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ORIGINAL PAPER

Trehalose accumulation from cassava starch and release by a highly thermosensitive and permeable mutant of *Saccharomycopsis fibuligera*

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Abstract Highly thermosensitive and permeable mutants are the mutants from which intracellular contents can be released when they are incubated both in low osmolarity water and at non-permissive temperature (usually 37°C). After mutagenesis by using nitrosoguanidine, a highly thermosensitive and permeable mutant named A11-b was obtained from *Saccharomycopsis fibuligera* A11-12, a trehalose overproducer in which the acid protease gene has been disrupted. Of the total trehalose, 73.8% was released from the mutant cells suspended in distilled water after they had been treated at 37°C overnight. However, only 10.0% of the total trehalose was released from the cells of *S. fibuligera* A11-12 treated under the same conditions. The cell volume of the mutant cells suspended in distilled water and treated at 37°C overnight was much bigger than that of *S. fibuligera* A11-12 treated under the same conditions. The cell growth and trehalose accumulation of the mutant were almost the same as those of *S. fibuligera* A11-12 during the cultivation at the flask level and in a 5-l fermentor. Both could accumulate around 28.0% (w/w) trehalose from cassava starch. After purification, the trehalose crystal from the aqueous extract of the mutant was obtained.

Keywords Trehalose extraction · Highly thermosensitive and permeable mutant · *S. fibuligera* · Mutagenesis · Cassava starch

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Introduction

The non-reducing disaccharide trehalose is widely present among microorganisms and invertebrates [6, 10]. Trehalose has many physiological roles within the cells. It not only primarily functions 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 concentrations of ethanol [8]. 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 foodstuffs [4, 5, 11, 18].

So far, at least five biosynthetic pathways are known for trehalose: the trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP) pathway, trehalose synthase (TS) pathway, maltooligosyl trehalose synthase TreY/TreZ pathway, trehalose phosphorylase (TreP) pathway and trehalose glycosyltransferring synthase (TreT) pathway [2]. *Saccharomycopsis fibuligera* is considered one of the best producers of trehalose, α-amylase, glucoamylase, acid protease, raw starch-digesting glucoamylase and β-glucosidase among ascomycetous yeast species, and yeast is known to synthesizes trehalose by the TPS/TPP pathway [8]. Soluble starch, corn starch and cassava starch can be effectively converted into trehalose by yeast cells, and the trehalose content accumulated by the yeast can reach over 23% (w/w) because of its high amylase activity [5, 9, 20]. However, the accumulated trehalose in the cells of *S. fibuligera* must be extracted using 0.5 M trichloroacetic acid (TCA) solution, which is toxic and erosive, or hot water (80°C), which is energy consuming [9, 20].

Yeast cells are known to have a rigid, thick cell wall of about 200 nm thickness outside of the plasma membrane [15]. The cell wall of yeast cells is mainly composed of mannoproteins and β-linked glucans, and has β-1,3- and β-1,6-linked glucose and a fibrillar or brush-like outer layer composed predominantly of mannoproteins [15]. Many mutants displaying a thermosensitive autolytic phenotype have been isolated in *Saccharomyces cerevisiae*, *Candida albicans*, *Schizosaccharomyces pombe*, *Cryptococcus aureus* and *Aspergillus nidulans* [1, 19]. One characteristic shared by all these mutant strains is the loss of viability when they are cultured at the non-permissive temperature (usually 37°C), resulting in the release of the intracellular contents into the medium [1, 19]. Therefore, it is very important to obtain the highly thermosensitive and permeable mutants from *S. fibuligera* cells with high trehalose content so that the trehalose can be released from the yeast cells when they are cultivated in low-osmolarity water and at elevated temperatures because of a weakening of their cell membrane. Therefore, the main purpose of this study is to isolate a highly thermosensitive and permeable mutant from *S. fibuligera* A11-12 with a high content of trehalose in order to simplify the process for trehalose extraction. After their cells had accumulated a high content of trehalose from cassava starch, the trehalose was extracted into aqueous phase from the cells incubated at 37°C. This is a simple and efficient way to extract trehalose from yeast cells.

