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High-level ethanol production from sago residue hydrolysate prepared with thermostable α-amylase and amyloglucosidase

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Abstract: Sago residue after starch extraction contains a considerable amount of starch. In this study, we aimed to develop a simple and effective method for producing bioethanol from sago residue. Starch in sago residue was efficiently liquefied with thermostable α-amylase at 90 °C using a mass ratio of sago residue to α-amylase solution of 1:6. The liquefied solution (approximately 100 g/L sugar) was subjected to both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes to produce ethanol. In SHF, glucose prepared with α-amylase and amyloglucosidase was almost consumed by yeast after 36 h of fermentation at 37 °C, and 34.2 g/L ethanol was produced with a yield of 66.0%. In SSF, 43.2 g/L ethanol was obtained with a yield of 86.4% after 72 h of saccharification and fermentation at 37 °C using the liquefied solution with added amyloglucosidase and yeast. This has been the highest ethanol concentration ever reported in bioethanol production from sago residue.

Keywords: amyloglucosidase, ethanol fermentation, sago residue, thermostable α-amylase, yeast

Introduction

Biofuels produced from renewable resources have attracted attention due to environmental pollution caused by fossil fuels and decreasing petroleum reserves (Demirbas, 2007). Bioethanol, the most common biofuel and an alternative to gasoline, can reduce greenhouse gas emissions. Most bioethanol is currently produced from corn grain (starch) and sugarcane (sucrose); however, competition between food and fuel occurs, since they are also food sources (Ho et al., 2014). Therefore, biomasses, such as agricultural residues and energy crops, are considered more favorable feedstocks for bioethanol production (Alvira et al., 2010). Though many studies have been carried out to efficiently produce ethanol from cell wall polysaccharides in these biomass materials, starch in agricultural residues (e.g., rice straw) is also an attractive source of bioethanol (Matsuki et al., 2010).

Producing bioethanol from polysaccharides requires two steps: (i) the conversion of polysaccharides (e.g., starch and cellulose) into monosaccharides (e.g., glucose) by hydrolysis, and (ii) the conversion of monosaccharides into ethanol by the fermentation of microorganisms (e.g., yeast). These two steps are carried out sequentially in the separate hydrolysis and fermentation (SHF) process, whereas fermentation together with enzymatic hydrolysis is performed in the simultaneous saccharification and fermentation (SSF) process. SSF has several advantages in ethanol production over SHF and is considered more time- and cost-effective (Olofsson et al., 2008; Sarkar et al., 2012; Zhu et al., 2012). For example, it has been reported that end-product (sugars) inhibition of some glycoside hydrolases used for polysaccharide hydrolysis is avoided in the SSF process. SSF can also decrease the osmotic pressure caused by a high initial sugar concentration, which is a stress for microorganisms, to yield a higher concentration of ethanol than SHF and contribute to the reduction of distillation costs (Bothast and Schlicher, 2005; Olofsson et al., 2008). In addition, SSF can also reduce the number of

reactors required (Sarkar et al., 2012). Actually, most bioethanol from corn starch is produced by SSF (Bothast and Schlicher, 2005).

Sago starch, one of the commercial starches, is extracted from the pith of the sago palm (*Metroxylon sagu*) (Singhal et al., 2008). The resulting sago residue is fibrous and contains cell wall components (e.g., cellulose, hemicellulose, and lignin); however, a considerable amount of starch (more than 50% on a dry weight basis) remains even after extraction (Abd-Aziz, 2002). Sago residue is generated abundantly and usually discarded into rivers near sago mills, causing river and air pollution (Wan et al., 2016). At present, many studies have attempted to utilize sago residue to produce useful materials, e.g., adsorbents, enzymes, and biodegradable foam (Singhal et al., 2008; Awg-Adeni et al., 2010; Utami et al., 2014).

