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RESEARCH PAPER

Optimization of Pretreatment Conditions and Use of a Two-stage Fermentation Process for the Production of Ethanol from Seaweed, *Saccharina japonica*

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Abstract *Saccharina japonica* (Sea tangle, Dasima), a seaweed, was fermented in order to produce bioethanol after thermal hydrogen peroxide (H_2O_2) hydrolysis pretreatment and enzymatic saccharification. The optimal pretreatment conditions of 1% (v/v) H_2O_2 (28%, Dustan Pure Chemicals Co., Ltd, Ansan, Korea) and 10% (w/v) seaweed slurry at 121°C for 60 min were determined using the Response Surface Method (RSM). A reducing sugar yield of 33.4% (w/w) and a viscosity of 520 cP were obtained. Enzymatic saccharification was then carried out; a monosaccharide concentration of 28.5 g/L with a 40.5% (w/w) theoretical yield was obtained after the addition of 2-mL Celluclast® 1.5L to 100 g/L of seaweed slurry after thermal H_2O_2 hydrolysis. Fermentation of a two-stage ethanol production was carried out using *Saccharomyces cerevisiae* KCCM 1129 in order to ferment glucose in the first stage, and a high level of mannitol-acclimated *Pichia angophorae* KCTC 17574 to ferment mannitol in the second stage. Acclimation of yeast effectively slowed the uptake of sugar in ethanol fermentation. The overall ethanol yield from *S. japonica* after the two-stage fermentation was 9.9 g/L.

Keywords: two-stage fermentation, ethanol, thermal H_2O_2 hydrolysis, enzymatic saccharification, *Saccharina japonica*

1. Introduction

The development of renewable bio-energy sources is an important step towards a sustainable-energy future. Ethanol is already a popular energy source in Brazil and is increasingly employed in the U. S. and Europe. Indeed, ethanol is expected to be one of the predominant renewable biofuels used in the transportation sector within 20 years [1,2].

Seaweed is considered a third-generation biomass for bioethanol production [3]. It grows quickly, is lignin-free, and it is not used as a primary food crop. Seaweeds, particularly *Saccharina japonica* (Sea tangle, Dasima), *Undaria pinnatifida* (Sea mustard, Miyuk) and *Porphyra* species (Purple laver, Gim), are cultured extensively in Korea; further, a well-established marine culture system is in place. Brown seaweeds have a high content of easily degradable carbohydrates, making them a potential source for the production of liquid fuels. Carbohydrates in brown seaweed comprise mainly of alginate, laminaran and mannitol. Mannitol and glucose from laminaran can be fermented to produce ethanol, and these sugars can be hydrolyzed from milled brown seaweed. Thus, *Saccharina japonica* was selected as the biomass for ethanol production in this study.

Several methods may be used for biomass pretreatment. The most widely used are thermal acid treatments, such as those using dilute acid and ammonia [4]. Hydrogen peroxide (H_2O_2) pretreatment of seaweed can also be used to improve the digestibility of seaweed biomass; H_2O_2 opens the structure of *S. japonica* prior to enzymatic saccharification, thereby facilitating the efficient production of sugars *via* enzymatic saccharification. No measurable production of furfural and hydroxymethylfurfural (HMF) is detected when H_2O_2 pretreatment is employed. Thus, H_2O_2 pretreatment is more favorable than that using dilute acid [5,6].

In this study, a response surface methodology (RSM)

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was used to optimize the pretreatment conditions as well as to assess the influence of pretreatment on the saccharification of *S. japonica*. Ethanol was produced using a two-stage fermentation process with one of two yeasts and was optimized in terms of maximum ethanol yield. The yeasts used were *Saccharomyces cerevisiae* KCCM 1129 and *Pichia angophorae* KCTC 17574. Ethanol production was evaluated in terms of total sugar utilization in the two-stage fermentation process. Using the optimized process, a higher ethanol yield was obtained from *S. japonica* compared to that using the traditional fermentation process.

2. Materials and Methods

2.1. Raw materials

Saccharina japonica (Sea tangle, Dasima) was obtained from Gijang Local Products Co., Ltd in Busan, Korea. The seaweed was dried in sunlight or under hot air and then ground using a hammer mill. Seaweed powder was filtered through a 200-mesh sieve prior to pretreatment. The composition and proximate analysis of *S. japonica* were carried out by the Feed and Foods Nutrition Research Center at Pukyong National University in Korea, according to the procedures recommended by AOAC [7].

