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Conversion of cassava starch to trehalose by *Saccharomycopsis fibuligera* A11 and purification of trehalose

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ABSTRACT

As one of the non-food crops, cassava is widely cultivated in some of the southern provinces of China as a source of starchy material for food and fermentation industry. In this study, cassava starch was used as the substrate for *Saccharomycopsis fibuligera* A11, a trehalose poor-assimilating mutant isolated from *S. fibuligera* sdu through chemical mutagenesis, to accumulate trehalose. The results indicated that trehalose accumulation by *S. fibuligera* A11 was optimal in the medium comprised of 20.0 g/l cassava starch, 20.0 g/l hydrolysate of soybean cake at pH 5.5 and 30 °C for 48 h. At the flask level, trehalose yield was 24.8 g per 100 g of cell dry weight. At the end of 2-l fermentation, trehalose yield was 25.8 g per 100 g of cell dry weight. This is the highest trehalose yield accumulated in the yeast cells reported so far. At the same time, 0.12 g/100 ml of reducing sugar and 0.21 g/100 ml of total sugar were observed in the fermented medium. After isolation and purification, the crystal trehalose was obtained from the culture extract.

1. Introduction

The non-reducing disaccharide trehalose is widely present among microorganisms and invertebrates. Trehalose has many physiological roles within the cells. It does not only primarily function as a reserve carbohydrate, but also as a highly efficient protectant, enhancing the resistance of cellular components against adverse conditions such as high temperature, freezing, low dehydration, high osmotic pressure and high concentration of ethanol. As a purified sugar, trehalose has several applications, for example as a cryoprotectant for preserving cells in medicine and microbiology, as an effective component in cosmetics, as a stabilizer for clinical reagents and bioproducts, or even as a preservative for fresh foodstuff (Chi, Liu, Ji, & Meng, 2003; Chi, Liu, & Zhang, 2001).

Different sugars and carbohydrates including glucose, soluble starch, maltose, lactose, dextrin and sucrose have been used as the substrates for trehalose production (Cardoso, Gaspar, Hugenholtz, Ramos, & Santos, 2004; Chi et al., 2001, 2003; Cho, Park, & Shin, 2006; Lernia, Schiraldi, Generoso, & Rosa, 2002; Miyazaki, Miyagawa, & Sugiyama, 1996; Mukai et al., 1997; Satto, Kase, Takahashi, & Horinouchi, 1998; Schick, Haltrich, & Kulbe, 1995). Among these substrates, starch is the best substrate for production of trehalose due to its low price and easy access as the raw material (Kobayashi, Komeda, Miura, Kettoku, & Kato, 1997; Mukai et al., 1997; Yoshida, Shizuoka, & Saitama, 1997). So far, most of starch is extracted from food products, such as corn, wheat, rice, potato and sweet potato. However, with subsequent rising food prices, trehalose production using such food products as feedstocks will contribute further to food insecurity. Thus, in order to reduce the use of food products for trehalose production, it is very important to use starch from non-food products that could sustainability grow on marginal and abandon lands (Li & Chan-Halbrendt, 2009).

Cassava, which starch content is higher than 30%, more than any other stem tuber plants, is a high-yielding crop that grows well at tropical and subtropical temperature. It is also cultivated in many provinces in the south of China and the cassava starch is being produced in a large scale in different regions of the country (Chi et al., 2009). The cassava plant is extremely robust, is resistant to disease, flood and drought and growing in relatively low-quality soils, such as light sandy soils and medium texture soils. Therefore, cassava starch is also a good raw material in food and fermentation industry in China. Cassava starch is composed of unbranched amylose (20 ± 5%) and branched amylopectin (80 ± 5%), both of which can be hydrolyzed enzymatically (either with pure enzymes or amylase-producing microorganisms) to release their constituent glucose and maltooligosaccharides (Ejiofor, Chisti, & Moo-Young, 1996). Because of their high starch debranching activity, amylases produced by *S. fibuligera* have been extensively used to hydrolyze cassava starch because they have very high activity against α-1,6-linkage in the starch molecules (González, Fariña, & de Figueroa, 2008; Reddy & Basappa, 1996). α-Amylase and

