J Ind Microbiol Biotechnol (2010) 37:19–25
DOI 10.1007/s10295-009-0644-6

ORIGINAL ARTICLE

Trehalose accumulation from corn starch by *Saccharomycopsis fibuligera* A11 during 2-l fermentation and trehalose purification

Zhe Chi · Ji-Ming Wang · Zhen-Ming Chi · Fang Ye

Received: 24 August 2009 / Accepted: 15 September 2009 / Published online: 5 December 2009

Abstract In this study, corn starch was used as the substrate for cell growth and trehalose accumulation by *Saccharomycopsis fibuligera* A11. Effect of different aeration rates, agitation speeds, and concentrations of corn starch on direct conversion of corn starch to trehalose by *S. fibuligera* A11 were examined using a Biostat B2 2-l fermentor. We found that the optimal conditions for direct conversion of corn starch to trehalose by this yeast strain were that agitation speed was 200 rpm, aeration rate was 4.0 l/min, concentration of corn starch was 2.0% (w/v), initial pH was 5.5, fermentation temperature was 30°C. Under these conditions, over 22.9 g of trehalose per 100 g of cell dry weight was accumulated in the yeast cells, cell mass was 15.2 g/l of the fermentation medium, 0.12% (w/v) of reducing sugar, and 0.21% (w/v) of total sugar were left in the fermented medium within 48 h of the fermentation. It was found that trehalose in the yeast cells could be efficiently extracted by the hot distilled water (80°C). After isolation and purification, the crystal trehalose was obtained from the extract of the cells.

Keywords Trehalose · Corn starch · *S. fibuligera* · Fermentation · Purification

Z. Chi · J.-M. Wang · Z.-M. Chi (✉) · F. Ye
Unesco Chinese Center of Marine Biotechnology,
Ocean University of China, Yushan Road,
No. 5, Qingdao, China
e-mail: zhenming@sdu.edu.cn

Introduction

Trehalose (1-α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing disaccharide composed of two molecules of glucose linked at their 1-carbons. It has been found that trehalose has many physiological roles in the cells. For example, 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 [7]. It also has been well documented that trehalose has several applications, for example as cryoprotectant for 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 [21].

Different sugars including glucose, soluble starch, maltose, lactose, dextrin, and sucrose have been used as the substrates for trehalose production [2, 4, 5, 8, 16, 18–20, 22, 23, 26]. However, it is thought that starch is the best substrate for production of trehalose due to its low price and easily obtained raw material [16, 20, 26]. So far, most of starch has come from food grains, such as corn, wheat, rice, and sweet potato. In China, corn starch is also widely available and easily obtained. In our previous studies [4], it was found that *S. fibuligera* sdu cells could accumulate 18.0% (w/w) trehalose from soluble starch in SSY medium. However, we think that corn starch as the substrate for trehalose production is better than soluble starch because corn starch is more easily obtained and much cheaper than soluble starch.

It has been shown that different strains of *S. fibuligera* can produce high activity of α-amylase and glucoamylase [3], and α-amylase and glucoamylase have been purified

and characterized from this yeast [11]. It was also found that glucoamylase produced by some strains of *S. fibuligera* can digest raw starch [13]. The α-amylase and glucoamylase can efficiently hydrolyze starch to yield glucose syrup for ethanol production by *S. cerevisiae* [15]. Therefore, starch can be directly converted into trehalose by *S. fibuligera*.

It has been reported that wild-type *S. fibuligera* contains high activities of acid and neutral trehalases, which have been reported to mobilize trehalose accumulated by the cells during fermentation, causing decreased trehalose yield in its cells [7]. In order to enhance the yield of trehalose, it is necessary to remove the trehalase activities from the cells. By mutagenesis of ethylmethanesulfonate, a mutant called *S. fibuligera* A11 that assimilates trehalose very slowly, but grows on other carbon sources as fast as its parent strain *S. fibuligera* sdu, has been isolated. The activities of acid and neutral trehalases of this mutant are much lower than those of its wild type, respectively [5]. We found that the trehalose poor-assimilating mutant also can efficiently convert soluble starch into trehalose in its cells. The reduction of acid and neutral trehalase activities was considered to be responsible for the increased yield of trehalose accumulated by the mutant [5]. However, as mentioned above, soluble starch is expensive and cannot be used as a material for trehalose production in a large scale. Therefore, corn starch was used to accumulate trehalose by the trehalose poor-assimilating mutant, *S. fibuligera* A11 in this study.