2.2. Optimization of the pretreatment procedure

The experimental data were analyzed according to the response surface regression procedure in order to fit the following second-order polynomial equation for each response variable:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

in which Y is the predicted response, $X_i X_j$ are the independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient. Thus, response surface experiments were designed to evaluate the relationship between three independent variables [H_2O_2 concentration (%, v/v medium, X_1), thermal hydrolysis time (min, X_2), slurry content (%, w/v medium, X_3)] and two dependent variables [reducing sugar yield (%) and viscosity (cP)]. The quality of fit for the polynomial model equation was expressed as the coefficient of determination (R^2). All statistical calculations were performed with RSM using SAS ver. 9.1 (SAS Institute, Cary, USA) [8].

2.3. Analytical methods

Reducing the sugar yields were determined according to RSM by the 3,5-dinitrosalicyclic acid (DNS) method with glucose (Sigma-Aldrich, Panama, USA) as the standard. Viscosity was measured using a Brookfield viscometer (BROOKFIELD DV-III Rheometer v3.1, Brookfield Eng. Inc., Middleboro, USA) equipped with spindles ULA, SC4-18, and SC4-34 at a temperature of 30°C [9]. Monosaccharide and organic acid concentrations in the fermentation samples were determined using high-performance liquid chromatography (HPLC) (Agilent 1100 Series, Agilent. Inc., Santa Clara, USA) equipped with a refractive index detector (RID). A Bio-Rad Aminex HPX-87H column (300.0 × 7.8 mm) was used with degassed 5 mM sulfuric acid at a flow rate of 0.6 mL/min and a temperature of 65°C.

2.4. Enzymatic saccharification

The *S. japonica* seaweed medium was thermally pretreated with H_2O_2 at 121°C for 60 min. Enzymatic saccharification was carried out after thermal H_2O_2 hydrolysis pretreatment. The influence of reaction conditions with various enzyme concentrations at constant slurry content (10%, w/v) are shown in Table 2. Commercial enzymes Viscozyme® L with final concentration of 1.2 FBG/mL (Beta-glucanase, Novozymes, Bagsvaerd, Denmark) and Celluclast® 1.5L with final concentration of 8.4 EGU/mL (Endo-glucanase, Novozymes, Bagsvaerd, Denmark) were used for enzymatic saccharification. Single and mixed enzymes (1 ~ 3 mL) were added to 100 g/L of seaweed slurry at 45°C and 150 rpm on a rotary shaker (Vision Co., Daejeon, Korea). After 48 h, the sugar concentrations of enzymatic hydrolysates were assessed by HPLC [10].

2.5. Two-stage ethanol fermentation

A two-stage fermentation process was used to maintain the optimum sugar consumption values during ethanol fermentation. The synergy of the two-stage process is due to maximum utilization of the source biomass, which is otherwise not completely converted to ethanol due to the narrow range of yeasts for ethanol production [11,12]. *Saccharomyces cerevisiae* KCCM 1129 and *Pichia angophorae* KCTC 17574 were used as glucose- and mannitol-fermenting yeasts, respectively. For acclimation to mannitol utilization, *P. angophorae* KCTC 17574 was cultured for 24 h in 30 mL YP-Man medium containing 20 g/L peptone, 10 g/L yeast extract, and 50 g/L mannitol (first seed culture). Next, 5% of the inoculation were transferred into a 250 mL Erlenmeyer flask containing 150 mL of the same medium and was held for 24 h (second seed culture). Cells were harvested during the late logarithmic growth phase by centrifugation at 3,000 × *g* and 4°C for 5 min; the collected cells were used as the inoculums to the main culture of the 5 L fermentor. The first stage of the two-stage fermentation process was carried out by inoculating 0.27-g dcw/L of the glucose-consuming yeast *S. cerevisiae* KCCM 1129 into the *S. japonica* culture under anaerobic

conditions. *P. angophorae* KCTC 17574 was not able to strictly carry out the anaerobic fermentation of mannitol. Mannitol which is the sugar alcohol corresponding to mannose is not readily fermented. It is oxidized to fructose by mannitol dehydrogenase, a reaction that generates NADH. Regeneration of NAD^+ requires oxygen (active electron transport chain) or transhydrogenase, which converts NADH to NADPH. Thus, the fermentation of sugar alcohols as mannitol by yeast requires a supply of oxygen in low concentration [13]. When glucose was consumed completely, 0.29-g dcw/L of mannitol-acclimated *P. angophorae* KCTC 17574 was added under aerobic conditions for ethanol production from mannitol.