Starch in sago residue appears an attractive source for the production of bioethanol. Many studies have reported on ethanol production from purified sago starch (Kim et al., 1992; Abd-Aziz et al., 2001; Bandaru et al., 2006; Saifuddin and Hussain, 2011); however, there have been very few reports on ethanol production using sago residue as a feedstock (Wan et al., 2016). Thangavelu et al. (2014) examined carbon dioxide–assisted microwave hydrothermal hydrolysis of sago residue and the subsequent ethanol fermentation. Awg-Adeni et al. (2013) reported the enzymatic hydrolysis of starch within sago residue using amyloglucosidase (glucoamylase) together with pullulanase for bioethanol production by SHF. This method involves cycles of three steps: (i) the heating of sago residue suspension at 80–90 °C to gelatinize starch, (ii) enzymatic hydrolysis at 60 °C, and (iii) the addition of another new sago residue to the enzymatic hydrolysate for the next cycle. They reported that a high concentration of glucose was obtained after three repeated cycles; however, this process, consisting of many steps, seems somewhat complicated. Furthermore, the method cannot be applied to SSF because the gelatinization and hydrolysis of starch occur at higher temperatures than fermentation.

In this study, we aimed to develop a simple and effective method for producing bioethanol from sago residue. We used thermostable α-amylase for the liquefaction of starch within sago residue and amyloglucosidase for saccharification. These enzymes are most generally used for glucose production on an industrial scale (Crabb and Shetty, 1999). Furthermore, we investigated ethanol production in both SHF and SSF using a brewing yeast.

Materials and Methods

1. Materials

Sago residue was obtained and dried as described previously (Utami et al., 2014).

Thermostable α-amylase from *Bacillus licheniformis* was purchased from Sigma-Aldrich (St. Louis, MO, USA). One unit of enzyme activity is defined as the amount of enzyme that liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C.

Amyloglucosidase from *Aspergillus niger* was purchased from Sigma-Aldrich. One unit of enzyme activity is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 3 min at pH 4.5 at 55 °C.

A brewing yeast strain, *Saccharomyces cerevisiae* Kyokai No.7, was obtained from the Brewing Society of Japan (Tokyo, Japan). The strain was precultured at 30 °C for 48 h with shaking at 160 rpm in 3 mL of YPD medium [10 g/L yeast extract (Nacalai Tesque, Kyoto, Japan), 20 g/L peptone (Nacalai Tesque), and 20 g/L glucose].

2. Liquefaction and saccharification

The liquefaction and saccharification of starch in sago residue were performed as follows unless otherwise noted.

Dry sago residue (8 g) was suspended in 48 g of a solution consisting of 20 mM sodium citrate buffer (pH 6.0), 2 mM $CaCl_2$, and 20 units/mL thermostable α-amylase to make the mass ratio of the sago residue to the α-amylase solution 1:6. The mixture was incubated at 90 °C for 3 h for liquefaction. The

liquefied solution (typically 30 mL) was collected by filtration through a filter paper under vacuum. When examining the mass ratio of sago residue to α-amylase solution for efficient liquefaction, the mass of dry sago residue varied in a range of 6–12 g.

Subsequently, 1/20 volume of 1 M sodium citrate buffer (pH 4.5) and 1/100 volume of 300 units/mL amyloglucosidase were added to the filtrate. The mixture was incubated at 50 °C for 72 h for saccharification.

3. Separate hydrolysis and fermentation (SHF)

After saccharification with amyloglucosidase was done as described above, 5.3 mL of the saccharified solution was mixed with 0.25 mL of the seed culture of *S. cerevisiae*. The mixture was incubated at 37 °C for 72 h.

4. Simultaneous saccharification and fermentation (SSF)

After liquefaction with α-amylase was done as described above, 5 mL of the liquefied solution was mixed with 0.25 mL of 1 M sodium citrate buffer (pH 4.5), 0.05 mL of 300 units/mL amyloglucosidase, and 0.25 mL of the seed culture of *S. cerevisiae*. The mixture was incubated at 37 °C for 72 h.

5. Analytical methods

Glucose was measured using a Glucose C2 kit (Wako Pure Chemical Industries, Osaka, Japan). The glucose yield (%) from starch in sago residue after liquefaction and saccharification was calculated as follows:

$$\textit{Glucose yield}\ (\%) = \frac{C \times V \times 100}{M \times 0.63 \times 1.11}$$

where C is the glucose concentration (g/L) of the saccharified solution, V is the volume (L) of the saccharified solution, M is the mass (g) of the sago residue used for liquefaction, 0.63 is the proportion of starch in sago residue, and 1.11 is the conversion factor of starch to glucose.

