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Ethanol Production from the Seaweed *Gelidium amansii*, Using Specific Sugar Acclimated Yeasts

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For the production of ethanol from seaweed as the source material, thermal acid hydrolysis and enzymatic saccharification were carried out for monosugars production of 25.5 g/l galactose and 7.6 g/l glucose using *Gelidium amansii*. The fermentation was performed with *Pichia stipitis* KCTC 7228 or *Saccharomyces cerevisiae* KCCM 1129. When wild *P. stipitis* and *S. cerevisiae* were used, the ethanol productions of 11.2 g/l and 6.9 g/l were produced, respectively. The ethanol productions of 16.6 g/l and 14.6 g/l were produced using *P. stipitis* and *S. cerevisiae* acclimated to high concentration of galactose, respectively. The yields of ethanol fermentation increased to 0.5 and 0.44 from 0.34 and 0.21 using acclimated *P. stipitis* and *S. cerevisiae*, respectively. Therefore, acclimation of yeasts to a specific sugar such as galactose reduced the glucose-induced repression on the transport of galactose.

Keywords: Fermentation, *Gelidium amansii*, thermal acid hydrolysis, enzymatic saccharification, acclimation

Introduction

Seaweed biomass has been regarded as an alternative to fossil fuel. As a renewable and eco-friendly biomass [2, 22], seaweed is a third-generation biomass that can be used for bioenergy production. Seaweed grows quickly, is lignin-free, but is not used as a primary food crop [2, 6]. In particular, *Gelidium amansii*, red seaweed, has the advantage of high carbohydrate contents. *G. amansii* is composed of cellulose and agar (galactan). Agar is composed of galactose and 3,6-anhydrogalactose (AHG) [7]. In ethanol production using *G. amansii*, thermal acid hydrolysis and enzymatic hydrolysis have been used for the saccharification. The agar is hydrolyzed to galactose and 3,6-AHG by thermal acid hydrolysis. Thermal acid hydrolysis is a simple process that has a short reaction time. Enzymatic saccharification has been used for the saccharification of cellulose in order to overcome the low yield of glucose production [9–11]. Therefore, galactose and glucose are monosaccharides for ethanol fermentation from *G. amansii*. However, glucose of hydrolysates causes the repression of galactose uptake. Because the repression decreases the yield of ethanol production, the repression has to be overcome for successful fermentation. The acclimation of galactose allows simultaneous utilization of glucose and galactose [5]. In many studies on mixed sugar fermentation, the yeast used has been improved by acclimation to a high concentration of sugar for a short time to enhance ethanol production. Therefore, acclimation of galactose is the key process of fermentation when *G. amansii* is used [5, 12, 14].

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

In order to produce ethanol using seaweed as the source material, this study conducted a thermal acid hydrolysis and enzymatic saccharification of *G. amansii*. *Pichia stipitis* has an ethanol yield of 82% and its able to ferment most sugars, including glucose, galactose, and cellobiose. *Saccharomyces cerevisiae* can generate a high yield of ethanol from about 90 % of glucose [16]. Therefore, fermentations were carried out using *Pichia stipitis* and *Saccharomyces*

cerevisiae. The yeasts were acclimated to produce a high concentration of ethanol and minimize the fermentation time by the prevention of repression on galactose uptake.

Materials and Methods

Raw Materials and Composition Analysis

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

Thermal Acid Hydrolysis

Milled *Gelidium amansii* was added to 91 mM H_2SO_4 to make solid/liquid (S/L) contents of 8% (w/v). Then, a thermal acid hydrolysis was carried out in the autoclave at 121°C for 45 min [6, 21]. The efficiency of thermal acid hydrolysis was calculated as follows:

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

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

Enzymatic Saccharification

The enzymatic saccharification of acid hydrolysate was performed by adding 8.4 EGU/ml of Celluclast 1.5 L (Novozyme) and 1.2 FBG/ml of Viscozyme L (Novozyme) with 2% (w/v) sodium azide to inhibit microbial growth during the enzymatic hydrolysis, after adjusting to pH 4.5 with 10 N NaOH [9, 24]. Viscozyme L has endo-beta-glucanase that hydrolyzes (1,3)- or (1,4)-linkages in beta-D-glucans with side activities of xylanase, cellulase, and hemicellulose. Celluclast 1.5 L has cellulase that hydrolyzes (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans.