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glucoamylase have been purified and characterized from *S. fibuligera* (Futatsugi, Ogawa, & Fukuda, 1993). It was also found that glucoamylase produced by *S. fibuligera* can digest raw starch (Hostinova, Solovicov, Dvorsky, & Gasperik, 2003). The α-amylase and glucoamylase can efficiently hydrolyze starch to yield glucose syrup for ethanol production by *Saccharomyces cerevisiae* (Knox, du Preez, & Kilian, 2004). We found that *S. fibuligera* A11, a trehalose poor-assimilating mutant obtained from *S. fibuligera* sdu by chemical mutagenesis also can efficiently convert soluble starch and corn starch into trehalose (Chi et al., 2001, 2003). In this study, cassava starch was used to accumulate trehalose by the trehalose poor-assimilating mutant *S. fibuligera* A11.

2. Materials and methods

2.1. Yeast strain

Saccharomycopsis fibuligera A11 is a trehalose poor-assimilating mutant isolated from *S. fibuligera* sdu by chemical mutagenesis (Chi et al., 2001, 2003).

2.2. Media

YPS medium used in this study contained 20.0 g/l soluble starch, 20.0 g/l polypeptone and 10.0 g/l yeast extract. YPD medium contained 20.0 g/l glucose, 20.0 g/l polypeptone and 10.0 g/l yeast extract.

2.3. Preparation of the hydrolysate of soybean cake

Thirty two grams of soybean cake were mixed with 250 ml of tap water containing 0.25 M HCl. The mixture was autoclaved at 121 °C for 25 min. After cooling, pH of the mixture was adjusted to 5.5 with 1.0 M NaOH solution and the suspension was filtered. The filtrate was diluted to 1600 ml (Duan, Chi, Wang, & Wang, 2008).

2.4. Trehalose accumulation in the shaking culture

Seed cultures were prepared by inoculating the yeast cells grown on a YPD agar slant into a 300-ml Erlenmeyer flask that contained 50 ml of the YPS liquid medium, with subsequent incubation at 30 °C for 24 h with shaking (200 rpm). Seed culture (10.0 ml) was then transferred into 300-ml flasks that contained 90.0 ml of the hydrolysate of soybean cake supplemented with 20.0 g/l cassava starch, corn starch, sweat potato starch, potato starch, soluble starch and rice starch, respectively, and the flasks were incubated at 30 °C for 48 h with shaking (180 rpm). In order to determine the optimal medium and cultivation conditions for trehalose accumulation by the yeast strain, different starch (20.0 g/l) and different concentrations of cassava starch (5.0–40.0 g/l) were added to the medium and the yeast strain was grown at different initial pH (4–7) and temperature (26–35 °C).

2.5. Fermentation

Seed cultures were prepared by inoculating the yeast cells grown on a YPD agar slant into 500-ml Erlenmeyer flask that contained 100 ml of YPS liquid medium, and cultivation at 30 °C for 24 h with vigorous shaking. The fermentation was carried out in a Biostat B2 2-1 fermentor (B. Braun, Germany) equipped with baffles, a stirrer, alkali pump, heating element, oxygen sensor and temperature sensor. Two hundred milliliters of the seed culture were transferred into 1800 ml of the hydrolysate of soybean cake containing 40 g of cassava starch. The fermentation was performed under the conditions of the agitation speed of 200 rpm, the aeration rate (sterile air volume input per min) of 4 l/min at 30 °C for 80 h.

2.6. Trehalose extraction and assay

The yeast cells were collected from the cultures obtained above and washed by centrifugation at 5000*g* and 4 °C for 10 min. Trehalose in the washed cells was extracted with 0.5 M trichloroacetic acid and trehalose content in the extract was assayed by the Anthrone method (Stewart, 1982).

2.7. Measurement of cell dry weight

The yeast cells from 5.0 ml of the culture were harvested and washed three times with distilled water by centrifugation at 5000*g* and 4 °C for 10 min. Then, cells in the tube were dried at 100 °C until the cell dry weight was constant.

2.8. Determination of reducing sugar and total sugar in the fermented media

Reducing sugar in the fermented media was determined by the Somogyi-Nelson method (Spiro, 1966). Residual total sugar was measured as reduction of sugar after hydrolysis of the fermented media (Chi et al., 2001).

