0.46	72	0.263

HMF, hydroxymethylfurfural
WT, wild-type

2.4. Conclusion

In this study, we evaluated ethanol production from *G. amansii* hydrolysate with detoxification of HMF. As a results, *G. amansii* slurry content of 12%, 182 mM H_2SO_4 at 121℃ for 45 min were used for thermal acid hydrolysis. Sixteen Units/mL Celluclast 1.5 L at 45℃ for 36 h were used for enzymatic saccharification. The HMF was reduced from 7.25 g/L to 0.74 g/L, 2.36 g/L and 1.72 g/L when detoxification methods of activated carbon, overliming and ion-exchange were used, respestively. Table 2.1 presents the overall fermentation of ethanol production from *G. amansii* via SHF. Activated carbon showed the highest efficiency reducing HMF by 89.5% and ethanol concentration of 20.28 g/L with Y_{EtOH} of 0.47 were obtained. These results indicate that activated carbon can be suitable for detoxification of *G. amansii* hydrolysate using for ethanol fermentation.

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CHAPTER III

ABE fermentation from the green seaweed *Enteromorpha intestinalis* via the separate hydrolysis and fermentation

3.1. Introduction

Ethanol and butanol have been developed as biofuels and chemicals in response to energy shortages and climate change over the past few decades. Butanol has an advantage over ethanol in that it can be blended with gasoline at any percentage. Moreover, butanol is less corrosive and absorbs less moisture. Finally, it has a higher energy content than ethanol and is more similar to gasoline fuel in terms of energy content [1 - 3].

Butanol can be produced from acetone, butanol and ethanol (ABE) fermentation of various substrates, such as sago [4], potato, soy molasses, cassava [5], and corn starch [6]. However, with the rising prices of these products, cost has become a major factor affecting the mass production of butanol [7]; a new generation of biomass sources has been developed for butanol production, including agriculture crop residuals, straw and lignocellulosic waste [8 - 10]. However, these types of biomass have low yields and high costs, and show inefficient hydrolysis with use of current technologies. For example, lignocellulosic biomass hydrolysate contains a mixture of inhibitors such as hydroxymethylfurfural, furfural, and lignin derivatives, which have severe inhibitory effects on both *Clostridium* growth and ABE production [11, 12]. Recently, seaweed biomass has become an attractive option as a bioresource for biofuel production. A large body of research has reported on ethanol production from red, green, and brown seaweed [13]. Pretreatment methods for the hydrolysis of

seaweed into sugars are less intensivethan those for lignocellulosic biomass pretreatment, probably due to the relatively lower content of crystalline sugar polymers and absence of lignin in their cell walls [14]. The main carbohydrates in green seaweed are starch and cellulose,which are composed of glucose, xylose, galactose, glucuronic acid and rhamnose [15].

Acetone, butanol and ethanol can be obtained via fermentation by many species of microorganisms, among which *Clostridium acetobutylicum* and *Clostridium beijerinckii* are the most promising for commercial and laboratory applications due to their high efficiencies [16]. *C. acetobutylicum* is a gram-positive, rod shaped obligate anaerobic bacterium that forms spores. In addition, it is one of the few microorganisms that can usea variety of sugars to produce desirable ABE products. For example, *C. acetobutylicum* can use sugars in biphasic fermentation processes, such as acidogenesis and solventogenesis. During acidogenesis, the rapid formation of acids causes a decrease in pH. Subsequently, solventogenesis begins when the acid content reaches a threshold level, beyond which acids are re-assimilated during ABE formation [17, 18]. Therefore, the pH of the medium is very important during ABE fermentation.

In the present study, thermal acid hydrolysis and enzymatic saccharification were employed to produce monosaccharides, and to support fermentation in the production of ABE from the green macroalgae *Enteromorpha intestinalis*. The optimal thermal acid hydrolysis parameters were determined using the one factor at a time

optimization method. Enzymatic saccharification was performed using the commercial enzymes Celluclast 1.5 L and Viscozyme L. Finally, ABE production was quantified under different fermentation conditions based on pH using *C. acetobutylicum* KCTC 1790.

3.2. Materials and Methods

3.2.1. Raw materials and composition analysis

Enteromorpha intestinalis was obtained from Wando county, Jeollanam province, Korea. Samples were oven-dried at 55°C and ground in a roller mill. The resulting *E. intestinalis* powder was passed through a 200-mesh sieve and storedin a sealed bag at room temperature until pretreatment. The approximate composition of *E. intestinalis* was analyzed at the Feed and Foods Nutrition Research Center of Pukyong National University (Busan, Korea) according to the American Organization of Analytical Chemists method [19].

Crude protein content was determined by Kjeldahl method. The content of nitrogen was calculated based on the determined ammonia amount. The protein content was calculated by multiplying the nitrogen content of the sample with a factor of 6.25.

Fiber was quantified using 0.3 g seaweed samples (S) with diluted H_2SO_4 by Henneberg-Stohmann method. Seaweed samples was boiled with diluted H_2SO_4 (0.4 N) previously. Then, the mixture was filtered and washed with 200 mL of distilled water and then boiled with NaOH (0.3 N). The residue was washed with boiling distilled water and finally dried at 135°C to constant weight (W_1). The material was heated at 550°C for two hours and the weight was recorded (W_2) [20]. The content of crude fiber was calculated using Eq. (1), as follows:

$$\text{Crude fiber (\%)} = \left(\frac{W_1 - W_2}{S} \times 100 \right) \times LC \qquad \text{Eq. (1)}$$

LC: Lipid correction coefficient = [(100 - lipid content of sample (%))/100]

The ash content was obtained by calcinations at 550°C for three hours in Muffle Furnace.