3. Results and Discussion

3.1. Determination of optimal conditions in terms of reducing sugar yield and viscosity

RSM was employed to determine the optimal pretreatment conditions (Table 1). The significant variables H_2O_2 concentration (X_1), thermal hydrolysis time (X_2) and slurry content (X_3) were assessed using a central composite design, and interactions with the reducing sugar yield and viscosity were determined. A total of 17 experiments, including three center points, were carried out using different combinations of these factors. The reported response values (Y_1 and Y_2) are the averages of triplicate measurements.

The regression coefficients were calculated, and the data were fitted to a second-order polynomial equation. The reducing sugar yield (Y_1) and viscosity (Y_2) were expressed in terms of the following regression equations:

$$Y_1 = -37.994 + 35.769\,X_1 + 1.364\,X_2 + 3.024\,X_3 - 15.524\,X_1^2 - 0.007\,X_2^2 - 0.148\,X_3^2 - 0.188\,X_1X_2 + 0.740\,X_1X_3 - 0.024\,X_2X_3$$

$$Y_2 = 22309 - 108828\,X_1 - 278.808\,X_2 + 10179\,X_3 + 42499\,X_1^2 - 1.391\,X_2^2 + 203.82\,X_3^2 + 1208.85\,X_1X_2 - 6192.25\,X_1X_3 - 120.98\,X_2X_3$$

Table 1. Central composite design of RSM experiments for the optimization of three variables in terms of reducing sugar yield and *S. japonica* slurry viscosity

Design point	Independent variable[a]			Dependent variable	
	H_2O_2 (%, v/v)	Thermal hydrolysistime (min)	Slurry content (%,w/v)	Yield of reducing sugar[b] (Y_1, %)	Viscosity[c] (Y_2, cP)
	X_1	X_2	X_3		
1	1(1.5)	1(60)	1(15)	24.63	581
2	1(1.5)	1(60)	-1(5)	25.34	153
3	1(1.5)	-1(30)	1(15)	21.63	455
4	1(1.5)	-1(30)	-1(5)	19.04	123
5	-1(0.5)	1(60)	1(15)	20.44	26,635
6	-1(0.5)	1(60)	-1(5)	32.76	677
7	-1(0.5)	-1(30)	1(15)	16.09	99,167
8	-1(0.5)	-1(30)	-1(5)	16.57	520
9[d]	0(1)	0(45)	0(10)	31.33	653
10	α(1.7)	0(45)	0(10)	30.20	177
11	-α(0.3)	0(45)	0(10)	18.06	43,884
12	0(1)	α(66)	0(10)	33.46	519
13	0(1)	-α(24)	0(10)	23.35	667
14	0(1)	0(45)	α(17.07)	21.45	22,615
15	0(1)	0(45)	-α(2.93)	27.13	159
16[d]	0(1)	0(45)	0(10)	31.33	510
17[d]	0(1)	0(45)	0(10)	31.33	512