2.9. Purification of trehalose

Ice-cold absolute ethanol was slowly added to 1000 ml of the extract obtained above with stirring until the precipitate appeared, to which saturated KCl solution (10.0–20.0 g/l) was also added at the same time. The mixture was kept at 4 °C overnight and centrifuged at 14,006*g* and 4 °C for 10 min. The precipitate obtained was washed by centrifugation with absolute ethanol, acetone and ether, sequentially. The washed precipitate was dried at 80 °C until its weight was constant. The dried precipitate was regarded as the crude trehalose. Crude trehalose (5.0 g) was dissolved in 25.0 ml of distilled water and pH of the solution was adjusted to 8.0. In order to remove the protein, 0.5 g of trypsin was added to the solution and incubated 37 °C for 24 h. The treated solution was centrifuged at 11,000*g* for 10 min and the protein in the supernatant obtained was further removed by Sevag method (Chi, Su, & Lu, 2007). In order to remove the pigment, pH of the treated supernatant was adjusted to 7.0 with ammonium and 25% (v/v) H_2O_2 solution was slowly added to the supernatant by mixing until slight yellow color appeared. The solution was dialyzed against distilled water for 24 h with changing distilled water. The trehalose in the dialyzed solution was precipitated by adding ice-cold absolute ethanol. The precipitate obtained was washed and dried as described above.

Twenty-five milligrams of the precipitate were dissolved in 0.1 M of NaCl solution. After centrifugation at 11,000*g* for 10 min, the supernatant was applied to a Sephadex G-50 column and the trehalose was eluted with 0.1 M of NaCl solution and the flow rate was 0.2 ml/min. The different fractions were collected automatically in glass tubes and the amount of sugar in each tube was assayed by using the Anthrone method (Stewart, 1982). The Anthrone positive fractions were combined and were applied to a Sephadex G-25 column to remove salts and the final elute was frozen at −20 °C for 2–3 days and lyophilized at −50 °C for 36 h. A small amount of the purified trehalose was dissolved in distilled water and UV spectra of the solution were recorded with spectrophotometer (Model UV-2102 PC, UNICO, USA) between 190 and 290 nm.

2.10. HPLC analysis

The purified trehalose from the yeast cells and standard trehalose from Sigma were analyzed by HPLC using Agilent Zorbax NH_2 column (5 μm) (4.6 × 250 mm) for determination of its purity. The HPLC conditions: flow rate was 1.0 ml/min; column temperature was 35 °C; the sample volume was 40 μl; detector was DAD (200 nm); mobile phase was acetonitrile–water (7:3); the sample concentration was 5.0 mg/ml.

3. Results

3.1. Trehalose accumulation of the yeast cells grown on different starch

As mentioned above, glucose, soluble starch, maltose, lactose, dextrin and sucrose have been used as the substrates for trehalose production by different microorganisms (Cardoso et al., 2004; Chi et al., 2001, 2003; Cho et al., 2006; Kobayashi et al., 1997; Lernia et al., 2002; Miyazaki et al., 1996; Mukai et al., 1997; Satto et al., 1998; Schick et al., 1995; Yoshida et al., 1997). *S. fibuligera* A11 has been confirmed to have ability to produce high amylase activity (Chen, Chi, Chi, & Li, 2009; Chi et al., 2003). Therefore, effects of cassava starch, corn starch, soluble starch, sweat potato starch, potato starch and rice starch on trehalose accumulation and cell growth by *S. fibuligera* A11 were examined. The results in Fig. 1 showed that when the yeast was grown in the medium containing cassava starch, the trehalose content in its cells was the highest. However, it could be seen from the results in Fig. 1 that the yeast grew best in the medium containing soluble starch. Therefore, cassava starch was subsequently used as the substrate for trehalose accumulation by *S. fibuligera* A11.