Materials and methods

Yeast strain

Saccharomycopsis fibuligera A11 is a trehalose poor-assimilating mutant isolated from *S. fibuligera* sdu [4, 5].

Media

1. YPS medium: 2.0% soluble starch, 2.0% polypeptone, 1.0% yeast extract.
2. YPD medium: 2.0% glucose, 2.0% peptone, and 1.0% yeast extract.

Corn starch

The corn starch used in this study was purchased from the local supermarket in Qingdao, China.

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 14 b for 25 min. After cooling, pH of the mixture was adjusted to 5.5 with 1 M NaOH solution and the suspension was filtered. The filtrate was diluted to 1,600 ml [4].

Trehalose accumulation in the shaking culture

The 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). Ten milliliter of the seed culture was then transferred into a 300-ml flask that contained 90 ml of the hydrolysate of soybean cake containing 1.0% corn starch or 1.0% soluble starch, and the flask was incubated at 30°C for 48 h with shaking (180 rpm).

Fermentation

The seed cultures were prepared by inoculating the yeast cells grown on a YPD agar slant into 500-ml Erlenmeyer flasks that contained 100 ml of YPS liquid medium and cultivating them 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 was transferred into 1,800 ml of the hydrolysate of soybean cake containing 40 g of corn starch in the fermentor. The fermentation was performed under the conditions of the agitation speed of 200 rpm, aeration rate of 4 l/min, the temperature of 30°C, and fermentation time of 72 h.

Trehalose extraction and assay

The yeast cells were collected from the yeast cultures obtained from the flask and fermentor and washed with sterile distilled water by centrifugation at 5,000×*g* and 4°C for 10 min for three times. Trehalose in the washed cells was extracted with 0.5 M trichloroacetic acid (TCA), and trehalose content in the extract was quantitatively assayed by the Anthrone Method [25].

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 5,000×*g* and 4°C for 10 min. Then, cells in the tube were dried at 100°C until the cell dry weight was constant.

Determination of reducing sugar and total sugar in the fermented media

Reducing sugar in the fermented media was determined by the Nelson–Somogyi method [24]. Residual total sugar was

measured as reduction of sugar after hydrolysis of the fermented media [4].

Purification of trehalose

The yeast cells were aerobically grown in the 2-l fermentor at 30°C for 48 h under the conditions as described above. The cells in the culture were collected and washed by centrifugation with sterile saline water (0.85% NaCl). One gram of the cell pellets obtained was mixed with 8.0 ml of sterile distilled water, and the mixture was incubated at 80°C for 30 min. The procedure for extraction of trehalose was repeated two times. Meanwhile, trehalose extraction was also carried out with TCA solution as described above. The suspension was centrifuged at 4,000×*g* for 10 min to yield a clear extract. The resulting extract was subsequently filtrated through an AmiconYM3 (MW cut-off 5 kDa) membrane to remove the water-soluble polysaccharides and proteins greater than 5 kDa.

In order to decolorize the filtrates and remove salts and other impurities from the filtrate, the filtrate was applied onto a column (717 cation-exchange resin), and the elute was continuously applied onto another column (201X7 anion-exchange resin). The final elute was collected at a flow rate of 3 ml/min. The fractions corresponding to trehalose were combined. The final elute, which contained trehalose was taken to be concentrated on a rotary evaporator (RE-52A, Yang Biochemical Instrumental Company, Shanghai) at 100°C under the reduced pressures. The crystallization of trehalose was performed by adding absolute ethanol to the concentrate (concentrate: absolute ethanol = 1:4 v/v) with stirring gently. The crystalline trehalose was collected and washed with absolute ethanol by filtration and dried under vacuum at 40–50°C for 25 h [4].