Lipid was extracted by a Randall modification of the Soxhlet method, using Foss Soxtec 2043 Extraction system. After extraction, the solvent was evaporated and the lipid content was determined gravimetrically after drying the recovered residues [21].

Carbohydrate content was determined as the weight difference using protein, lipid, fiber and ash content data [22].

3.2.2. Bacterial strains and culture medium

The anaerobic strain *C. acetobutylicum* KCTC 1790 was purchased from the Korean Collection for Type Cultures (KCTC), Biological Resource Center (Daejeon, Korea). The strain was cultured in PGY + P2 medium containing 20 g/L glucose, 3 g/L peptone, 1 g/L yeast extract, and 0.15 g/L cysteine hydrochloride with 0.001% (v/v) of 0.1% resazurin solution and 0.01% (v/v) of P2 solution. The P2 stock solution containing vitamin, minerals, and buffers had the following composition: 50 g/L KH_2PO_4, 50 g/L K_2HPO_4, 220 g/L ammonium acetate, 0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamin, 0.001 g/L biotin, 20 g/L $MgSO_4$ $7H_2O$, 1 g/L $MnSO_4$ H_2O, 1 g/L $FeSO_4$ $7H_2O$

and 1 g/L NaCl. The initial pH was adjusted to approximately 6.0. Bacteria were cultured for approximately 18 h at 37°C before inoculation in the ABE production medium. All experiments were conducted in triplicate.

3.2.3. Thermal acid hydrolysis pretreatment

The one factor at a time optimization method was used to optimize thepretreatment of *E. intestinalis*. The conditions for thermal acid hydrolysis were optimized in terms of slurry content (6 - 16%, w/v), H_2SO_4 concentration (90 - 450 mM) and thermal hydrolysis time (15 - 75 min). Thermal acid hydrolysis was carried out at 121°C. *E. intestinalis* hydrolysate was neutralized to pH 5 using 10 N NaOH. After thermal acid hydrolysis, the monosaccharide concentrations of each sample were determined using high-performance liquid chromatography (HPLC) (1100 Series; Agilent Technologies, Santa Clara, CA, USA). The thermal acid hydrolysis efficiency was calculated using Eq. (2), as follows:

$$E_p\ (\%) = \frac{\triangle S_p}{TC} \times 100 \qquad \text{Eq. (2)}$$

where E_p is the pretreatment efficiency (%), $\triangle S_p$ is the increase in monosaccharides (g/L) during thermal acid hydrolysis, and TC is the total carbohydrate concentration (g/L) of *E. intestinalis*.

3.2.4. Enzymatic saccharification

After thermal acid hydrolysis, the optimal conditions for the enzymatic saccharification of *E. intestinalis* were evaluated. Celluclast 1.5 L (854 endoglucanase units/mL; Novozymes, Bagsværd, Denmark) and Viscozyme L (121 fungal β-glucanase units/mL; Novozymes) were used to hydrolyze fiber. Enzymes at a 1:1 ratio (4 - 32 Units/mL) were added to 100 g/L of *E. intestinalis* slurry after thermal acid hydrolysis at pH 5.0, 45°C, and 150 rpm for 0 - 60 h. Celluclast 1.5 L contains cellulase, which hydrolyzes the β(1, 4)-D-glucosidic linkages of cellulose and other β-D-glucans. Viscozyme L contains endo-β-glucanase, which hydrolyzes the β(1,3)- and β(1,4)-linkages in β-D-glucans, with xylanase, cellulase and hemicellulase as side activities. The β-glucosidase and cellulase activities were determined according to Mandels *et al.* [23] and Kubicek *et al.* [24]. The monosaccharide content of the samples was measured using HPLC (1100 Series; Agilent Technologies). The thermal acid hydrolysis and saccharification efficiency was calculated using Eq. (3), as follows:

$$E_{ps}\ (\%) = \frac{\triangle S_{ps}}{TC} \times 100 \qquad \text{Eq. (3)}$$

where E_{ps} is the efficiency of the pretreatment (%), $\triangle S_{ps}$ is the increase in monosaccharides (g/L) during thermal acid hydrolysis and

saccharification, and TC is the total carbohydrate concentration (g/L) of *E. intestinalis*.

3.2.5. ABE fermentation

The hydrolysates obtained from the saccharification process were used as the ABE production medium, and were supplemented with the following nutrients: 1 g/L yeast extract, 3 g/L peptone, 0.15 g/L cysteine hydrochloride and 0.01% P2 solution. Fermentation was conducted in 125-mL screw-capped bottles containing 70 mL of medium. The medium was purged with N_2 for 10 min to maintain anaerobic conditions and the pH of the medium was adjusted to 5.5 with 10 N NaOH before fermentation. *C. acetobutylicum* KCTC 1790, with an inoculation percentage of 10% and 2.9 g dry cell weight (dcw)/L, was cultivated in PGY medium at 37°C for 18 h. Fermentation was carried out in an N_2 incubator (JS-N2-AT100; Johnsam Corporation, Bucheon, Korea) filled with 96% nitrogen and 4% hydrogen at 37°C, and at 120 rpm in a shaker (VS-201D; Vision Scientific, Daejeon, Korea). Culture trials under three conditions were carried out to study fermentation under different pH conditions. The ABE yield (Y_{ABE}) (g/g) was determined according to the following equation:

$$Y_{ABE}\ (g/g) = \frac{[\text{ABE concentration}]_{max}}{[\text{Sugar concentration}]_{ini}} \qquad \text{Eq. (4)}$$

where [ABE concentration]$_{max}$ is the highest ABE concentration (g/L) obtained during fermentation and [Sugar concentration]$_{ini}$ is the total initial sugar concentration (g/L) at the start of fermentation [25]. Fermentation samples were collected daily for analysis.