Materials and methods

Yeast strains and media

Saccharomycopsis fibuligera A11-12 in which the acid protease gene had been disrupted was obtained in the previous study and accumulated over 28.3% (w/w) trehalose in its cells [3, 4]. The highly thermosensitive and permeable mutant of *S. fibuligera* A11-12 was obtained as described below. These yeast strains were maintained in YPD medium containing 2.0% (w/v) glucose, 1.0% (w/v) yeast extract and 2.0% (w/v) polypeptone at 4°C or in YPS medium containing 2.0% soluble starch, 1.0% yeast extract and 2.0% polypeptone at 4°C. The trehalose accumulation medium contained 100.0 ml of the hydrolysate of soybean meal supplemented with 2.0% (w/v) cassava starch [9].

Isolation of a highly thermosensitive and permeable mutant

The cells of *S. fibuligera* A11-12 were aerobically grown in 10.0 ml of YPD at 28°C overnight, and 3.5×10^8 cells in 1.0 ml of the yeast cell suspension were collected and washed with sterile phosphate buffer (0.1 M and pH 7.5) by centrifugation at 5,000×*g* and 4°C for 5 min. The cell pellets were suspended in 1.0 ml of the same phosphate buffer, and 0.25 ml of 2.5 mg/ml of nitrosoguanidine (NTG) was thoroughly mixed with the cell suspension. The mixture was incubated at 28°C for 30 min, which was sufficient to kill 80% of the yeast cells. Then 1.25 ml of the sterile $Na_2S_2O_3$ solution (10% w/v) was added to the mixture immediately to cease the reaction. The treated cells were collected and washed with sterile saline water (0.85% NaCl) two times by centrifugation at 5,000×*g* and 4°C. The washed cell pellets were resuspended in fresh sterile saline water, and the suspension was diluted suitably. The diluted suspension with 50–80 cells/ml was spread on YPD plates, and the plates were incubated at 28°C for 3–5 days. The plates were transferred to a 37°C incubator and further incubated overnight [3]. A solution containing 0.5% (w/v) agar, 0.05 M glycine hydrochloride (pH 9.7) and 10 mM sodium β-nitrophenylphosphate was prepared as follows: agar and the glycine buffer were autoclaved separately and then mixed. A 0.1-M solution of sodium ρ-nitrophenylphosphate was filter-sterilized and stored at −20°C. Before use, the agar was melted by heating briefly in boiling water, and then cooled and maintained at 40°C. Sodium ρ-nitrophenylphosphate was added, and 3.3 ml of the liquid mixture was pipetted onto the plates. After 15 and 60 min overlay, the colonies containing the cells losing semi-permeability acquired a yellow color, and a yellow halo gradually spread out from them [3]. The colonies that had the largest yellow halo were selected and purified. One of the mutants was named A11-b.

Treatment of the yeast cells under different conditions

The mutant strains A11-b and *S. fibuligera* A11-12 were grown in the trehalose accumulation medium by shaking at 28°C for 60 h, respectively. The cells in the cultures were harvested and washed with the sterile saline water by centrifugation at 5,000×*g* and 4°C. One part of the washed cell pellets was suspended in the sterile distilled water, and cell density in the suspension was adjusted to 10^8 cells/ml. Another part of the washed cell pellets was suspended in 1.0 M of sorbitol solution, and cell density in the suspension was also adjusted to 10^8 cells/ml. The cell suspensions were incubated at 28 and 37°C overnight, respectively. The released trehalose concentration was quantitatively determined as described below.

Phase contrast microscopy

The yeast cells treated at 28 and 37°C as described above were observed under a phase contrast microscope (Olympus BH-2) and photographed, respectively.

Cassava starch

The cassava starch used in this study was purchased at the local cassava starch company in Qingdao, China.

Preparation of the hydrolysate of soybean meal

Thirty-two grams of soybean meal was mixed with 250.0 ml of tap water containing 0.25 M HCl. The mixture was autoclaved at 121°C for 25 min. After cooling, the pH of the mixture was adjusted to 5.5 with 1.0 M of NaOH solution, and the suspension was filtered. The filtrate was diluted to 1,600 ml [4].

Trehalose accumulation in the shaking culture

Seed cultures were prepared by inoculating the yeast cells of *S. fibuligera* A11-12 and the mutant strain A11-b grown on a YPD agar slant into a 300-ml Erlenmeyer flask that contained 50.0 ml of the YPS liquid medium, respectively, with subsequent incubation at 28°C for 24 h with shaking (200 rpm). The seed cultures (5.0 ml) were then transferred into 300-ml flasks that contained 45.0 ml of the hydrolysate of soybean meal supplemented with 2.0% (w/v) cassava starch, respectively, and the flasks were incubated at 30°C for 96 h with shaking (180 rpm).