UV analysis

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.

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 were as follows: 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.

Results and discussion

Trehalose accumulation from corn starch and soluble starch

In our previous study [4], soluble starch was used as the substrate for cell growth and trehalose accumulation by *S. fibuligera* sdu. In another study [5], we found that *S. fibuligera* A11, a trehalose poor-assimilating mutant from *S. fibuligera* sdu, could produce higher amount of trehalose than *S. fibuligera* sdu. As mentioned above, soluble starch is not feasible for trehalose production due to its high price. Therefore, in this study, trehalose accumulation from soluble starch and corn starch by *S. fibuligera* A11 was compared, trying to use the latter as the substrate for trehalose production. The results in Fig. 1 showed that the yeast cells could accumulate a little more trehalose from corn starch than from soluble starch when the media contained 1.0% corn starch or soluble starch; although, cell growth in the medium containing soluble starch was a little better than that in the medium containing corn starch. This meant that corn starch could be used to replace soluble starch for trehalose accumulation by *S. fibuligera* A11.

Corn starch is widely available in every supermarket in China, and its price is much lower than the price of soluble starch. Therefore, it is feasible to use the substrate as the material for trehalose accumulation by *S. fibuligera* A11. As mentioned above, glucose, soluble starch, maltose, lactose, dextrin, and sucrose have been used as the substrates for trehalose production by different microorganisms [2, 4, 5, 8, 16, 18–20, 22, 23, 26]. Here, we reported that corn starch also could be used as the substrate for trehalose accumulation by *S. fibuligera* A11.

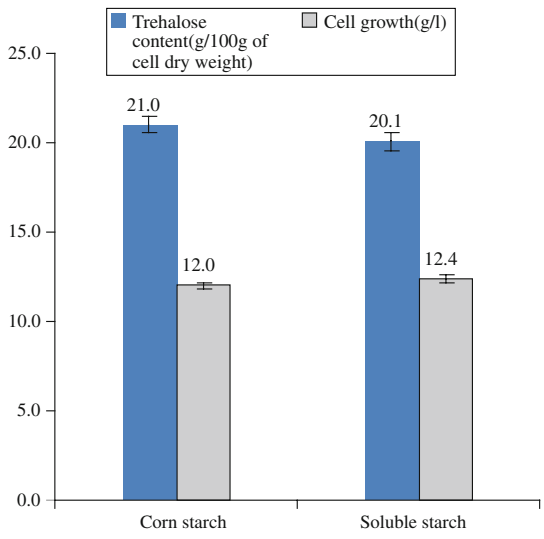

Fig. 1 Trehalose accumulation and cell growth by *S. fibuligera* A11 grown in the media containing 1.0% of corn starch and soluble starch. Data are given as mean ± SD, $n = 3$

Effects of different concentrations of corn starch on trehalose accumulation and cell growth

In order to know the optimal concentration of corn starch for trehalose accumulation by *S. fibuligera* A11, different concentrations of corn starch on trehalose accumulation and cell growth by *S. fibuligera* A11 were examined in the 2-l fermentor as described in Materials and methods. The results in Fig. 2 indicated that the more corn starch in the media, the better cell growth during the fermentation. However, the results in Fig. 3 revealed that when the yeast cells grew in the medium containing 2.0% corn starch, they could accumulate the highest trehalose (22.9 g trehalose per 100 g of cell dry weight) within the shortest period (48 h). Therefore, corn starch with 2.0% (w/v) was subsequently used as the substrate for trehalose accumulation by *S. fibulegira* A11. However, it was found that 1.0% (w/v) soluble starch in the medium was the most suitable for trehalose accumulation by its wild type. Under such conditions, only 16.5 g trehalose per 100 g of cell dry weight accumulated in the yeast cells (cell dry weight) was attained [4].