[a]Experimental codes, ranges, and levels of the independent variables:
X_1= H_2O_2 (%, v/v) [-α = 0.3%, -1 = 0.5%, 0 = 1%, 1 = 1.5%, α = 1.7%]
X_2= Thermal hydrolysis time (min) [-α = 24 min, -1 = 30 min, 0 = 45 min, 1 = 60 min, α = 66 min]
X_3= Slurry content (%, w/v) [-α = 2.93%, -1 = 5%, 0 = 10%, 1 = 15%, α = 17.07%]
The second-order polynomial equations (reducing sugar yield[b] and viscosity[c]):
$Y_1 = -37.994 + 35.769\,X_1 + 1.364\,X_2 + 3.024\,X_3 - 15.524\,X_1^2 - 0.007\,X_2^2 - 0.148\,X_3^2 - 0.188\,X_1X_2 + 0.740\,X_1X_3 - 0.024\,X_2X_3$, $R^2 = 0.9138$, *F-Value* = 8.25, *Probability of F* = 0.0055
$Y_2 = 22309 - 108828\,X_1 - 278.808\,X_2 + 10179\,X_3 + 42499\,X_1^2 - 1.391\,X_2^2 + 203.82\,X_3^2 + 1208.85\,X_1X_2 - 6192.25\,X_1X_3 - 120.98\,X_2X_3$, $R^2 = 0.9031$, *F-Value* = 7.25, *Probability of F* = 0.0080
[d]Central points.

Table 2. Influence of reaction conditions on monosaccharide yield from *S. japonica* slurry by enzymatic saccharification*

Chemical treatment (Monosaccharide, g/L)		Enzymes (Activity)	Enzyme treatment (Monosaccharide, g/L)		
Before	After		After		
Carbohydrate of *S. japonica* (100 g/L)	Thermal H_2O_2 hydrolysis		Enzyme 1 mL/100 g slurry	Enzyme 2 mL/100 g slurry	Enzyme 3 mL/100 g slurry
59.7	11.3	1.2 FBG/mL Viscozyme L (Beta-glucanase)	22.3	23.3	25.6
59.7	11.8	8.4 EGU/mL Celluclast 1.5L (Endo-glucanase)	27.6	28.5	27.8
59.7	12.1	Celluclast® 1.5L, Viscozyme® L	29.2	29.8	29.7

*The medium contained hydrolysates from 100 g of *S. japonica* slurry (dried biomass) per L. For pretreatment, the slurry (10%, w/v) was treated with 1% H_2O_2 (%, v/v) at 121°C for 60 min. Single and mixed enzymes were added at levels of 1 ~ 3 mL in 100 g/L of seaweed slurry at 45°C and 150 rpm for 48 h.

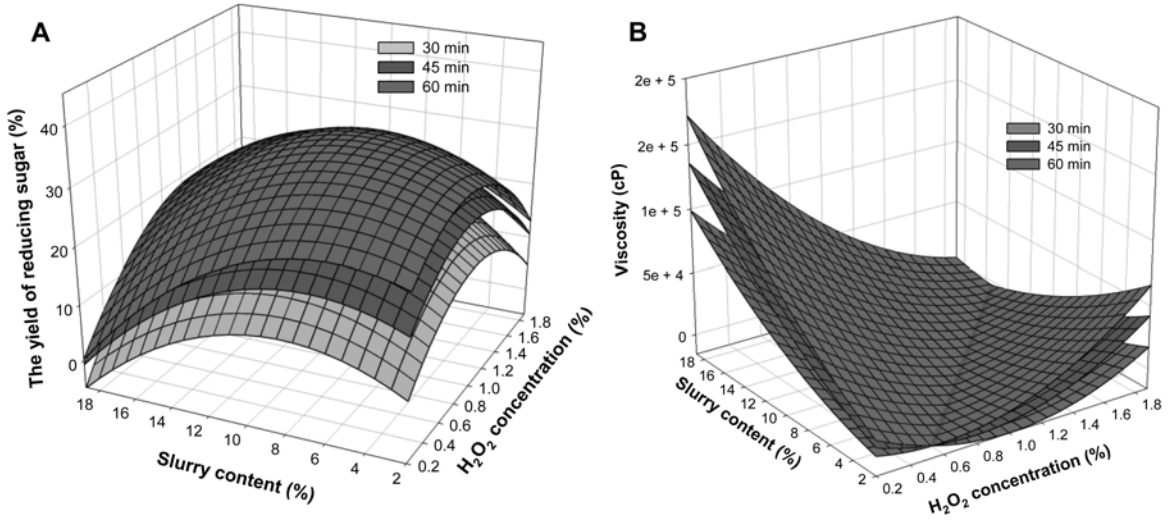

Fig. 1. Response surface showing the effects of *S. japonica* slurry content, H_2O_2 concentration, and thermal hydrolysis time on (A) reducing sugar yield, and (B) viscosity.