3.2. Effects of different concentrations of cassava starch on trehalose accumulation and cell growth

The results in Fig. 2 indicated that cell dry weight was continuously increased as the concentrations of cassava starch were increased from 5.0 to 40.0 (g/l). However, it could be observed from the results in Fig. 2 that when the concentrations of cassava starch were increased from 5.0 to 20.0 (g/l), trehalose content in the yeast cells was increased from 12.9 to 23.3 g per 100 g of cell dry weight. In contrast, when the concentrations of cassava starch were increased from 20.0 to 40.0 (g/l), trehalose content in the yeast cells was decreased from 23.3 to 12.5 g per 100 g of cell dry weight. This meant that 20.0 g/l cassava starch in the medium

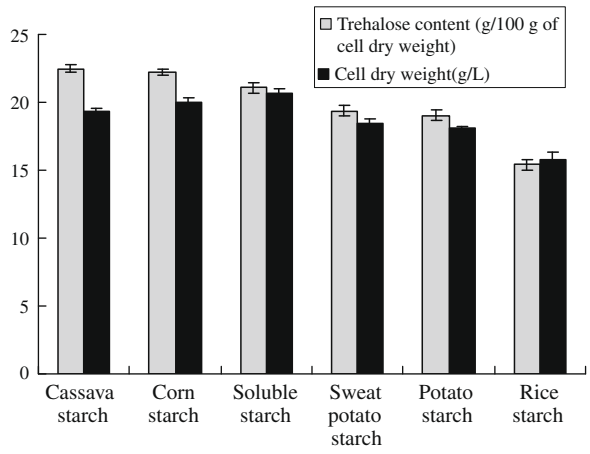

Fig. 1. Effects of different starch on trehalose accumulation and cell growth by *S. fibuligera* A11. Data are given as means ± SD, $n = 3$.

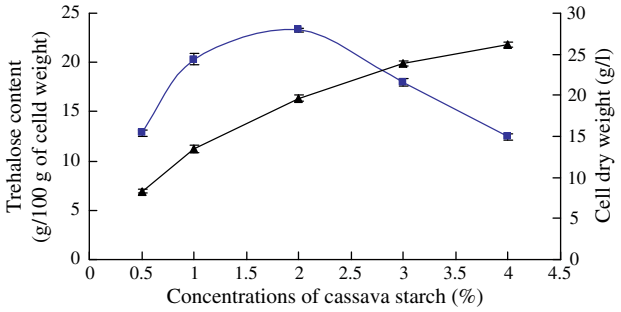

Fig. 2. Effects of different concentrations of cassava starch on trehalose accumulation (■) and cell growth (▲) by *S. fibuligera* A11. Data are given as means ± SD, $n = 3$.

was the most suitable for trehalose accumulation by the yeast strain.

3.3. Effects of different pHs and temperatures on trehalose accumulation and cell growth

The results in Fig. 3A revealed that trehalose accumulation reached the highest level (24.5% w/w) when initial pH of the growth medium was 5.5. However, cell growth was the best when initial pH of the growth medium was 6.0. It could be seen from the results in Fig. 3B that trehalose accumulation reached the highest level (24.5% w/w) when the cultivation temperature was 30 °C. In contrast, cell growth was the best when the cultivation temperature was 28 °C. At the end of the cultivation at flask level, only 0.16 g/100 ml of reducing sugar and 0.62 g/100 ml of total sugar were left in the fermented medium (Table 1), suggesting that over 84.5% of the added sugar was used by the yeast cells for growth and trehalose accumulation.

3.4. Time course of cell growth and trehalose accumulation during 2-l fermentation

The results above at 300-ml flask level showed that when the yeast strain was cultivated in the medium containing 20.0 g/l cassava starch and 20.0 g/l hydrolysate of soybean cake, pH 5.5, and at 30 °C, its cells could accumulate 24.5% (w/w) trehalose within 48 h. Therefore, trehalose accumulation and cell growth by *S. fibuligera* A11 during 2-l fermentation were examined as described in Section 2. The results in Fig. 4 showed that the cells could accumulate 25.8% (w/w) trehalose from cassava starch and cell dry weight reached 22.8 g/l within 48 h of the fermentation when cell growth reached early stationary phase. At the end of the fermentation, only 0.12 g/100 ml of reducing sugar and 0.24 g/100 ml of total sugar were left in the fermented medium (Table 1), suggesting that over 94.0% of the added sugar was used by the yeast cells for growth and trehalose accumulation.