The total sugar was determined by the phenol-sulfuric acid method, using glucose as the standard (Dubois et al., 1956).

Ethanol was measured using an alcohol densitometer (Alcomate AL-2, Riken Keiki, Tokyo, Japan).

The ethanol yield (%) in fermentation was calculated as follows:

$$\textit{Ethanol yield}\ (\%) = \frac{C_e \times 100}{C_c \times 0.51}$$

where C_e is the final ethanol concentration (g/L) of the culture, C_c is the consumed glucose concentration (g/L) of the culture in the SHF experiment or total sugar concentration (g/L) of the culture in the SSF experiment, and 0.51 is the theoretical ethanol yield.

Results and Discussion

1. Liquefaction of starch in sago residue

The sago residue used in this study consisted of starch (63%), cellulose (11%), hemicellulose (7.6%), lignin (12%), and minor components, as previously determined (Utami et al., 2014). The composition was similar to that reported by Abd-Aziz (2002). In this study, we used thermostable α-amylase and amyloglucosidase to hydrolyze starch within sago residue. A suspension of sago residue containing thermostable α-amylase was heated at 90 °C for 3 h to gelatinize and liquefy the starch, and the resultant filtrate was then incubated with amyloglucosidase to produce glucose.

Initially, we evaluated the effect of the amount of sago residue used for liquefaction on the final glucose concentration after saccharification. The mass ratio of sago residue to α-amylase solution was set from 1:4 to 1:8. Figure 1 shows the glucose concentrations and yields after saccharification at 50 °C by amyloglucosidase. In any case, there was only a slight difference in the glucose concentrations and yields between 24-h and 72-h saccharification, indicating that saccharification was nearly complete in 24 h. High concentrations of glucose (approximately 80–100 g/L) were obtained when the mass ratio was in the range of 1:4–1:6 (Fig.

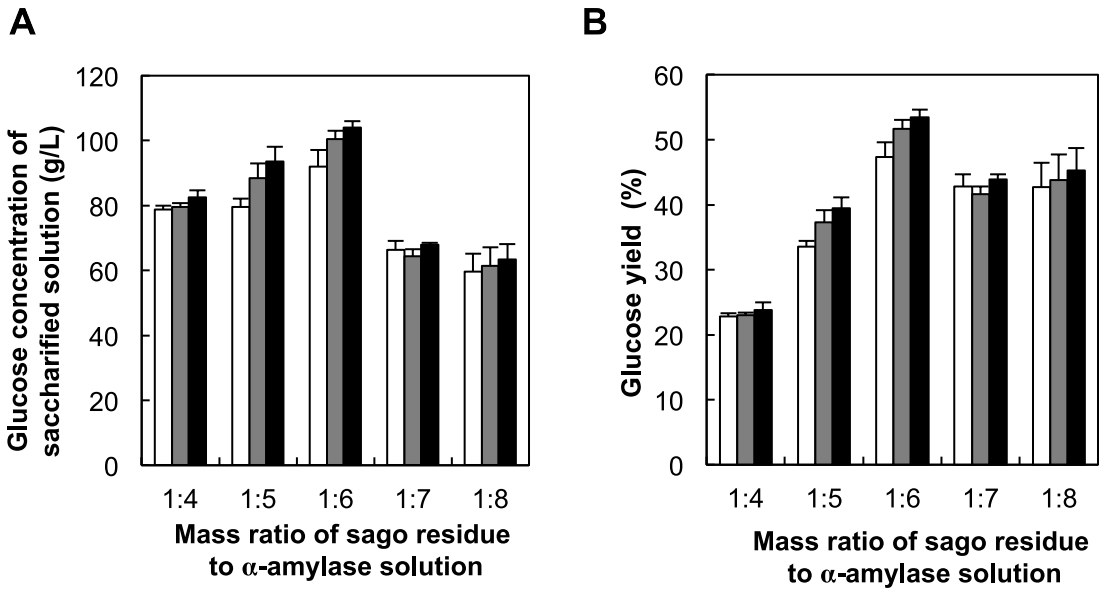

Fig. 1. Liquefaction and saccharification of starch in sago residue
Sago residue and an α-amylase solution were mixed in various mass ratios and incubated at 90 °C for 3 h. The liquefied solution was collected and subjected to saccharification with amyloglucosidase. Saccharification was performed at 50 °C for up to 72 h, and samples were collected at intervals of 24 h. The glucose concentration of saccharified solution (A) and the glucose yield from starch in sago residue after liquefaction and saccharification (B) were measured. White, gray, and black bars indicate the values of 24, 48, and 72 h of saccharification, respectively. The average of three experiments is shown; error bars indicate the standard deviation.