Effects of different agitation speed on trehalose accumulation and cell growth

In our previous study [5], it has been confirmed that *S. fibuligera* A11 accumulates the highest trehalose when it is cultivated in the medium with pH 5.5 and at 30°C. It has been reported that different agitation speeds have significant influence on yeast cell growth and product formation [6]. Therefore, effects of different agitation speeds on cell growth and trehalose accumulation from corn starch during

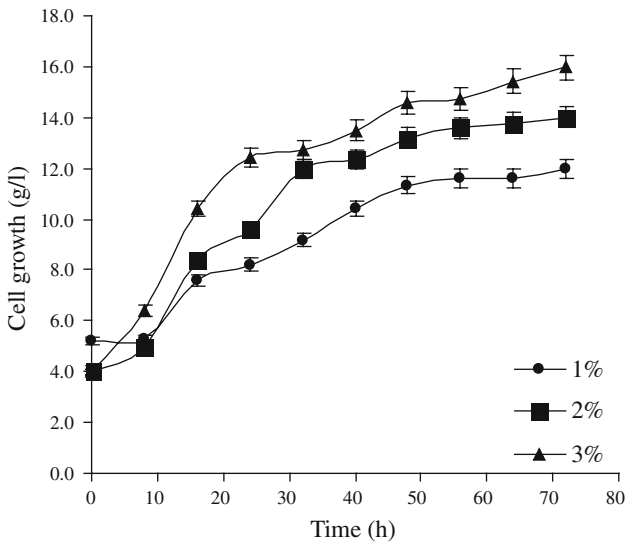

Fig. 2 Effects of different concentrations of corn starch on cell growth. Data are given as mean ± SD, $n = 3$

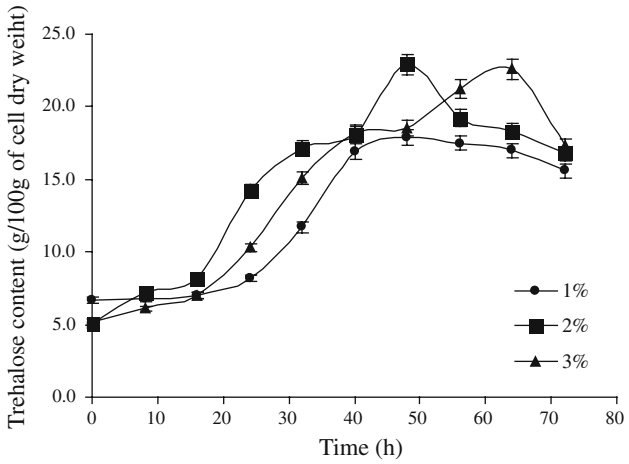

Fig. 3 Effects of different concentrations of corn starch on trehalose accumulation. Data are given as mean ± SD, $n = 3$

the 2-l fermentation were examined in the medium with pH 5.5 and at 30°C. It could be seen clearly from the data in Fig. 4 that when agitation speeds were increased from 150 to 300 rpm, cell growth was also increased. However, the results in Fig. 5 demonstrated that trehalose content in the yeast cells was the highest when the agitation speed was 150 rpm during the fermentation. This may suggest that the more cell growth, the less trehalose accumulated in the cells. This may be due to the fact that the more cell growth, the less sugar that could be transformed into trehalose in the yeast cells. In another study [10], it has been found that *Aureobasidium pullulan* Y68 synthesizes less pullulan when it grows better. However, when the agitation speed was 150 rpm, cell growth was the poorest (Fig. 4). In this case, low trehalose yield per liter of the culture existed in the fermented medium. For example, 2.45, 3.29, and 3.17 g of trehalose per liter of the culture were obtained when the agitation speeds were 150, 200, and 300 rpm, respectively, and fermentation period was 48 h. Therefore, the trehalose yield per liter of the culture was the highest when the agitation speed was 200 rpm.