3.5. Purification of trehalose

After isolation and purification of trehalose from the yeast cells as described in Section 2, the crystal trehalose was obtained (Fig. 5). Our results demonstrated that the protein and nucleic acids in the sample had been removed as there was no absorption peak of the sample at around 260–290 nm (data not shown). Fig. 6 presents the results of HPLC of standard trehalose from Sigma (Fig. 6A) and crystalline trehalose obtained from the yeast cells (Fig. 6B). This demonstrated that the sample extracted from the yeast cells only contained one component of trehalose. These find-

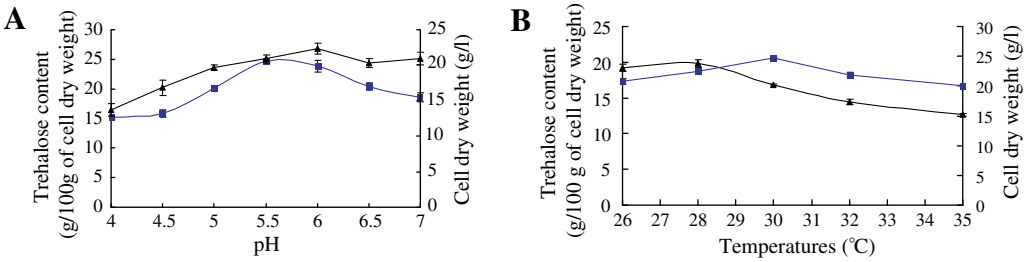

Fig. 3. Effects of different pHs and temperatures on trehalose accumulation (■) and cell growth (▲) by *S. fibuligera* A11. Data are given as means ± SD, $n = 3$.

Table 1
The amount of reducing sugar and total sugar left in the fermented media.

Samples	Reducing sugar (g/100 ml)	Total sugar (g/100 ml)
From 300-ml flask culture	0.16 ± 0.04	0.62 ± 0.06
From 2-l fermentor culture	0.12 ± 0.02	0.24 ± 0.03

Data are given as means ± SD, $n = 3$.

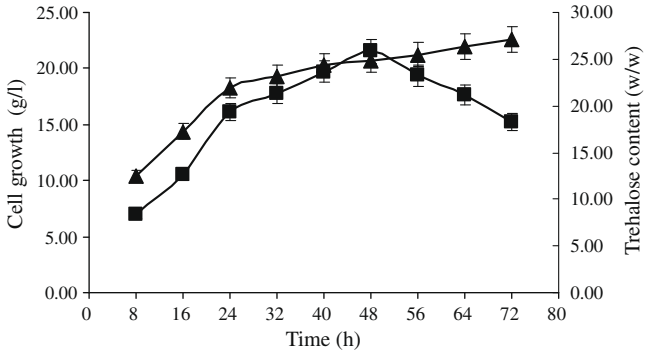

Fig. 4. Time course of trehalose accumulation (■) and cell growth (▲) during 2-l fermentation. Data are given as means ± SD, $n = 3$.

Fig. 5. The crystal trehalose.

ings identified the crystalline trehalose and standard trehalose as the same substance.

4. Discussion

In this study, 20.0 g/l cassava starch in the medium was found to be the most suitable for trehalose accumulation by *S. fibuligera* A11 (Figs. 1 and 2). Cassava is widely cultivated in the south of China and cassava starch is easily available in most parts of China. Furthermore, cassava is one of the non-food crops in China (Chi et al., 2009). In another study (Chen et al., 2009), it has been confirmed that the amylases produced by the yeast indeed have very high ability to hydrolyze cassava starch. So, it is feasible to use cassava starch as the substrate for trehalose production by the yeast. In our previous studies (Chi et al., 2001, 2003), it was observed that the same yeast strain can transform soluble starch into trehalose. At the same time, it was found that 10.0 g/l soluble starch in the medium was the most suitable for trehalose accumulation by its wild type. Under such conditions, only 16.5% (w/w) trehalose accumulated in the yeast cells (cell dry weight) was attained.

The data presented in Fig. 3 indicated that the yeast strain accumulated trehalose best at pH 5.5 and 30 °C. In our previous study (Chi et al., 2001), we also found that *S. fibuligera* sdu (the wild type of the yeast strain used in this study) accumulated the highest trehalose from soluble starch when it was grown in the medium with pH 5.5 at 30 °C. *S. cerevisiae* growing in fed-batch cultivations accumulated the highest trehalose when temperature was 30 °C and pH was 5.0 (Aranda, Salgado, & Taillandier, 2004). This meant that the results obtained in this study were consistent with those obtained by other researchers.