Fermentation

Seed cultures were prepared by inoculating the yeast cells of the mutant strain A11-b grown on a YPD agar slant into a 500-ml Erlenmeyer flask that contained 100.0 ml of the YPS liquid medium and cultivating them at 28°C for 24 h with vigorous shaking. The fermentation was carried out in a SY-3005 B fermentor (Shiyuan Bio-engineering Equipment, Co., Ltd.) equipped with baffles, a stirrer, alkali pump, heating element, oxygen sensor and temperature sensor. Four hundred milliliters of the seed culture was transferred into 4,000 ml of the hydrolysate of soybean meal containing 88.0 g of cassava starch. The fermentation was performed under the conditions of an agitation speed of 200 rpm, aeration rate (sterile air volume input per min) of 4 l/min and temperature of 28°C for 96 h.

Trehalose extraction and assay

The yeast cells were collected from the cultures obtained above and washed by centrifugation at 5,000×*g* and 4°C for 10 min. Trehalose in the washed cells was extracted with both 0.5 M TCA solution at 4°C and sterile distilled water at 37°C. Trehalose content in the extracts was assayed by the Anthrone method [14].

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 Somogyi-Nelson method [13]. Residual total sugar was measured as the reduction of sugar after hydrolysis of the fermented media [4].

Purification of trehalose

Ice-cold absolute ethanol was slowly added to 1,000 ml of the aqueous extract obtained above with stirring until the precipitate appeared, to which saturated KCl solution (1.0–2.0%) 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. The crude trehalose (5.0 g) was dissolved in 25.0 ml of distilled water, and the 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 at 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 [7]. In order to remove the pigment, the 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 a 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 was 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; 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 using the Anthrone method [14]. 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 a 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 the Agilent Zorbax NH_2 column (5 μm) (4.6 × 250 mm) for determination of its purity. The HPLC condition:flow rate was 1.0 ml/min; column temperature was 35°C; the sample volume was 40 μl; the detector was DAD (200 nm); mobile phase was acetonitrile-water (7:3); the sample concentration was 5.0 mg/ml.

Results and discussion

Mutagenesis and screening for the highly thermosensitive and permeable mutants

It has been reported that the colonies containing the cells losing semi-permeability acquire a yellow color, and a yellow halo gradually spreads out from them on the plates containing sodium *ρ*-nitrophenylphosphate [3]. As mentioned above, trehalose in yeast cells are usually extracted with 0.5 M TCA solution. TCA is a protein denaturant that is toxic and erosive. Therefore, its use must be avoided in the food and pharmaceutical industries. In our previous studies [19, 21], it was found that most of the protein in the highly thermosensitive and permeable mutant cells suspended in distilled water can be released at 37°C. In order to know if trehalose in the highly thermosensitive and permeable mutant cells suspended in distilled water can also be released at 37°C, the cells of *S. fibuligera* A11-12 that contained over 28.3% (w/w) trehalose were mutated by using NTG as described in "Materials and methods," and over 1,000 mutants that had clear yellow halos on the plates were obtained after they were incubated at 37°C overnight. After determination of the released trehalose at 37°C, it was found that the mutant strain A11-b obtained could release the highest amount of trehalose (data not shown). As shown in Fig. 1, we found that the colonies of the mutant strain A11-b had large yellow halos (Fig. 1b), whereas the colonies of its wild type had no yellow halos (Fig. 1a). The results demonstrate that the mutant strain A11-b incubated at elevated temperature indeed liberated alkaline phosphatase, which gave rise to a yellow color on and around the colonies. This means that the methods used in this study for mutagenesis and screening of the highly thermosensitive and permeable mutants from *S. fibuligera* A11-12 were simple and sensitive. Therefore, the mutant strain A11-b was used for the subsequent investigations. After the cells of the mutant strain A11-b and *S. fibuligera* A11-12 were grown in the trehalose accumulation media for 60 h, the trehalose in the washed cells was extracted with both 0.5 M TCA at 4°C and sterile distilled water at 37°C, respectively, as described in "Materials and methods," and the trehalose release with 0.5 M TCA was regarded as 100%. The results in Fig. 2 indicate that over 73.8% of trehalose in the cells of the mutant strain A11-b was extracted with distilled water at 37°C, whereas only 9.98% of the trehalose in the cells of *S. fibuligera* A11-12 was extracted under the same conditions, suggesting that trehalose in the cells of the mutant strain A11-b was much more easily released in distilled water at 37°C than in its wild-type cells.