3.2.6. Analytical methods

The cell concentration was determined via optical density measurement at 600 nm (OD_{600}) using a standard curve of OD_{600} versus dry cell weight and a spectrophotometer (Amersham Biosciences Ultrospec 6300 Pro; Biochrom, Cambridge, UK). Monosaccharides (glucose and xylose) concentrations were measured using HPLC (1100 Series; Agilent Technologies) with a refractive index detector. An Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) was used with filtered and degassed 5 mM H_2SO_4 as the eluent at a rate of 0.6 mL/min and temperature of 65°C.

The fermentation products (acetone, butanol, ethanol, acetic acid, and butyric acid) were analyzed by gas chromatography (YL 6100; Young Lin Instrument Co. Ltd., Anyang, Korea) equipped with a flame ionization detector and silica capillary column (30 m × 0.32 mm × 0.5 ìm; HP-INNOWax; Agilent Technologies, USA). The oven temperature was programmed to increase from 80°C to 150°C at a rate of 30°C/min. Both the injector and detector temperatures were set at 250°C. Before analysis, aqueous samples were centrifuged at 14,240 ×

g for 10 min and the supernatant was filtered through a 0.2-μm syringe filter.

3.2.7. Statistical analysis

All experiments were performed in triplicate. The statistical significance of the differences among the pretreatment, saccharification, and monosaccharide contents were evaluated using one-way analysis of variance and Duncan's multiple range test ($P < 0.05$) in SPSS software (ver. 23; SPSS Inc., Chicago, IL, USA).

3.3. Results and Discussion

3.3.1. Composition of *Enteromorpha intestinalis*

The green seaweed *E. intestinalis* was composed of 35.7% crude protein, 5.1% crude fiber, 2.26% crude lipid, 18.1% crude ash and 38.8% carbohydrate. The total carbohydrate content of *E. intestinalis* used in this experiment was 43.9% (including crude fiber as cellulose on a dry solid basis). The carbohydrates in green seaweed are composed mainly of glucose, xylose, galactose, rhamnose, and glucuronic acid [15].

3.3.2. Monosaccharide production via thermal acid hydrolysis

Thermal acid hydrolysis is necessary to convert carbohydrates into fermentable sugars. Three factors (slurry content, H_2SO_4 concentration and treatment time) were evaluated for their effects on monosaccharide production by thermal acid hydrolysis. Figure 1 presents the results of the thermal acid hydrolysis analysis.

The slurry content was varied within a range of 6 - 16% (w/v), with 182 mM H_2SO_4 at 121°C for 60 min, to determine the optimal slurry content. The sugar concentration increased with increasing slurry content and a slurry content of 16% (w/v) yielded a monosaccharide concentration of 13.0 g/L, equivalent to an efficiency of

E_p = 18.5% (Fig. 1(a)). However, increasing the slurry content above 10% during thermal acid hydrolysis resulted in a decrease in pretreatment efficiency from 25.0% to 18.5%. A previous study similarly reported that the green seaweed *Ulva lactuca* was used at a concentration of 10% for alkaline pretreatment with the addition of NaOH, and acid pretreatment with the addition of H_2SO_4 [14]. Therefore, a slurry content of 10% (w/v), yielding 11.0 g/L of monosaccharides and E_p = 25.5%, was selected as the optimal content for ABE production.

According to Feng *et al.* [15], H_2SO_4 is the most effective acid among various acids, such as H_2SO_4, HCl, H_3PO_4 and maleic acid, for the hydrolysis of *Enteromorpha*. Therefore, we assessed H_2SO_4 concentrations in the range of 90 - 450 mM, with a 10% (w/v) slurry content at 121°C for 60 min, to determine the optimal acid concentration (Fig. 1(b)). In a previous study, high acid concentrations resulted in high monosaccharide yields from coastal Bermuda grass [26]. In this study, H_2SO_4 concentrations in the range of 270 - 450 mM resulted in similar E_p values. Therefore, 270 mM H_2SO_4 was selected as the optimal acid concentration, yielding 15.1 g/L of monosaccharides and E_p = 34.4%.

The treatment time was varied from 15 min to 75 min, with a slurry content of 10% (w/v) and an H_2SO_4 concentration of 270 mM at 121°C. The monosaccharide content increased from 10.9 g/L to 15.1 g/L after increasing the treatment time from 15 min to 60 min (Fig. 1(c)). However, monosaccharide production did not increase further

when the treatment time was extended from 60 min to 75 min. Therefore, 60 min was selected as the optimal hydrolysis time for thermal acid hydrolysis. From these results, the optimal conditions for thermal acid hydrolysispretreatment were 10% (w/v) slurry and 270 mM H_2SO_4 at 121°C for 60 min.