1A). However, the glucose yields were low (20–40%) at mass ratios of 1:4 and 1:5 (Fig. 1B). This may be because fibrous sago residue liquefied by α-amylase holds a significant amount of liquid containing starch hydrolysate even after filtration. It is also possible that high viscosity leads to the incomplete enzymatic hydrolysis of starch, as mentioned by Wang et al. (2008). In contrast, glucose yields were relatively high (approximately 45–55%) when the mass ratio was in a range of 1:6–1:8.

Considering both the concentration and yield of glucose, the optimal mass ratio of sago residue to α-amylase solution for liquefaction was found to be 1:6; this ratio was then used for the following experiments. The glucose concentration after 72 h of saccharification at a mass ratio of 1:6 was the highest (104.0 g/L) of the conditions used in this experiment. The value was smaller than the concentration of glucose obtained from sago residue after three cycles of the hydrolysis process (138.4 g/L) but larger than that after two cycles (73.0 g/L) in the study by Awg-Adeni et al. (2013), where each cycle consisted of the gelatinization of starch within sago residue, the hydrolysis of starch using amyloglucosidase and pullulanase, and the feeding of another new sago residue to the hydrolysate. It was also higher than the reducing sugar solution (46 g/L) produced after 96 h of solid substrate fermentation of sago residue by *Trichoderma* sp. (Shahrim et al., 2008) and glucose solution (33.1 g/L) prepared by the carbon dioxide–assisted microwave hydrothermal hydrolysis of sago residue at 900 W for 2 min (Thangavelu et al., 2014). The glucose yield after 72 h of saccharification at a mass ratio of 1:6 was 53.5%, which is comparable to the yield (52.7%) after three cycles of the hydrolysis process using amyloglucosidase and pullulanase (Awg-Adeni et al., 2013) and higher than the maximum glucose yield (43.8%) obtained by microwave hydrothermal hydrolysis (Thangavelu et al., 2014).

2. SHF from sago residue

The hydrolysate prepared with thermostable α-amylase and amyloglucosidase using the optimal mass ratio of sago residue to α-amylase solution was subjected to fermentation by a commercial brewing

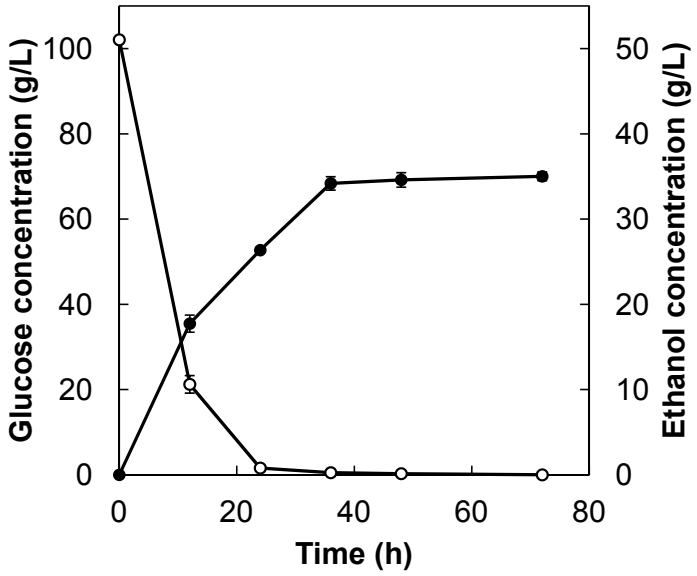

Fig. 2. SHF from sago residue
The saccharified solution prepared with amyloglucosidase was subjected to fermentation by *S. cerevisiae*. Samples were collected at intervals and briefly centrifuged. The glucose concentration (open circle) and ethanol concentration (solid circle) of the supernatant were measured. The average of three experiments is shown; error bars indicate the standard deviation.