So far, many processes for trehalose production from starch have been developed [12, 16]. For example, when trehalose production from starch was carried out by using thermostable enzymes from *Sulfolobus acidocaldarius*, the two thermostable enzymes maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase) from *Sulfolobus acidocaldarius* ATCC 33909 had to be produced by cultivating the bacterial

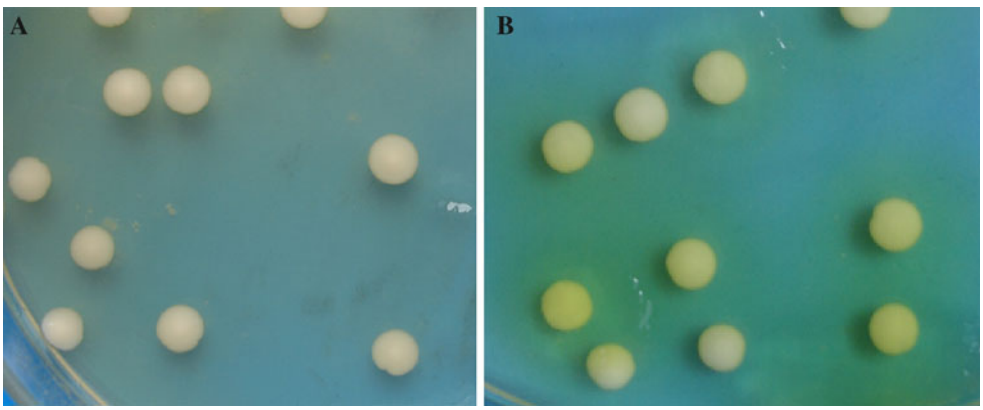


Fig. 1 The colonies of *S. fibuligera* A11-12 (**a**) and the *yellow halo* around the colonies of its highly thermosensitive and permeable mutants (**b**)

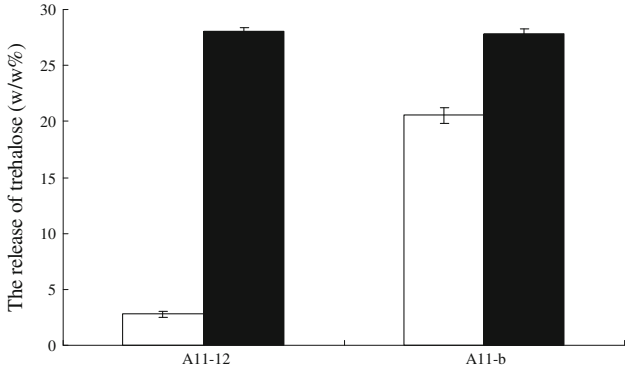


Fig. 2 Trehalose release from *S. fibuligera* A11-12 and the highly thermosensitive and permeable mutant (A11-b), which were suspended in an aqueous system (in *white*) and 0.5 M TCA (in *black*), respectively. Trehalose release in 0.5 M TCA solution was regarded as 100%. Data are given as means ± SD, $n = 3$

strain. Furthermore, isoamylase from *Pseudomonas amyloderamosa* as a debranching enzyme also must be used. Then, the concentrated thermostable enzymes are used to transform starch into trehalose [16]. In another study [12], the maltose phosphorylase (MP), trehalose phosphorylase (TP) and β-amylase produced by *Plesiomonas* sp. and a starch debranching enzyme (pullulanase, isoamylase) were used to develop a more efficient method for preparing trehalose from starch. Therefore, these processes are very complex, and at least three enzymes must be used. However, in the technique developed in this study, after the mutant had been cultivated in the cheap media containing cassava starch at 28°C and the cassava starch was directly converted into trehalose by one-step fermentation, then trehalose was extracted from the concentrated cells with water at 37°C. Therefore, we think the technique developed in this study is very simple.