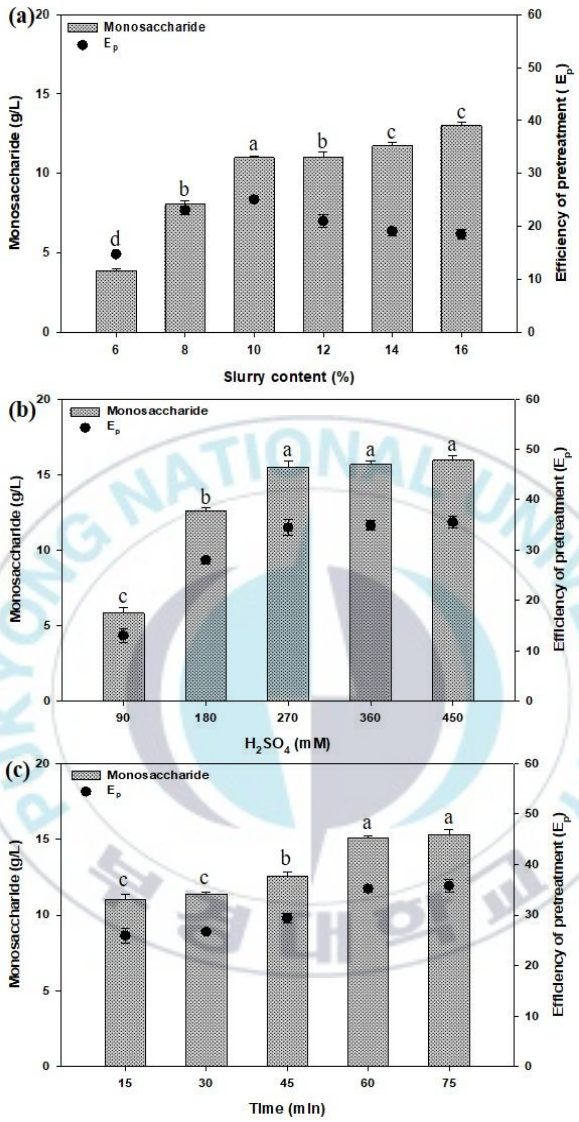

Fig. 3.1. Results of thermal acid hydrolysis based on (a) slurry content, (b) H_2SO_4 concentration, and (c) hermal hydrolysis time. Different letters indicate a significant difference in seaweed concentration ($P < 0.05$, Duncan's test).

3.3.3. Enzymatic saccharification

Glucose production from cellulose was achieved viaenzymatic saccharification, resulting in the degradation of *E. intestinalis* fiber after thermal acid hydrolysis. Enzymatic saccharification was performed using 16 Units/mL of Viscozyme L, Celluclast 1.5 L, or a mixture of the two enzymes at various ratios, at 45°C for 48 h (Table 1). The initial monosaccharide concentration was 15.1 g/L after thermal acid hydrolysis. When either Viscozyme L or Celluclast 1.5 L was applied, the monosaccharide concentration was increased from 15.1 g/L to 19.0 g/L and 19.6 g/L, respectively. When mixtures of Viscozyme L and Celluclast 1.5 L were applied at ratios of 2:1, 1:1 and 1:2, the monosaccharide concentration increased to 20.9 g/L, 24.2 g/L and 22.13 g/L, respectively. Thus, a mixture of enzymes resulted in more effective enzymatic saccharification than either of the enzymes alone and a mixture of 16 Units/mL of Celluclast 1.5 L and Viscozyme L at a ratio of 1:1 was selected for enzymatic saccharification. A previous study similarly reported that a mixture of enzymes had a synergistic effect and yielded a higher monosaccharide content than single enzymes [27].

Table 3.1. Enzymatic saccharification of *E. intestinalis* using the commercial enzymes Viscozyme L and Celluclast 1.5 L

Enzyme	Monosaccharide (g/L)	E_{ps}
Control	15.10	34.40
Viscozyme L (Vis)	19.00	43.28
Celluclast 1.5 L (Cell)	19.60	44.65
Vis:Cell (2:1)	20.90	47.61
Vis:Cell (1:1)	24.20	55.13
Vis:Cell (1:2)	22.13	50.41

Control: hydrolysate by thermal acid hydrolysis under optimal conditions

Next, various concentrations (4 - 32 Units/mL) of Celluclast 1.5 L and Viscozyme L at a ratio of 1:1 were assessed for saccharification at 45°C for 0 - 60 h. The monosaccharide concentration increased with increasing enzyme concentration and reaction time (Fig. 2). Enzyme mixture concentrations of 16 Units/mL, 24 Units/mL and 32 Units/mL resulted in more efficient saccharification than lower concentrations; however, the monosaccharide concentration did not increase beyond 36 h and at 36 h, 16 - 32 Units/mL of mixed enzymes yielded the same monosaccharide concentrations (24.2 g/L). Therefore, the optimal enzymatic saccharification conditions were considered to be a mixture of Celluclast 1.5 L and Viscozyme L at ratio of 1:1 and concentration of 16 Units/mL at 45°C for 36 h.