Effects of different aeration rate on trehalose accumulation and cell growth

Because *S. fibuligera* is an obligate aerobe, different aeration rate must have great effects on the yeast cell growth and product formation [17]. Therefore, effects of different aeration rates on cell growth and trehalose accumulation from corn starch by *S. fibuligera* A11 during the 2-l fermentation were examined in the medium with pH 5.5 and at 30°C when the agitation speed was 200 rpm. It could be observed from the results in Fig. 6 that the cell growth (15.2 g/l) was the best when the aeration rate was 4 l/min. In contrast, the cell growth (11.2 g/l) was the poorest when

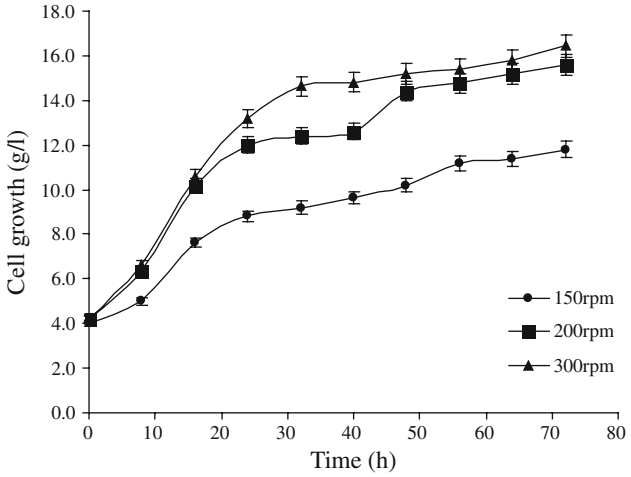

Fig. 4 Effects of different agitation speed on cell growth. Data are given as mean ± SD, $n = 3$

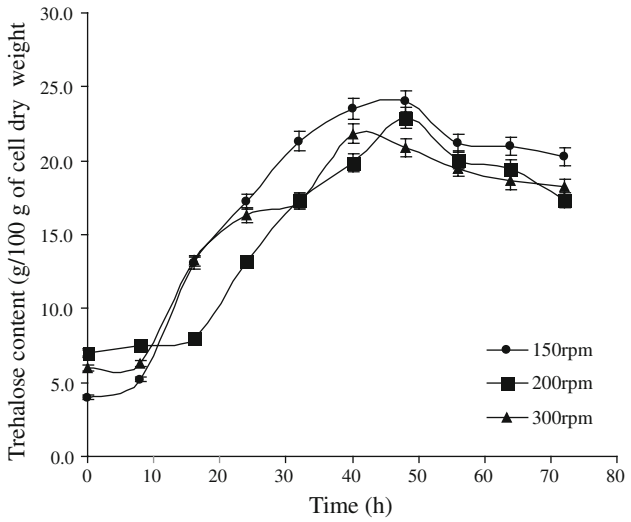

Fig. 5 Effects of different agitation speed on trehalose accumulation. Data are given as mean ± SD, $n = 3$

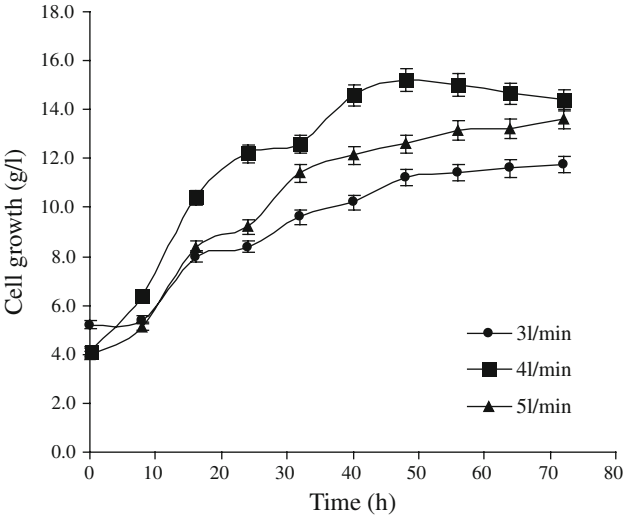

Fig. 6 Effects of different aeration rate on cell growth. Data are given as mean ± SD, $n = 3$