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

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

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

Fermentation

Seed culture and fermentation medium. Stocked *Pichia stipitis* KCTC 7228 and *Saccharomyces cerevisiae* KCCM 1129 were cultured in YPG agar plates composed of 10 g/l yeast extract, 20 g/l peptone, 20 g/l galactose, and 15 g/l agar for 24 h [19]. Each colony of yeasts was inoculated with 15 ml of YPG (yeast extract, peptone, and galactose) medium containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l galactose. The mixture was cultured at 30°C, 30 rpm for 24 h. Five milliliters of cultures was transferred to 50 ml of YPG medium and cultured under the same condition. The OD_{600} of *P. stipitis* and *S. cerevisiae* reached 23.6 and 27.3, respectively. The acclimation of *P. stipitis* and *S. cerevisiae* to high concentration of galactose was carried out. Five milliliters of yeasts was inoculated in 50 ml of YPHG (yeast extract, peptone, and high concentration of galactose) medium composed of 10 g/l yeast extract, 20 g/l peptone, and 120 g/l galactose, and cultured for 18 h until the OD_{600} of *P. stipitis* and *S. cerevisiae* reached 26.2 and 24.8 [19]. Each cell of 25 ml was centrifuged at 3000 rpm for 5 min to remove the YPG medium and cell pellets were inoculated, respectively.

Separated hydrolysis and fermentation. The fermentation was carried out in 500 ml flasks with a working volume of 250 ml [17]. After enzymatic saccharification and final neutralization to pH 6.4 were carried out, fermentations were carried out with acclimated and non-acclimated yeasts *P. stipitis* and *S. cerevisiae.* The fermentation was carried out at 30°C, 30 rpm for 96 h. The efficiency of ethanol yield was calculated as follows:

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

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

Analytical Methods

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

Results and Discussion

Composition of *G. amansii*

G. amansii is a red alga and has the highest carbohydrate contents among seaweeds [11]. The analytical results indicated that the total carbohydrate content was 74.4% (w/w). Agar and fiber in the total carbohydrate were 62.8% (w/w) and 11.6% (w/w), respectively. The ratio of galactose