At the end of the 2-l fermentation, 25.8% (w/w) trehalose from cassava starch was accumulated, cell dry weight reached 22.8 g/l within 72 h of the fermentation and over 94.0% of the added sugar was used by the yeast cells for growth and trehalose accumulation (Fig. 4 and Table 1). It also could be observed from the results in Fig. 4 that the rate of trehalose biosynthesis in the yeast cells was paralleled with that of cell growth. Fig. 7 shows the changes in reducing sugar and total sugar during the 2-l fermentation. It could be clearly seen from the results in Fig. 7 that the culture contained a large amount of reducing sugar from 8 to 24 h of the fermentation. However, it could observed from the results in Fig. 4 that even when the medium contained high content of the reducing sugar, the yeast cells still actively synthesized trehalose from 8 to 24 h of the fermentation. The results suggested that trehalose biosynthesis in the yeast cells were not sensitive to reducing sugar available in the culture. This may be due to the fact that *S. fibuligera* A11 used in this study is a poor trehalose-assimilating mutant (Chi et al., 2003). However, many results from other research have shown that trehalose immobilization can be enhanced by glucose available in the medium as TPS1 (trehalose-6-phosphate synthase) activity responsible for trehalose biosynthesis is inhibited by glucose while NTH1 (neutral trehalase) activity and mRNA responsible for trehalose immobilization is activated by glucose because of the presence of stress responsive elements STRE (CCCCT) in its promoter region (Francois & Parrou, 2001; Soto, Fernandez, Vicente-Soler, Cansado, & Gacto, 1995; Zaëhringer, Burgert, Holzer, & Nwak,

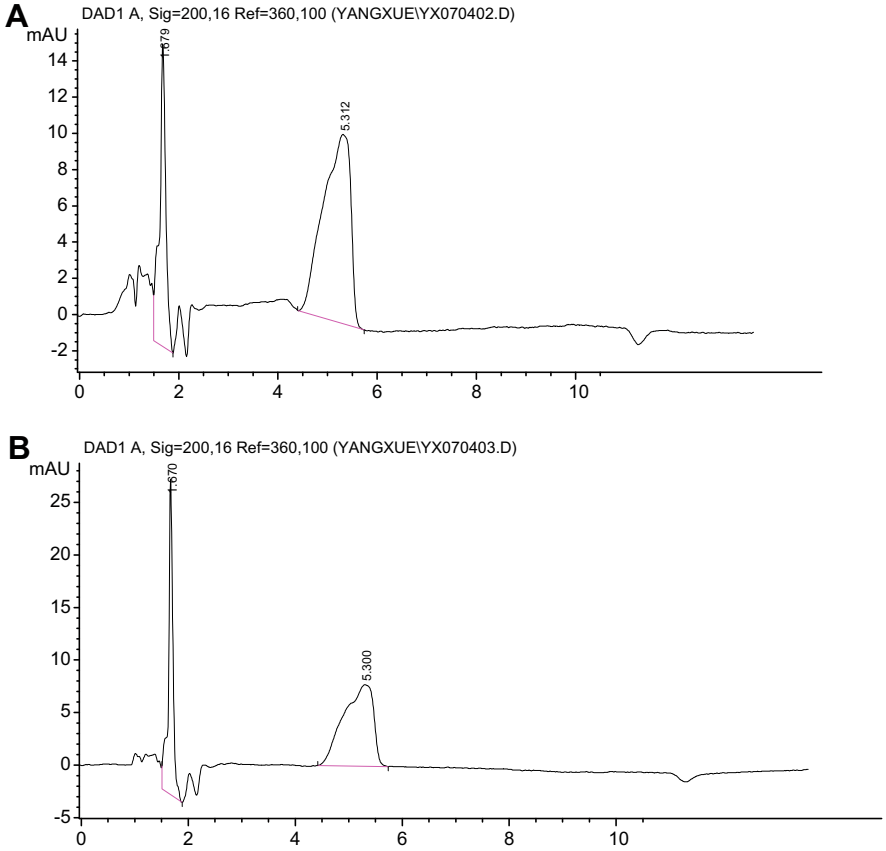

Fig. 6. The results of HPLC of standard trehalose (A) and the crystalline trehalose (B) obtained from the yeast cells.