The regression equations generated using SAS ver. 9.1 (SAS Institute, Cary, USA) indicated that the R^2 (multiple correlation coefficient) values for the reducing sugar yield and the decrease in viscosity were 0.9138 and 0.9031, respectively (Table 1). The response surface plots according to the second-order polynomial equation are shown in Fig. 1. In the evaluation of optimal conditions, H_2O_2 concentrations were 0.2 ~ 1.8% (v/v), durations of thermal hydrolysis were 30 ~ 60 min, and slurry contents were 2 ~ 18% (w/v). The optimal conditions for slurry content and H_2O_2 concentration at various thermal hydrolysis times are presented in Fig. 1A. The reducing sugar yield increased with the thermal hydrolysis time. However, energy consumption also increased with thermal hydrolysis time [9]. Therefore, the conditions that resulted in the maximum reducing sugar yield were an H_2O_2 concentration of 1% (v/v), a thermal hydrolysis time of 60 min, and a slurry content of 10% (w/v). Viscosity was an important parameter for ethanol fermentation and was related to slurry content, as shown in Fig. 1B. High slurry content resulted in high viscosity, engendering the fermentation mixture difficult to handle [11]. The viscosity decreased from 99,167 to 520 cPs when the slurry content and H_2O_2 concentration were decreased to 10% (w/v) and 1% (v/v), respectively. Based on the RSM experiments, the optimal pretreatment conditions for obtaining a reducing sugar yield of 33.4% and a viscosity of 520 cP were 1% (v/v) H_2O_2 and 10% (w/v) slurry with a 60 min thermal hydrolysis time.

3.2. *S. japonica* composition and enzymatic saccharification

The composition of *S. japonica* was 59.7% carbohydrate, 6.3% crude fiber, 10.6% crude protein, 1.6% crude lipid, and 21.8% crude ash.

The influence of the reaction conditions on the monosaccharide produced by enzymatic saccharification was evaluated (Table 2). The optimum enzyme reaction time

was 48 h, and an increase in reaction time up to 72 h had no significant effect on saccharification efficiency (data not shown). Thermal H_2O_2 hydrolysis increased monosaccharide formation, as portrayed in Table 2. Thus, H_2O_2 pretreatment was effective for thermal hydrolysis [5,6]. A monosaccharide content of 29.8 g/L was obtained when both Viscozyme® L and Celluclast® 1.5L were added to the pretreated slurry. A minimal difference in the monosaccharide conversion between single (Celluclast® 1.5L) and mixed enzyme (Celluclast® 1.5L and Viscozyme® L) treatment was observed; thus, a single-enzyme treatment with 2-mL Celluclast® 1.5L to 100 g/L of the seaweed slurry was preferable. A total of 28.5 g/L monosaccharide was produced with a 40.5% theoretical yield.

3.3. Ethanol production by a two-stage fermentation process

The two-stage fermentation was carried out at 30ºC and 150 rpm using a 5-L fermenter (KF-5, KFC, Incheon, Korea) with a 3-L working volume. Fermentation profile with mixed sugars was shown in Fig. 2. First stage fermentation with *S. cerevisiae* KCCM 1129 and a second stage with *P. angophorae* KCTC 17574 were carried out in order to produce ethanol in the two-stage fermentation. Glucose was converted to ethanol with a yield of 0.41 g ethanol/g glucose by *S. cerevisiae* KCCM 1129, with a glucose consumption rate of 0.2 g/h under anaerobic conditions in the first stage, as shown in Fig. 2A. However, in the second stage, mannitol was consumed at a low rate, and only 0.13 g ethanol/g mannitol was produced by non-acclimated *P. angophorae* KCTC 17574. Mannitol consumption rate of 0.03 g/h ceased after 108 h, with 10.3 g/L mannitol still remaining in the medium. The uptake of mannitol during ethanol production can be increased using acclimated yeast to the mannitol and supplying oxygen to the system. Acclimation of *P. angophorae* KCTC 17574 was carried out using a high concentration of mannitol for 24 h. During the second stage of the fermentation, aerobic conditions were maintained by supplying air into the culture broth at 0.2 vvm. However, further increase in an aeration rate over 0.2 vvm adversely resulted in a low concentration of ethanol production (data not shown). Therefore, aeration rate of 0.2 vvm was chosen as the optimal condition.