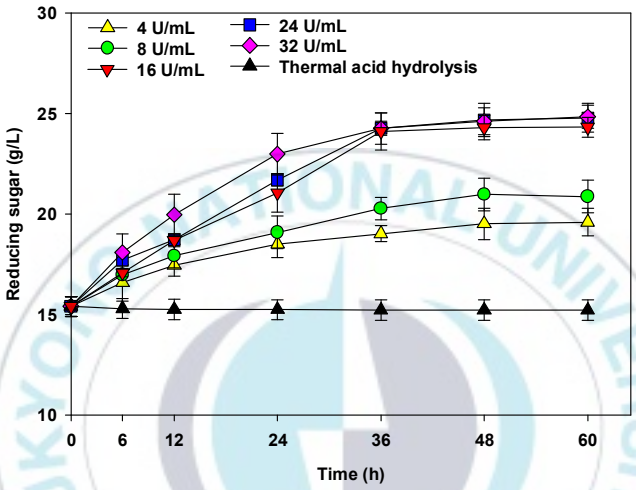

Fig. 3.2. Saccharification of *E. intestinalis* using a 1:1 mixture of Celluclast 1.5L and Viscozyme L. The initial monosaccharide concentration was 15.1 g/L after thermal acid hydrolysis pretreatment.

3.3.4. Effect of hydrolysate pH on *C. acetobutylicum* growth

Clostridium acetobutylicum cannot maintain a constant pH gradient in the intracellular transmembrane under drastic changes inexternal pH, which affects its cellular growth and metabolism [28]. Thus, the initial pH of the fermentation medium for cell growth is an important factor in the fermentation process. Initial pH values of 4.5, 5.0, 5.5, 6.0 and 6.5, adjusted with the addition of 5 N HCl or 5 N NaOH, were evaluated for their effect on *C. acetobutylicum* growth. Figure 3 shows *C. acetobutylicum* growth in *E. intestinalis* hydrolysate at various pH levels. Cell growth was severely inhibited at low pH levels (e.g., pH 4.5) and increased with increasing pH. Cell growth reached a maximum of 3.56 g dcw/L at a pH of 6.0 after 3 days. A previous study showed that the optimal pH for butanol fermentation using different carbohydrates was usually between 5.0 and 6.5 [29] and Li *et al.* [30] showed that an initial pH of 6.0 resulted in the highest butanol production. Moreover, the salt is generated from acid-base reaction during pH adjustment prior to fermentation which inhibits cell growth and fermentation by *C. acetobutylicum* and more NaOH is required to bring the pH of the seaweed hydrolysates to pH 6.5 [12]. Therefore, an initial pH of 6.0 was selected as the optimal pH for *C. acetobutylicum* growth.

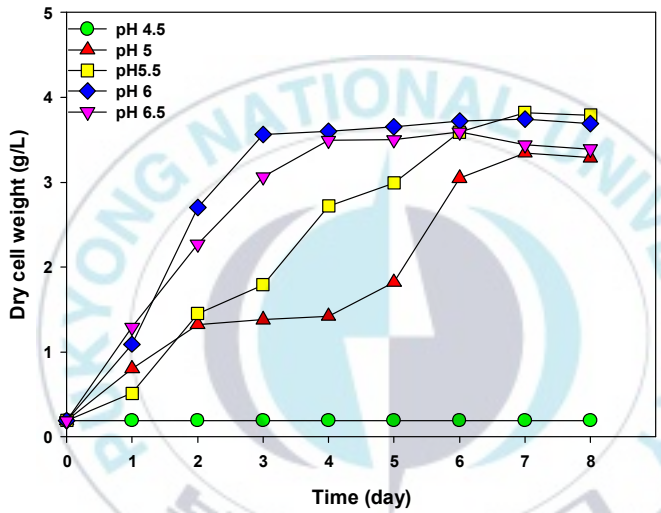

Fig. 3.3. Optimal conditions for *C. acetobutylicum* growth in *E. intestinalis* hydrolysate at various pH levels.

3.3.5. ABE production using the separate hydrolysis and fermentation method

To assess ABE production via the separate hydrolysis and fermentation (SHF) method, *E. intestinalis* hydrolysate was used as a substrate for fermentation with the anaerobic bacteria *C. acetobutylicum* KCTC 1790 as the ABE fermentation strain (Fig. 4). The initial pH of the hydrolysate was adjusted to 6.0 for the evaluationof fermentation with and without pH control on ABE production.

Hydrolysate-based medium was fermentable by *C. acetobutylycum* without pH control (Fig. 4(a)). Glucose was completely consumed within 3 days; however, 2.2 g/L of xylose was not consumed until the end of the fermentation. The pH decreased from 6.0 to 4.3 during the first 3 days, and then remain steady until the end of fermentation. The cell density decreased due to the decrease in pH, and 5.7 g/L of ABE was produced with a Y_{ABE} of 0.24. A similar study showed that cell growth ceased, and galactose uptake and synthesis of all products were inhibited when the pH decreased below 4.5 [31]. Another study reported that fermentation by *C. acetobutylicum* without pH control had a low product yield without the consumption of monosaccharides, due to the low pH [32]. Therefore, fermentation without pH control not only affects cell growth, but also causes a low product yield, and it is important to control the pH to produce a high cell density and improve ABE production during fermentation.