yeast. We found that the saccharified solution (102.1 g/L glucose) obtained by the present method did not contain compounds to severely limit fermentation because a considerable amount of ethanol was produced even at an early stage of cultivation. Glucose was almost consumed after 36 h of fermentation at 37 °C, and 34.2 g/L ethanol was produced with a yield of 66.0% (Fig. 2). The yield was not so high, suggesting that glucose was converted to compounds other than ethanol. For example, it is well known that a high sugar concentration causes an increase in the synthesis and accumulation of glycerol by *S. cerevisiae* to compensate for osmotic stress and decreases the production of ethanol (Scanes et al., 1998). On the other hand, the ethanol concentration was higher than the maximum value (15.6 g/L) in SHF from a hydrolysate (33.1 g/L) prepared by microwave hydrothermal hydrolysis of the sago residue (Thangavelu et al., 2014), and was only slightly lower than the maximum value (40.3 g/L) in SHF from a hydrolysate (84.8 g/L) obtained by three cycles of enzyme hydrolysis (Awg-Adeni et al., 2013).

3. SSF from sago residue

SSF was carried out by adding amyloglucosidase and yeast to the filtered solution after liquefaction by thermostable α-amylase. In this SSF experiment, glucose production by amyloglucosidase from the liquefied solution and glucose consumption to generate ethanol by yeast proceeded concurrently. The concentration of total sugar (100.8 g/L) at the beginning of the SSF in the liquefied solution that included maltooligosaccharides and glucose was very similar to the concentration of glucose (102.1 g/L) of the hydrolysate used for the SHF experiment (Fig. 2 and Fig. 3). After 72 h of saccharification and fermentation at 37 °C, 43.2 g/L ethanol was produced with a yield of 86.4% (Fig. 3). Although ethanol production in SSF proceeded more slowly than that in SHF, the ethanol concentration and yield were higher. The ethanol concentration has been the highest ever reported of bioethanol production from sago residue.

Conclusion

We found that the starch in sago residue can be efficiently liquefied with thermostable α-amylase at 90 °C using a mass ratio of sago residue to α-amylase

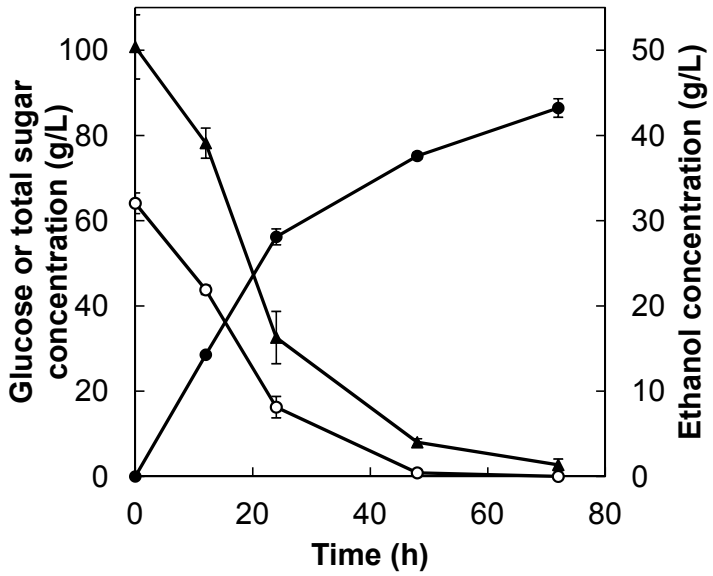

Fig. 3. SSF from sago residue
The liquefied solution prepared with α-amylase was subjected to simultaneous saccharification with amyloglucosidase and fermentation by *S. cerevisiae*. Samples were collected at intervals and briefly centrifuged. The glucose concentration (open circle), ethanol concentration (solid circle), and total sugar concentration (solid triangle) of the supernatant were measured. The average of three experiments is shown; error bars indicate the standard deviation.

solution of 1:6. This hydrolyzing method is very simple and can be applied to SSF as well as SHF to produce ethanol. In this study, we did not examine culture factors that affect ethanol production (e.g., yeast strains, culture temperatures, and medium additives). The ethanol concentration and yield would be increased further by optimizing culture conditions.

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