The maintenance of optimum conditions for ethanol production from glucose by *S. cerevisiae* KCCM 1129 under anaerobic conditions, followed by the addition of mannitol-acclimated *P. angophorae* KCTC 17574 under aerobic conditions in the second-stage are shown in Fig. 2B. In the first stage of fermentation, a yield of 0.41 g ethanol/g glucose by *S. cerevisiae* KCCM 1129 was obtained. Glucose consumption rate was 0.2 g/h. In the second stage,

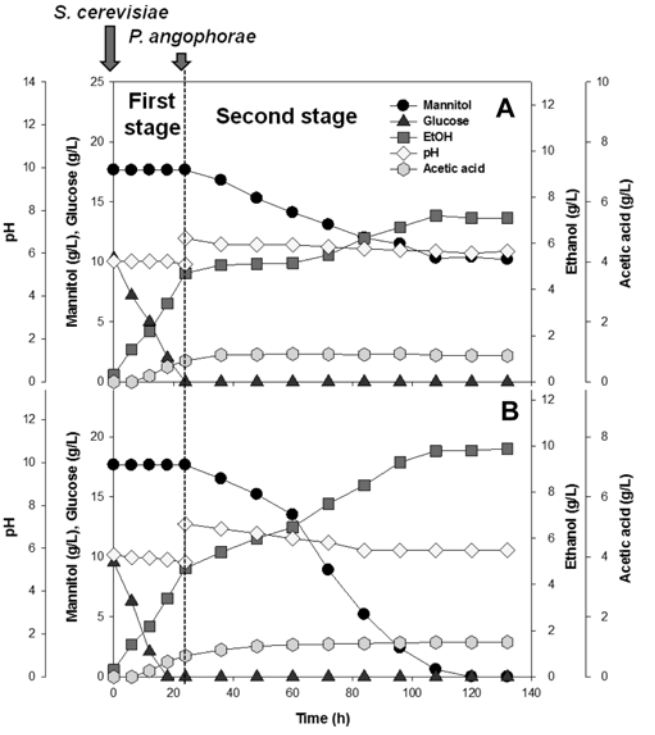

Fig. 2. Ethanol production from 10% (w/v) *S. japonica* slurry by *S. cerevisiae* KCCM 1129 and *P. angophorae* KCTC 17574 in a two-stage fermentation process at 30ºC and 150 rpm for 108 h by (A) non-acclimated *P. angophorae* KCTC 17574 and (B) *P. angophorae* KCTC 17574 acclimated to a high concentration of mannitol.

acclimated *P. angophorae* KCTC 17574 utilized mannitol to produce 5.2 g/L of ethanol with a yield of 0.30 g ethanol/g mannitol. Mannitol was consumed sequentially after glucose consumption. Mannitol consumption rate was 0.06 g/h using acclimated *P. angophorae* KCTC 17574. The productivity of acclimated *P. angophorae* KCTC 17574 (1.49 g ethanol/L/day) was higher than that of non-acclimated *P. angophorae* KCTC 17574 (0.72 g ethanol/L/day). Jang [9] reported that an ethanol production of 7.7 g/L was obtained by *P. angophorae* KCTC 17574 from *S. japonica.* However, the acclimation of *P. angophorae* KCTC 17574 to a high concentration of a specific sugar (mannitol) improved sugar utilization and also led to 9.9 g/L of ethanol production in the two-stage fermentation.

4. Conclusion

Thermal H_2O_2 hydrolysis of *S. japonica* was optimized in terms of reducing sugar production and viscosity decrease using RSM. Monosaccharides at a level of 28.5 g/L with a 40.5% theoretical yield were obtained *via* enzymatic saccharification of pretreated *S. japonica* slurry with 2-mL

Celluclast® 1.5L in 100 g/L of seaweed slurry. The two-stage fermentation using yeast *S. cerevisiae* KCCM 1129 and mannitol-acclimated *P. angophorae* KCTC 17574 was conducted, resulting in the production of 9.9 g ethanol/L. These results suggest that the use of acclimated yeast is effective for the fermentation of mixed sugars from hydrolyzed *S. japonica*.

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