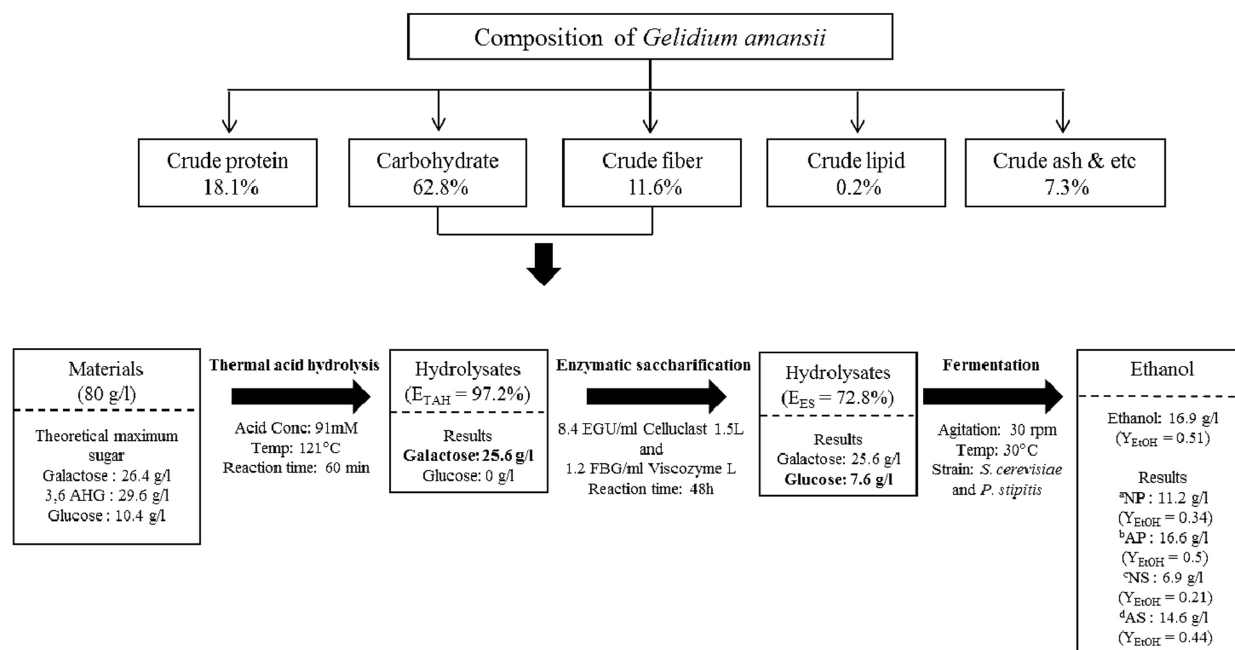

Fig. 1. Compositional analysis data and mass balance flow chart of bioethanol production process from *Gelidium amansii*.
(E_{TAH}, efficiency of thermal acid hydrolysis; E_{ES}, efficiency of enzymatic saccharification; Y_{EtOH}, efficiency of ethanol yield; [a]NP, non-acclimated *P. stipitis*; [b]AP, acclimated *P. stipitis*; [c]NS, non-acclimated *S. cerevisiae*; [d]AS, acclimated *S. cerevisiae*).

to 3,6-anhydrogalactose was reported as 1:1.13 (0.47:0.53) [8]. Therefore, the maximum galactose content was calculated as 33%: (0.628 g agar/g *G. amansii*) × (0.47 g galactose unit/g agar) × (180 g galactose/162 g galactose unit) × 100. The maximum glucose content was calculated as 13%: (0.116 g cellulose/g *G. amansii*) × (180 g glucose/162 g unit of cellulose) × 100. The total initial fermentable sugar content was calculated as 46% of *G. amansii*. Therefore, the maximum galactose concentration can reach 26.4 g/l: (80 g *G. amansii* /l liquid) × (0.33 g galactose/g *G. amansii*); and glucose concentration can reach 10.4 g/l: (80 g *G. amansii* /l liquid) × (0.13 g glucose/g *G. amansii*) from 80 g/l of slurry [19]. Moreover, the components in *G. amansii* were composed of 18.1% (w/w) crude protein, 0.2% (w/w) crude lipid, and 7.3% (w/w) crude ash, as shown in Fig. 1.

Thermal Acid Hydrolysis

The agar can be hydrolyzed by acid, but cannot be hydrolyzed by the enzyme [7]. Sulfuric acid of 91 mM was added to 8% (w/v) slurry of *G. amansii* and treated at 121°C for 45 min. As a result, the galactose concentration of 25.6 g/l was obtained and the E_{TAH} was 97.2%, as shown in Fig. 1. Because the physical morphology of the agar is softer than that of cellulose, the optimal reaction conditions for thermal acid hydrolysis would be milder than that of cellulose [15]. Therefore, the glucose was not found in the thermal acid hydrolysis. When thermal acid hydrolysis was used, 5-hydroxymethyl furfural (5-HMF) was generated from the degradation of 3,6-anhydrogalactose owing to its acid-labile character [11]. In this study, 4.8 g/l of 5-HMF as inhibitor in the fermentation process was found in the thermal acid hydrolysis. However, according to a previous study, a 5-HMF concentration above 5 g/l shows the crucial role of inhibiting the fermentation process when using red seaweed hydrolysates [11, 20]. Therefore, the 5-HMF concentration of thermal acid hydrolysis did not affect the fermentation process.

Enzymatic Saccharification

For the hydrolysis of fiber, Celluclast 1.5 L, Viscozyme L, and mixed enzymes of Celluclast 1.5 L and Viscozyme L were used. After a thermal acid hydrolysis, enzymatic saccharification was carried out at pH 4.5, 130 rpm, 45°C for 60 h, as shown in Fig. 2. The glucose was released until 48 h after the addition of enzymes. Specifically, the mixed enzymes (Celluclast 1.5 L + Viscozyme L) produced 7.6 g/l and 7.8 g/l glucose at 48 and 60 h, respectively. In the case of Celluclast 1.5 L treatment, the glucose was released until 48 h of saccharification. The final glucose concentration of 5.5 g/l with E_{ES} of 52.9% was obtained. When Viscozyme L