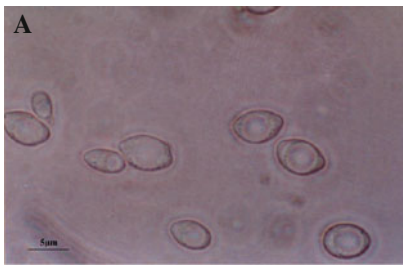
After the cells of *S. fibuligera* A11-12 and the mutant strain A11-b were treated at 28 and 37°C, respectively, they were observed under a phase contrast microscope and photographed. After they had been suspended in distilled water, the results in Fig. 3 demonstrate that the cell volume of the mutant strain A11-b treated at 37°C was increased compared to that of its wild-type treated at 28 and 37°C and that of the mutant strain A11-b treated at 28°C, respectively. This may be due to increased trehalose release from the mutant strain A11-b treated both at 37°C and in distilled water, and differences in osmolarity inside and outside of the cell membrane (Fig. 2). It also can be clearly seen from the results in Fig. 2 that no big changes in cell volume of the wild type were detected after being treated at 28 and 37°C, respectively. However, after suspension in 1.0 M of sorbitol solution, the results in Fig. 3 demonstrate that the cell volume of the mutant strain A11-b treated at 37°C was not changed compared to that of its wild type treated at 28 and 37°C and that of the mutant strain A11-b treated at 28°C, respectively. In another study [19], it was found that the intracellular density of the highly thermosensitive and permeable mutant of *Cryptococcus aureus* G7a with a high content of protein treated at 37°C was greatly decreased, and cell volume of the mutant treated at 37°C was increased because of the increased protein release. However, no significant changes in the intracellular density and cell volume of the mutant of *C. aureus* G7a were observed when its cells suspended in 1.0 M sorbitol solution were treated at 37°C.

Trehalose accumulation from cassava starch and cell growth by the mutant strain A11-b and its wild type during the cultivation at flask level

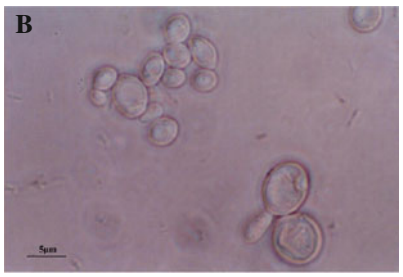
In order to know if the amount of accumulated trehalose in the mutant strain A11-b was negatively affected, the mutant and its wild type were cultivated in the trehalose accumulation media by shaking for 96 h, respectively, and the amount of accumulated trehalose and cell growth were examined. It can be clearly observed from the data in Fig. 4 that trehalose accumulation and cell growth of the mutant strain A11-b were almost the same as those of its wild type, suggesting that trehalose accumulation in the mutant strain A11-b and cell growth were not affected. In another study [19], the results showed that the crude protein contents in the highly thermosensitive and permeable mutant of *C. aureus* G7a and its wild type both could accumulate high contents of protein, which were also almost the same (over 53% crude protein).

Trehalose accumulation from cassava starch and cell growth by the mutant strain A11-b and its wild type during 5-l fermentation

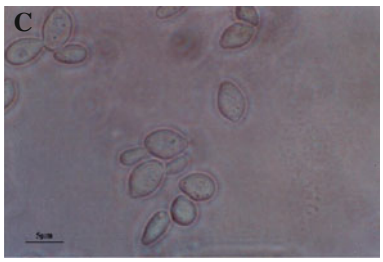
In order to scale up trehalose production from cassava starch by the mutant strain A11-b, the 5-l fermentation was performed as described in "Materials and methods." It can be observed from the data in Fig. 5 that within 48 h of fermentation, the rate of trehalose accumulation from cassava starch was very rapid, and the amount of accumulated trehalose reached the highest level (28.3% w/w) at 60 h of fermentation when cell growth (17.8 g of cell dry weight/l of the fermented medium) reached the stationary phase. It can be seen from the results in Fig. 6 that the total sugar and reducing sugar in the fermented media were 0.201% (w/v) and 0.121% (w/v), respectively. This means that almost all the added cassava starch was converted into trehalose and cell mass at the end of the fermentation. During 2-l fermentation, over 22.9 g of trehalose per 100 g of cell dry weight was accumulated from corn starch (2.0%) by *S. fibuligera* A11, the cell mass was 15.2 g/l of