the aeration rate was 3 l/min. The results in Fig. 7 showed that the yeast cells accumulated the highest amount of trehalose (24.0 g trehalose per 100 g of cell dry weight) when the aeration rate was 3 l/min, while the yeast cells accumulated 22.9 g of trehalose per 100 g of cell dry weight when the aeration rate was 4 l/min. This again demonstrated that the more cell growth, the less trehalose accumulated in the cells. However, when the aeration rate was 4 l/min, the highest trehalose yield per liter of the culture was obtained. For example, 2.7, 3.2, and 2.4 g of trehalose per liter of the cultures were obtained when the aeration rates were 3, 4, and 5 l/min rpm, respectively, and fermentation period was 48 h. Therefore, the optimal aeration rate for trehalose accumulation by *S. fibuligera* A11 was 4 l/min under the conditions used in this study. In our previous study [4], it was observed that 18.0 g trehalose per 100 g of cell dry weight was accumulated from soluble starch in the cells of *S. fibuligera* sdu within 48 h of the fermentation, and cell dry weight only reached 11.1 g/l when agitation speed was 200 rpm. In contrast, the high ethanol producing yeast *Saccharomyces* sp. W0 and other *S. cerevisiae* only produce 12.0–15.0% trehalose (g/100 g of cell dry weight) from glucose [12]. *S. cerevisiae* EC1118 can accumulate trehalose up to 13% of biomass dry weight (0.13 g trehalose per gram biomass) under carbon or nitrogen starvation [1]. 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 [14]. This meant that *S. fibuligera* A11 could accumulate much higher trehalose from corn starch and grow much better than any other yeast strains.

At the end of the fermentation, only 0.12% (w/v) of reducing sugar and 0.21% (w/v) of total sugar were left in the fermented medium within 48 h of the fermentation (data not shown). This meant that most of corn starch in the medium was converted into cell mass and trehalose. However, the reducing sugar concentration in the culture of *S. fibuligera* sdu was also maintained very low (65 μg/ml), and a considerable amount (3.67 mg/ml) of residual total sugar was left in the culture of *S. fibuligera* sdu at the end of the fermentation [4].

Isolation and purification of trehalose

The 0.5 M TCA solution is commonly used to extract trehalose from yeast cells [4, 5, 9, 25]. However, TCA is very toxic and erosive. Therefore, it is not feasible to use the toxic chemical to extract trehalose. In this study, it was

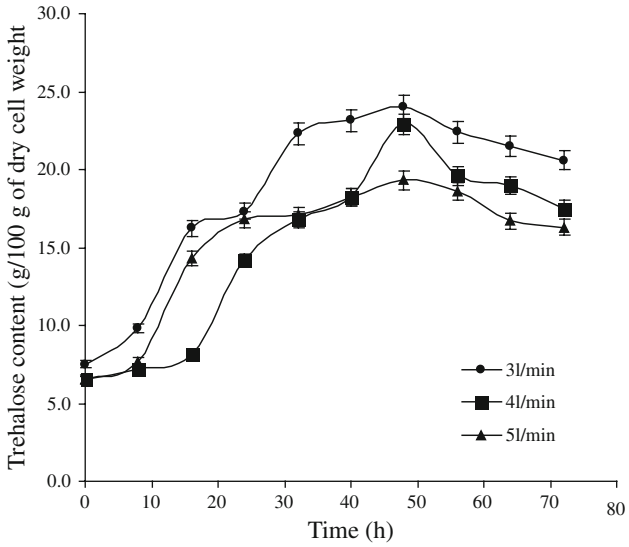

Fig. 7 Effects of different aeration rate on trehalose accumulation. Data are given as mean ± SD, $n = 3$