ABE fermentation with pH controlled at 6.0 was carried out to produce a high cell density and complete xylose consumption (Fig. 4(b)). *C. acetobutylicum* consumed 16.6 g/L of glucose and 7.6 g/L of xylose from the *E. intestinalis* hydrolysate. Glucose was completely consumed within 3 days and xylose consumption began later on day 2 and was completely consumed by day 4. This was in good agreement with previous studies [33, 34] showing that *C. acetobutylicum* preferred glucose over xylose with a high consumption rate, and consumed glucose first when both sugars were present in the medium. The pH of the medium decreased after 1 day and was adjusted to 6.0 using 10 N NaOH. It did not change after xylose was completely consumed. The dry cell weight reached a maximum of 3.80 g dcw/L, which was maintained until the end of fermentation. The maximum ABE concentration was 4.8 g/L with a Y_{ABE} of 0.2 on day 4, which was lower than the results in Fig. 4(a); however, ahigh concentration of butyric acid (5.9 g/L) was produced. A previous study reported that fermentation mostly produced organic acids with a small amount of ABE at an initial culture pH of 6.0 or greater [35]. Therefore, it is important to employ novel pH control strategies to improve ABE production during fermentation.

To improve ABE production, pH was controlled from 6.0 for acidogenesis to 4.5 for solventogenesis (Fig. 4(c)). Aprevious study reported that *C. acetobutylicum* preferentially produces acetate and butyrate during acidogenesis at high pH values, whereas solvents such as ABE are produced during solventogenesis at a low pH [17, 18]. As

a result, the pH was initially adjusted to 6.0 to support glucose and xylose uptake for acidogenesis. Glucose was completely consumed by day 3, while xylose consumption began on day 2 and was completed by day 4. After the complete consumption of xylose, the pH was reduced to 4.5 to support solventogenesis. The dry cell weight was similar to that shown in Fig. 4(b); however, a decrease in dry cell weight was observed under solventogenesis. After acidogenesis, 5.1 g/L of ABE and 5.8 g/L of butyric acid were produced; however, conversion of butyric acid into butanol occurred during solventogenesis. Moreover, the ABE concentration increased to 8.5 g/L with a Y_{ABE} of 0.36, while the butyric acid concentration decreased to 0.6 g/L. Another study reported that the conversion of butyric acid into butanol was catalyzed by butyl aldehyde dehydrogenase and butanol dehydrogenase [36].

Compared to ABE production in previous studies based on various biomasses (Table 2), ABE production from *E. intestinalis* with a controlled pH was slightly lower than that from mixed grain, barley straw, corncob and *U. lactuca*, but higher than that from potato, switchgrass and sago pith residues. The differences in production might be due to differences in the pretreatment methods employed and biomass characteristics, because pretreated hydrolysate is a complex mixture of various components, including both sugars and inhibitory compounds [12].

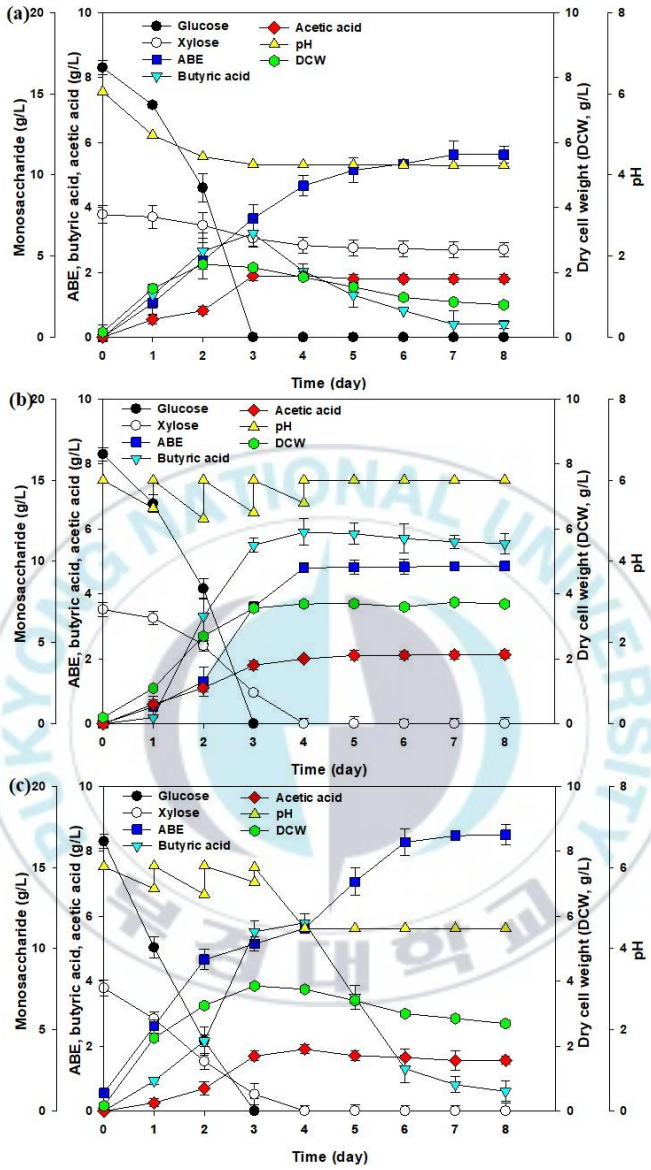

Fig. 3.4. Acetone, butanol and ethanol (ABE) fermentation of *E. intestinalis* hydrolysate using *C. acetobutylicum* with (a) uncontrolled pH, (b) pH controlled at 6.0, and (c) pH controlled initially at 6.0 and then 4.5 on day 4.

Table 3.2. Comparision of ABE fermentation with various biomass.