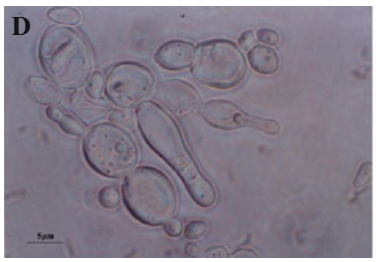
The cells of the strain A11-12 at 28°C and in the distilled water



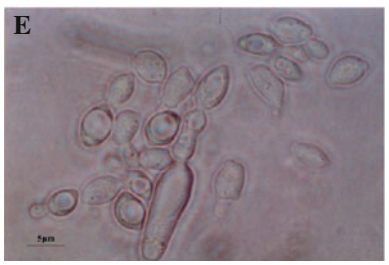
The cells of the mutant strain A11-b at 28°C and in the distilled water



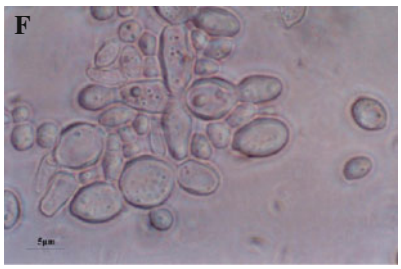
The cells of the strainA11-12 at 37°C and in the distilled water



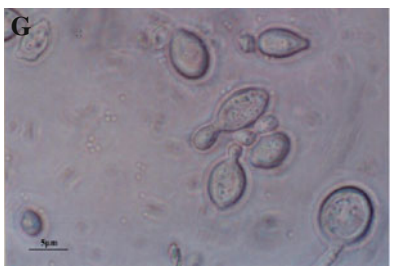
The cells of the mutant strain A11-b at 37°C and in the distilled water



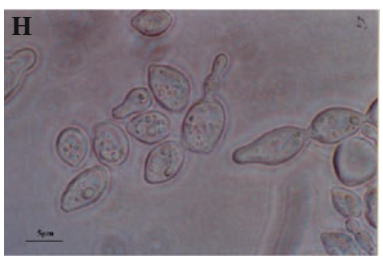
The cells of the strain A11-12 at 28°C and in1.0 M sorbitol solution



The cells of the mutant strainA11-b at 28°C and in 1.0 M sorbitol solution



The cells of the strain A11-12 at 37°C and in1.0 M sorbitol solution



The cells of the mutant strain A11-b at 37°C and in 1.0 M sorbitol soltion

Fig. 3 Microphotographs of *S. fibuligera* A11-12 and the mutant strain A11-b treated at 28 and 37°C, respectively. All the photos were taken at 100 × 10 magnification. **a** Strain A11-12 at 28°C and in sterile distilled water; **b** the mutant at 28°C and in sterile distilled water; **c** strain A11-12 at 37°C and in sterile distilled water; **d** the mutant strain A11-b at 37°C and in sterile distilled water; **e** strain A11-12 at 28°C and in 1.0 M sorbitol solution; **f** the mutant strain A11-b at 28°C and in 1.0 M sorbitol solution; **g** strain A11-12 at 37°C and in 1.0 M sorbitol solution; **h** the mutant strain A11-b at 37°C and in 1.0 M sorbitol solution

the fermented 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 fermentation [8]. In another study [20], the accumulated trehalose from cassava starch (2.0% w/v) by *S. fibuligera* A11 reached 24.8 g per 100 g of cell dry weight at the flask level. At the end of 2-l fermentation, the trehalose yield produced from cassava starch (2.0% w/v) by *S. fibuligera* A11 was 25.8 g per 100 g of cell dry weight, and the cell dry weight reached 22.8 g/l within 48 h of the fermentation. 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. This means that the mutant strain A11-b obtained in this study could accumulate very high trehalose contents.