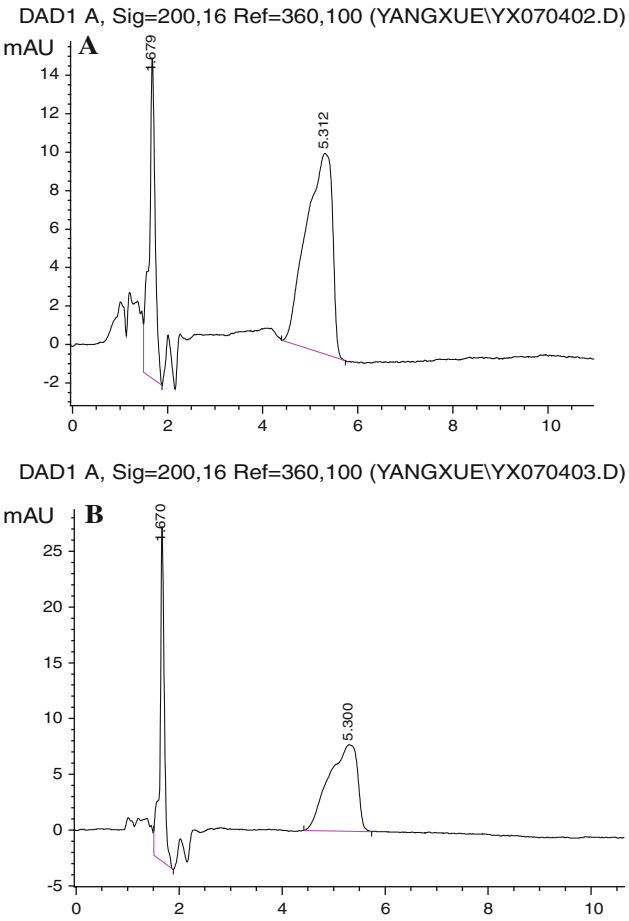

Fig. 8 The results of HPLC of standard trehalose (**a**) and the crystalline trehalose (**b**) obtained from the yeast cells

found that the hot water (80°C) also could be used to extract trehalose from the yeast cells as efficiently as 0.5 M TCA solution (data not shown). After isolation and purification of trehalose from the yeast cells as described in "Materials and methods", the crystal trehalose was obtained (data not shown). Our results demonstrated that the protein and nucleic acids in the sample were completely removed as there was no absorption peak of the sample around 260–290 nm after UV analysis (data not shown). Figure 8 presents the results of HPLC of standard trehalose from Sigma (Fig. 8a) and crystalline trehalose obtained from the yeast cells (Fig. 8b). The results in Fig. 8 demonstrated that the trehalose sample extracted from the yeast cells only contained one component of trehalose. These findings identified the crystalline trehalose and standard trehalose as the same substance.

Acknowledgments The authors would like to thanks Qingdao Municipal Science and Technology Commission, Qingdao, China for providing financial support to carry out this work. The grant no. is 06-2-2-22-jch.

References

1. Aranda JS, Salgado E, Taillandier P (2004) Trehalose accumulation in *Saccharomyces cerevisiae* cells: experimental data and structured modeling. Biochem Eng J 17:129–140
2. Cardoso FS, Gaspar P, Hugenholtz J, Ramos A, Helena Santos H (2004) Enhancement of trehalose production in dairy propionibacteria through manipulation of environmental conditions. Int J Food Microbiol 91:195–204
3. Chen L, Chi ZM, Chi Z, Li M (2009) Amylase production by *Saccharomycopsis fibuligera* A11 in solid-state fermentation for hydrolysis of cassava starch. Appl Biochem Biotechnol. doi: 10.1007/s12010-009-8744-3
4. Chi ZM, Liu J, Zhang W (2001) Trehalose accumulation from soluble starch by *Saccharomycopsis fibuligera* sdu. Enzyme Microb Technol 38:240–246
5. Chi ZM, Liu J, Ji JR, Meng ZL (2003) Enhanced conversion of soluble starch to trehalose by a mutant of *Saccharomycopsis fibuligera* sdu. J Biotechnol 102:135–141
6. Chi ZM, Zhao SZ (2003) Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast strain. Enzyme Microb Technol 33:206–211
7. Chi ZM, Chi Z, Liu GL, Wang F, Ju L, Zhang T (2009) *Saccharomycopsis fibuligera* and its applications in biotechnology. Biotechnol Adv 27:423–431
8. Cho YJ, Park OJ, Shin HJ (2006) Immobilization of thermostable trehalose synthase for the production of trehalose. Enzyme Microb Technol 39:108–113
9. Deegenaars MI, Kenneth Watson K (1998) Heat shock response in psychrophilic and psychrotrophic yeast from Antarctica. Extremophiles 2:41–49
10. Duan XH, Chi ZM, Wang L, Wang XH (2008) Influence of different sugars on pullulan production and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase involved in pullulan synthesis in *Aureobasidium pullulans* Y68. Carbohydr Polym 73:587–593
11. Futatsugi M, Ogawa T, Fukuda H (1993) Purification and properties of two forms of glucoamylase from *Saccharomycopsis fibuligera*. J Ferment Bioeng 76:521–523
12. Gao J, Chi ZM (2001) Relationship between trehalose content and ethanol tolerance in high ethanol tolerant yeast and ethanol sensitive yeast. Food Ferment Ind 27:4–7