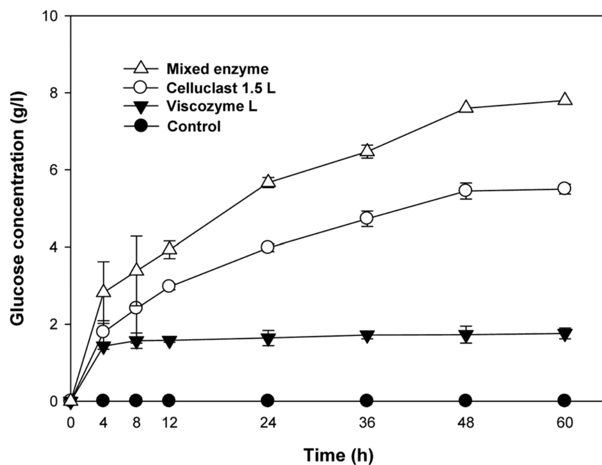

Fig. 2. Effects of various enzymes on the production of glucose from *G. amansii* by enzymatic saccharification at 45°C, 30 rpm for 60 h.

was treated, the glucose concentration of 1.7 g/l with E_{ES} of 16.3% was obtained at 4 h. Enzyme saccharification of Celluclast 1.5 L to *G. amansii* hydrolysate was preferable to that of Viscozyme L. Among those treatment methods, the mixed enzyme treatment showed a synergistic effect and the maximum efficiency of enzymatic saccharification [20]. Therefore, mixed enzymes were used as an optimal saccharification of fiber to glucose for 48 h, and the glucose concentration of 7.6 g/l with E_{ES} = 72.8% was obtained, as shown in Fig. 1.

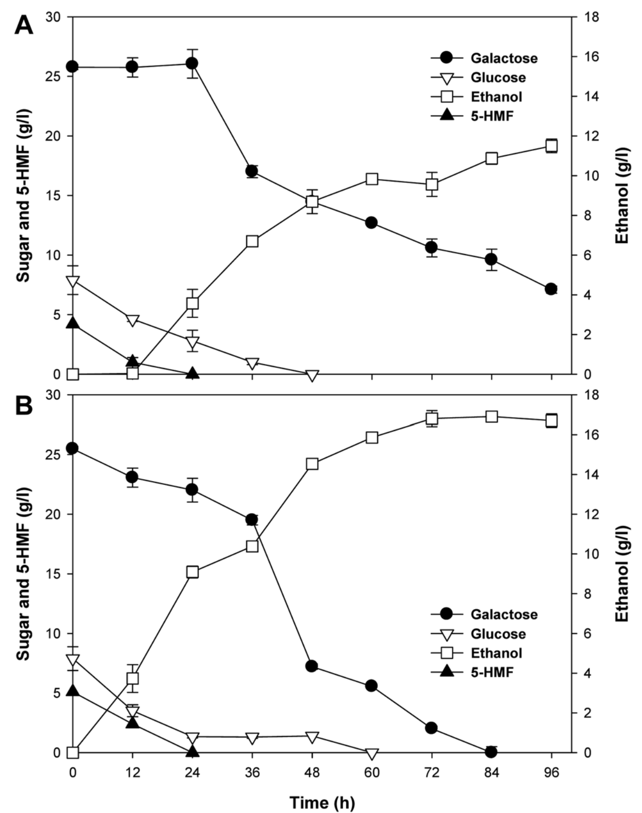

Fig. 3. Ethanol production from hydrolysis of *G. amansii* by SHF with *P. stipitis* KCTC 7228.

(**A**) Non-acclimated *P. stipitis* and (**B**) acclimated *P. stipitis* to high concentration of galactose.