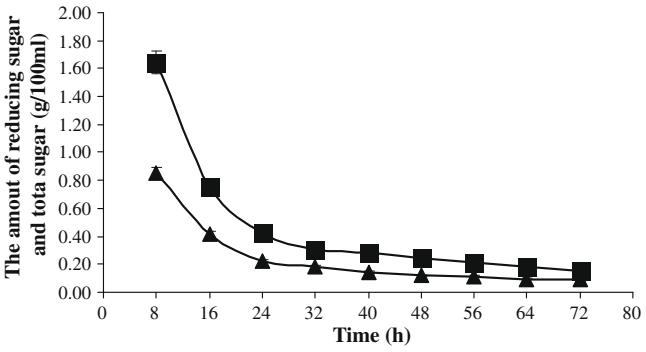

Fig. 7. Changes in reducing sugar (▲) and total sugar (■) during 2-l fermentation. Data are given as means ± SD, $n = 3$.

1997). These results imply that regulation of trehalose biosynthesis in the cells of *S. fibuligera* A11 is different from that of trehalose biosynthesis in the cells of other yeasts. At the same time, it could be noted from the results in (Figs. 4 and 7) that the trehalose synthesized in the yeast cells still could be degraded when most of reducing sugar in the culture was used after 48 h of the fermentation as the yeast cells kept low activities of neutral trehalase and acid trehalase (Chi et al., 2003). Therefore, it is necessary to delete the genes required for trehalose degradation in the yeast cells in order to further improve trehalose yield, their tolerance to freezing, cell viability and carbohydrate utilization (Argüelles, 2000).

In our previous study (Chi et al., 2001), it was found that 18.0% (w/w) trehalose was accumulated from soluble starch in the yeast cells (cell dry weight) within 48 h of the fermentation when agitation speed was 200 rpm while cell dry weight only reached 11.1 g/l. *S. cerevisiae* EC1118 can accumulate trehalose up to 13% of biomass dry weight (0.13 g trehalose/g biomass) under carbon or nitrogen starvation (Aranda et al., 2004). A rise of growth temperatures from 45 to 50 and 52.5 °C led to a significant accumulation of intracellular trehalose in the thermophilic fungus *Chaetomium thermophilum* var. *coprophilum* from 0.17% of dry weight (% dw) to 1.4% and 1.5% (dw), respectively (Jepsen & Jensen, 2004). Pitching yeasts grown in 10.8°P brewers wort at 14 °C for 96 h only accumulated 2.7 g/100 g of cell dry weight (Guldfeldt & Arneborg, 1998). This meant that *S. fibuligera* A11 could accumulate much higher trehalose and could grow much better than any other yeast strains. So, we think that the yeast strain and the process for trehalose accumulation developed in this study have many potential applications. However, the highest total yield of extracellular trehalose (34.0 g/l) from glucose was obtained by *Cellulosimicrobium cellulans* when ammonium sulfate was added at 8.0 g/l (Seto et al., 2004).

It has been well documented that trehalose accumulation and trehalose-synthesizing gene expression in *S. cerevisiae* cells are greatly enhanced when they are stressed by high temperature, low temperature, high ethanol concentration and high oxidative conditions (Aranda et al., 2004). However, it was found that neither activation of TPS1 (trehalose-6-phosphate synthase) nor change in trehalose content was observed under the stress exposure of *S. fibuligera* A11 cells (Chi, Liang, Zhu, & Zhang, 2006). Under normal growth conditions, the genes *ZrTPS1* encoding TPS1 and *ZrTPS2* encoding TPS2 (trehalose-6-phosphate phosphatase) in *Zygosaccharomyces rouxii* were also highly and constitutively expressed, unlike *S. cerevisiae* TPS1 and TPS2. However, salt stress and heat stress reduced the expression of the *ZrTPS1* and *ZrTPS2* genes, respectively (Kwon et al., 2003). Therefore, it is impossible to fur-

ther promote trehalose accumulation in *S. fibuligera* A11 cells using such stress treatment.

The results in (Figs. 5 and 6) demonstrated that the sample extracted from the yeast cell was indeed trehalose. This is the first report that cassava starch can be actively converted into trehalose by *S. fibuligera* A11 cells.

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