13. Hostinova V, Solovicov A, Dvorsky R, Gasperik J (2003) Molecular cloning and 3D structure prediction of the first raw-starch-degrading glucoamylase without a separate starch-binding domain. Arch Biochem Biophys 411:189–195
14. Jepsen HF, Jensen B (2004) Accumulation of trehalose in the thermophilic fungus *Chaetomium thermophilum* var. coprophilum in response to heat or salt stress. Soil Biol Biochem 36:1669–1674
15. Knox AM, du Preez JC, Kilian SG (2004) Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Enzyme Microb Technol 34:453–460
16. Kobayashi K, Komeda T, Miura Y, Kettoku M, Kato M (1997) Production of trehalose from starch by novel trehalose-producing enzymes from *Sulfolobus solfataricus* KM1. J Ferment Eng 83:296–298
17. Kurtzman CP, Fell JW (eds) (2000) The yeasts. A taxonomic study, 4th revised and enlarged edn. Elsevier, Amsterdam, pp 377–378
18. Lernia ID, Schiraldi C, Generoso M, Rosa MD (2002) Trehalose production at high temperature exploiting an immobilized cell bioreactor. Extremophiles 6:341–347
19. Miyazaki JI, Miyagawa KI, Sugiyama Y (1996) Trehalose accumulation by a basidiomycotious yeast, *Filobasidium floriforme*. J Ferment Bioeng 4:315–319
20. Mukai K, Tabuchi A, Nakada T, Shibuya T, Chaen H, Fukuda S, Kurimoto M, Tsujisaka Y (1997) Production of trehalose from starch by thermostable enzymes from *Sulfolobus acidocaldarius*. Starch 49:26–30
21. Plourde-Owobi L, Durner S, Goma G, Francois J (2000) Trehalose reserves in *Saccharomyces cerevisiae*: phenomenon of transport, accumulation and role in cell viability. Int J Food Microbiol 55:33–40
22. Satto K, Kase T, Takahashi E, Takahashi E, Horinouchi S (1998) Purification and characterization of a trehalose synthase from the basidiomycete *Grifola frondosa*. Appl Environ Microbiol 64:4340–4345
23. Schick I, Haltrich D, Kulbe KD (1995) Trehalose phosphorylase from *Pichia fermentans* and its role in the metabolism of trehalose. Appl Microbiol Biotechnol 43:1088–1095
24. Spiro RG (1966) Analysis of sugars found in glycoproteins. Methods Enzymol 8:3–26
25. Stewart PR (1982) Trehalose extraction and determination. In: Prescott DM (ed) Methods in cell biology, vol 12. Academic Press, London, pp 111–147
26. Yoshida M, Shizuoka NN, Saitama KH (1997) Production of trehalose from starch by maltose phosphorylase and trehalose phosphorylase from a strain of *Plesiomonas*. Starch 49:21–26