Substrate	Microorganisms	Technology	ABE (g/L)	Yield (g/g)	References
Potato	*C. acetobutylicum* P262	Gelatinization	4.6	0.14	[4]
Mixture of grain and barley straw	*C. acetobutylicum* DSM 1731	Dilute acid pretreatment	11.3	0.34	[37]
Corncob	*C. acetobutylicum SE-1*	Wet disk milling pretreatment, enzymatic hydrolysis	14.12	0.36	[33]
Switchgrass	*C. beijerinckii* P262	Dilute H_2SO_4, enzymatic hydrolysis	1.48	0.08	[38]
Sago pith residues	*C. acetobutylicum ATCC 824*	Enzymatic hydrolysis	4.2	0.2	[39]
Green seaweed (*U. lactuca*)	*C. beijerinckii* NCIMB 8052	Hot-water treatment, enzymatic saccharification, supplemented with glucose and xylose	5.5	0.35	[14]
Green seaweed *(E. intestinalis)*	*C. acetobutylicum* KCTC 1790	Thermal acid hydrolysis, enzymatic saccharification, fermentation without pH control	5.7	0.24	This study
Green seaweed *(E. intestinalis)*	*C. acetobutylicum* KCTC 1790	Thermal acid hydrolysis, saccharification, fermentation with pH controlled at 5.5	4.8	0.20	This study
Green seaweed *(E. intestinalis)*	*C. acetobutylicum* KCTC 1790	Thermal acid hydrolysis, saccharification, fermentation with pH controlled at 5.5 (initial) and 4.5 (day 4)	8.5	0.36	This study

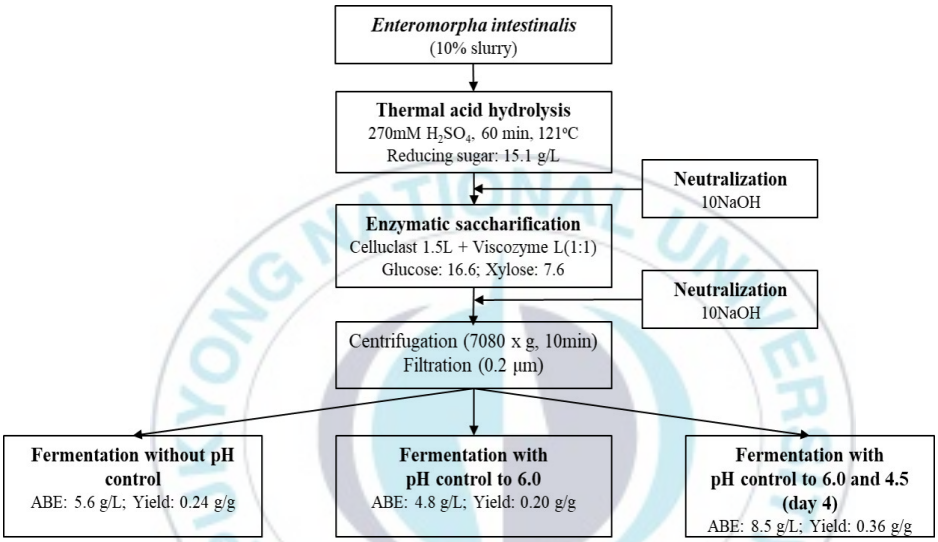

Fig. 3.5. Schematic diagram of ABE production from *E. intestinalis* using *C. acetobutylicum*.

3.4. Conclusion

In this study, we optimized ABE production from *E. intestinalis* via SHF. The overall process of ABE production from *E. intestinalis* via SHF. A slurry content of 10% *E. intestinalis* was used for thermal acid hydrolysis. A reducing sugar content of 15.1 g/L was obtained under optimal thermal acid hydrolysis conditions of 270 mM H_2SO_4, 121°C and 60 min. Enzymatic saccharification was performed using a mixture of the commercial enzymes Celluclast 1.5 L and Viscozyme L in a 1:1 ratio. Glucose and xylose contents of 16.6 g/L and 7.6 g/L, respectively, were obtained from enzymatic saccharification, which were used for subsequent ABE fermentation. Fermentation was carried out with uncontrolled pH, pH controlled at 6.0 and pH controlled initially at 6.0 and then at 4.5 on day 4, which produced an ABE content of 5.6 g/L with a Y_{ABE} of 0.24 g/g, 4.8 g/L with a Y_{ABE} of 0.20 g/g and 8.5 g/L with a Y_{ABE} 0.36 g/g, respectively. These results indicate that *E. intestinalis* can be used as biomass for ABE production.

3.5. References

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SUMMARY (in Korean)

분리 당화발효 통한 다양한 바이오매스로부터 바이에너지 생산

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부 경 대 학 교 대 학 원 생물공학과

요 약

바이오 에너지는 전분 또는 셀룰로오스와 같은 단당류로 분해 될 수 있는 물질을 함유한 재료로부터 발효를 통해 생산 될 수 있다. 전통적으로 바이오 에너지는 사탕수수, 밀, 옥수수를 사용하는 전분이나 당과 같은 1 세대 바이오매스로 생산되었다. 그러나 1 세대 바이오매스는 인간의 음식이나 동물 사료로도 사용될 수 있어서 도덕적인 문제와 가격 상승에 대한 우려가 있다고 한다. 제 2 세대 바이오매스로서 리그노셀룰로오스 바이오매스 및 농산물 폐기물로부터 바이오 에너지가 생산되었다. 그러나 공급원료는 현재의 기술을 사용하는 효율적인 가수 분해 공정으로 낮은 수율과 높은 비용을 가지고 있다. 따라서 비지와 해조류는 바이오 에너지 생산을 위한 본 연구에서 새로운 바이오매스로 사용되었다.