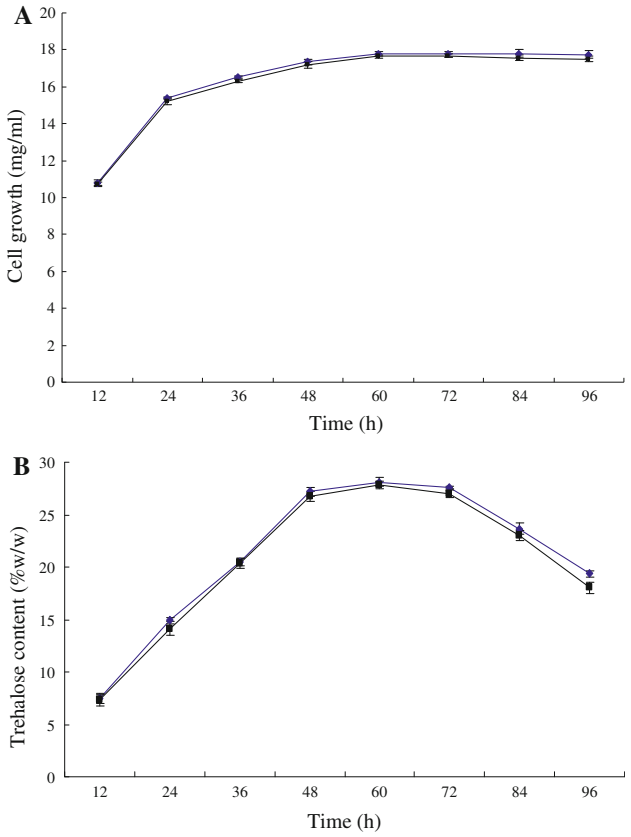


Fig. 4 Trehalose accumulation (**b**) and cell growth (**a**) by the mutant strain A11-b (*filled square*) and its wild type (*filled diamond*) at flask level. Data are given as means ± SD, $n = 3$

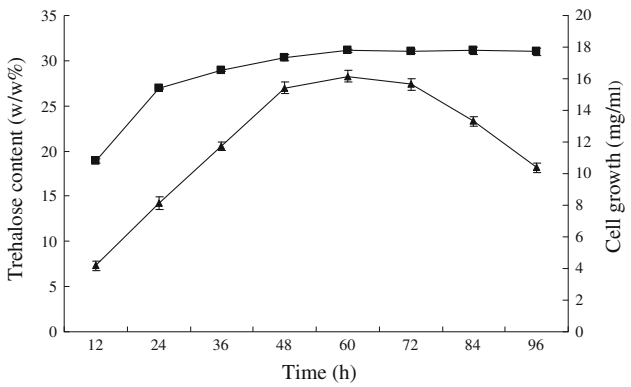


Fig. 5 Trehalose accumulation (*filled triangle*) and cell growth (*filled square*) of the mutant strain A11-b during the 5-l fermentation. Data are given as means ± SD, $n = 3$

Purification and crystallization of the produced trehalose

The yeast cells in the 5-l fermentor were harvested and washed by centrifugation, and the trehalose was extracted with sterile distilled water at 37°C. After purification of

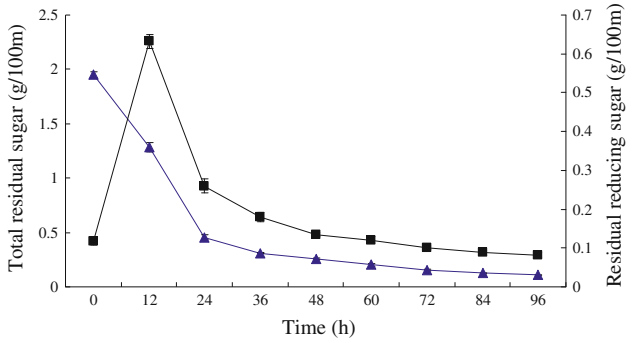


Fig. 6 The changes in residual total sugar (*filled triangle*) and residual reducing sugar (*filled square*) during the 5-l fermentation. Data are given as means ± SD, $n = 3$

trehalose in the aqueous extract as described in "Materials and methods," crystal trehalose was obtained (data not shown). 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). The results of HPLC of standard trehalose from Sigma and crystalline trehalose obtained in this study demonstrated that the sample extracted from the yeast cells with sterile distilled water only contained one component of trehalose. These findings identified the crystalline trehalose and standard trehalose as the same substance.

So far, TCA (0.5 M), ethanol, heat shock treatment and hot water (80°C) have been used for extraction of trehalose from fungal cells [9, 10, 14, 17]. However, none of these methods is economical, and they need a large amount of toxic TCA. Therefore, trehalose extraction from the mutant strain A11-b with distilled water at 37°C may be a simple and economical method. However, how to extract all the trehalose in the yeast mutant cells within a short time period is still a problem.

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