Separated Hydrolysis and Fermentations

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

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

The fermentation with non-acclimated *S. cerevisiae* to high concentration of galactose produced ethanol concentration of 6.9 g/l with Y_{EtOH} = 0.21 for 96 h, as shown in Fig. 4A. As the result of saccharification, 25.6 g/l galactose was hydrolyzed by thermal acid hydrolysis and 7.6 g/l glucose was obtained by enzymatic hydrolysis. *S. cerevisiae* preferred glucose to galactose. The glucose consumption rate of *S. cerevisiae* with 0.33 was faster than that of *P. stipitis* with 0.16, as shown in Fig. 4A. The glucose was consumed in 24 h; however, galactose was rarely consumed because of the repression of galactose uptake by glucose. Since glucose directly enters glycolysis, which is the main metabolic pathway in ethanol fermentation, glucose is taken by yeasts in preference to galactose (requiring conversion to glucose prior to the use for the glycolysis) [2]. Therefore, the ethanol production from the mixture of galactose and glucose was inhibited owing to the glucose repression to galactose consumption. The utilization of galactose by

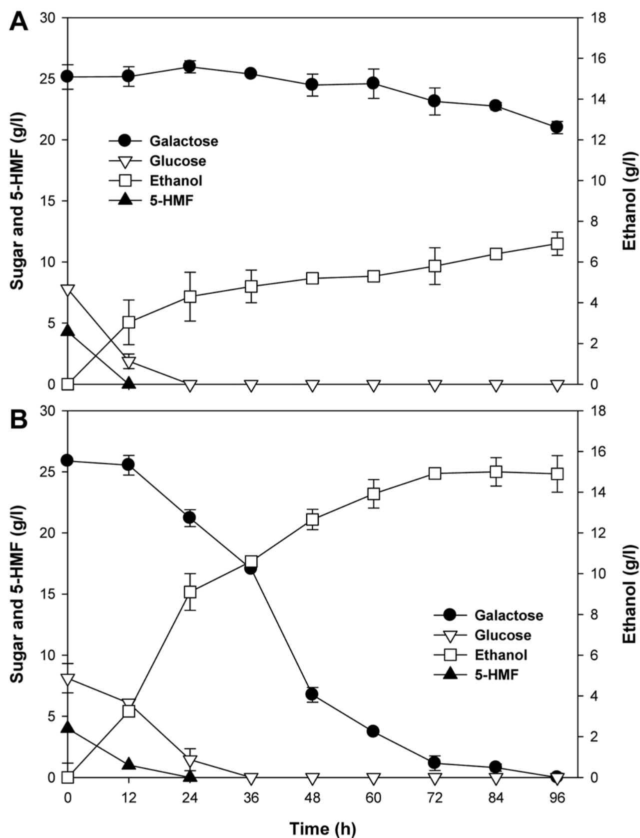

Fig. 4. Ethanol production from hydrolysis of *G. amansii* by SHF with *S. cerevisiae* KCCM 1129.

(**A**) Non-acclimated *S. cerevisiae* and (**B**) acclimated *S. cerevisiae* to high concentration of galactose.

yeast requires the enzymes of the Leloir pathway, which catalyze the transformation of galactose to glucose-6-phosphate. These enzymes are encoded by a family of the GAL gene, and their expression is induced by growth in galactose and repressed during growth in glucose. When galactose is absent from the medium, the GAL gene inhibits the function of the transcriptional activator. Therefore, the acclimation of yeasts to galactose diminishes the repression of galactose consumption [3, 4]. The fermentation with acclimated *S. cerevisiae* utilized 25.8 g/l galactose and 8.1 g/l glucose, as shown in Fig. 4B. The galactose was rarely consumed owing to the repression of non-acclimated *S. cerevisiae*, as shown in Fig. 4A. However, when *S. cerevisiae* was acclimated to high concentration of galactose, the glucose and galactose were simultaneously consumed, as shown in Fig. 4B. Diauxic fermentation was observed in acclimated *S. cerevisiae* on galactose. Fermentation using glucose produced ethanol for 24 h. When glucose was exhausted, ethanol fermentation had temporarily slowed down, resulting in a lag that is called the diauxic shift, from 24 to 36 h. After a short lag at the diauxic shift, the galactose was totally consumed for 96 h [22]. An ethanol concentration of 14.6 g/l with Y_{EtOH} = 0.44 was obtained from 25.6 g/l galactose and 7.6 g/l glucose, as shown in Fig. 4B. As results, the overall mass balance and ethanol yields are described in Fig. 1. Therefore, the acclimation of yeasts to high concentration of galactose could make simultaneous utilization of galactose and glucose for the production of ethanol from the seaweed *G. amansii*. Acclimated yeasts produced a higher ethanol concentration than non-acclimated yeasts.

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