비지로부터 분리 가수 분해 및 발효 (SHF)를 통해 바이오 에탄올 생산이 진행되었다. 이 연구는 전처리, 효소의 당화 및 발효에 중점을 두었다. 단당을 얻기 위한 전처리는 비지 슬러리 20% (w/v)와 121 ℃에서 60 분 동안 270 mM H_2SO_4를 사용하여 진행 하였다. 더 많은 당을 얻기 위해

효소 C-Tec 2와 Viscozyme L의 16 Units/mL 혼합물을 45 ℃에서 48 시간 동안 효소 가수 분해시켜 얻었다. 20% (w/v) 비지의 가수 분해물을 이용한 에탄올 발효는 플라스크 및 5 L 발효기를 사용하여 wild-type 및 고농도의 갈락토오스에 순치된 *Saccharomyces cerevisiae* KCTC 1126을 사용하여 수행 하였다. *S. cerevisiae*의 wild-type을 사용했을 때 에탄올 생산량은 20.77 g/L이었고 효율이 0.31이었다. 플라스크 및 5 L 발효기에서 고농도 갈락토오스에 순치된 *S. cerevisiae*를 사용하여 33.89 g/L 및 31.64 g/L의 에탄올을 생산하였다. 또한 그 수율은 0.49 및 0.47으로 각각 생산 되였다. 결과로서, 갈락토스에 순치한 *S. cerevisiae*는 wild-type *S. cerevisiae*와 비교하여 에탄올 생산량을 증가시켰다.

해조류 *Gelidium amansii*를 바이오매스로 사용하여 분리 가수 분해 및 발효 (SHF) 과정을 사용하여 바이오에탄올을 생산하였다. 이 연구는 열산 가수 분해 전처리, 효소의 당화, 해독 및 발효에 중점을 두었다. 열산 가수 분해는 다양한 황산 농도 (90 ~ 450 mM), 슬러리 함량 (8 ~ 16 %) 및 처리 시간 (15 ~ 75 분)으로 수행되었다. 결과로 121 ℃에서 45 분 동안 12 % (w/v) 해조류 슬러리, 182 mM H_2SO_4를 열산 가수 분해를 위한 최적 조건으로 선택하여 6.8 g/L 포도당과 26.1 g/L 갈락토스를 얻었다. 단당류 (주로 포도당)는 16 Units/mL Celluclast 1.5 L 사용하여 36 시간 동안 열산 가수 분해물을 효소 가수 분해하여 얻은 것이다. 해독은 활성탄 흡착 법, $Ca(OH)_2$를 이용한 overliming 법, polyethylenimine (PEI)을 이용한 이온 교환법으로 실시한 결과, 활성탄 흡착 법은 HMF 제거에 가장 좋은 결과를 나타냈다. 해조류 가수 분해물 12% (w/v)를 함유 한 에탄올 발효는 wild-type과 갈락토스에 순치한 *Saccharomyces cerevisiae*를 사용하여 수행하였다.

Acetone, butanol과 ethanol (ABE)은 *Enteromorpha intestinalis*로부터 분

리 가수분해 및 발효(SHF) 방법으로 생산되었다. *E. intestinalis*의 발효를 위한 전처리와 효소 당화의 최적화에 중점을 두었다. 전처리는 10 % (w/v) 해조류 슬러리와 270 mM H_2SO_4를 사용하여 121 ℃에서 60 분간 수행 하였다. Celluclast 1.5 L와 Viscozyme L의 16 Units/mL 혼합물을 사용하여 45 ℃에서 36 시간 동안 효소 가수 분해로부터 단당을 더 얻었다. 혐기성 박테리아 인 *Clostridium acetobutylicum*을 사용하여 pH를 조절하지 않고 pH 6.0으로 조절하거나 pH를 처음 6.0으로 조절 한 후 4 일 후에 4.5로 조정하여 10 % (w / v) *E. intestinalis* 가수 분해물을 이용한 ABE 발효를 수행 하였다. 그에 따라 0.24 g/g의 Y_{ABE}를 갖는 5.6 g/L, 0.2 g/g의 Y_{ABE}를 갖는 4.8 g/L 및 0.36 g/g의 Y_{ABE}를 갖는 8.5 g/L의 ABE 함량이 각각 생산되었다.

결과적으로 비지로부터 갈락토스에 순치한 *S. cerevisiae*를 이용해 분리 가수 분해 및 발효 (SHF)를 통한 최대 에탄올 농도는 33.89 g/L 이었고, Y_{EtOH}는 0.49 이었다. *G. amansii*로부터 에탄올을 생산했을때 활성탄은 해독에 적합할 수 있고, 에탄올 농도가 20.28 g/L, Y_{EtOH}가 0.47로 가장 높은 효율이었다. *E. intestinalis*로부터의 ABE 발효는 pH를 6.0으로 조절한 다음 4 일에 pH 4.5로 수행하였을때 가장 높은 ABE 함량이 8.5 g/L 이고 Y_{ABE}가 0.36 g/g인 것으로 나타났다.

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