Mol Biotechnol
DOI 10.1007/s12033-013-9683-3

RESEARCH

Expression of *TPS1* Gene from *Saccharomycopsis fibuligera* A11 in *Saccharomyces* sp. W0 Enhances Trehalose Accumulation, Ethanol Tolerance, and Ethanol Production

Tian-Shu Cao · Zhe Chi · Guang-Lei Liu · Zhen-Ming Chi

Abstract It has been reported that trehalose plays an important role in stress tolerance in yeasts. Therefore, in order to construct a stably recombinant *Saccharomyces* sp. W0 with higher ethanol tolerance, the *TPS1* gene encoding 6-phosphate-trehalose synthase cloned from *Saccharomycopsis fibuligera* A11 was ligated into the 18S rDNA integration vector pMIRSC11 and integrated into chromosomal DNA of *Saccharomyces* sp. W0. The transformant Z8 obtained had the content of 6.23 g of trehalose/100 g of cell dry weight, while *Saccharomyces* sp. W0 only contained 4.05 g of trehalose/100 g of cell dry weight. The transformant Z8 also had higher ethanol tolerance (cell survival was 25.1 % at 18 ml of ethanol/100 ml of solution) and trehalose-6-phosphate synthase (Tps1) activity (1.3 U/mg) and produced more ethanol (16.4 ml of ethanol/100 ml of medium) than *Saccharomyces* sp. W0 (cell survival was 12.1 % at 18 ml of ethanol/100 ml of solution, Tps1 activity was 0.8 U/mg and the produced ethanol concentration was 14.2 ml of ethanol/100 ml of medium) under the same conditions. The results show that trehalose indeed can play an important role in ethanol tolerance and ethanol production by *Saccharomyces* sp. W0.

Keywords *TPS1* gene · Trehalose · Ethanol tolerance · *Saccharomyces* sp. W0 · *Saccharomycopsis fibuligera*

Introduction

Trehalose is a non-reducing disaccharide in which two glucose molecules are linked in an α,α-1,1-glycosidic linkage. In yeasts, trehalose is synthesized in a two-step process. The enzyme trehalose-6-phosphate synthase (Tps1) is identified to transfer one glucose residue from uridine diphosphate glucose (UDPG) to glucose-6-phosphate to produce trehalose-6-phosphate and UDP. Trehalose-6-phosphate phosphatase (Tps2) is identified from the same source to cause de-phosphorylation of trehalose-6-phosphate to yield trehalose [1]. Trehalose acts not only as a reserve carbohydrate, but also functions as a highly efficient protectant [2]. In yeasts and filamentous fungi, trehalose-6-phosphate also has many functions, such as control of glycolysis in yeasts [3].

In our previous studies [4–7], *Saccharomycopsis fibuligera* A11 was found to accumulate more than 25 g trehalose/100 g of cell dry weight in its cells and secrete high level of acid protease and amylases, suggesting that the yeast has very high Tps1 activity. The *TPS1* gene encoding Tps1 in this strain was also cloned by degenerate PCR using the CoDeHOP strategy [8]. We found that the expression of the *TPS1* gene in *S. fibuligera* A11 was kept constant under the various stress conditions [8]. Neither activation of Tps1 nor change in trehalose content was observed under the stress exposure of *S. fibuligera* A11 cells [8].

Electronic supplementary material The online version of this article (doi:10.1007/s12033-013-9683-3) contains supplementary material, which is available to authorized users.

T.-S. Cao · Z. Chi · G.-L. Liu · Z.-M. Chi (✉)
UNESCO Chinese Center of Marine Biotechnology and Institute of Biodiversity and Evolution, Ocean University of China, Yushan Road, No. 5, Qingdao, China
e-mail: zhenming@sdu.edu.cn

T.-S. Cao
e-mail: tian19850113@yahoo.cn

Z. Chi
e-mail: chizhe1108@yahoo.cn

G.-L. Liu
e-mail: lglouc@yahoo.com.cn

Published online: 09 July 2013

Mahmud et al. [9] thought that an increase in trehalose content was correlated with the increase in stress tolerance in *Saccharomyces cerevisiae*. It has been confirmed that *Saccharomyces* sp. W0 can produce high concentration of ethanol (more than 16.0 ml of ethanol/100 ml of medium) from glucose and sucrose [10, 11]. In this study, the *TPS1* gene cloned from *S. fibuligera* A11 was expressed in *Saccharomyces* sp. W0, and trehalose content in the transformants obtained was determined and the transformants with the enhanced trehalose synthesis was used to produce ethanol from sucrose.

Materials and Methods

Yeast Strains and Media

Saccharomyces sp. W0 which was a high ethanol-producing yeast [10] was used as the ethanol producer. The uracil mutant *Saccharomyces* sp. W12d was isolated from *Saccharomyces* sp. W0 as described below. *Saccharomycopsis fibuligera* A11 is the trehalose poor-assimilating mutant isolated from *S. fibuligera* sdu [2]. Growth medium was yeast–peptone–dextrose (YPD) medium containing 20 g glucose/l, 20 g peptone/l, and 10 g yeast extract/l. The ethanol fermentation medium was the synthetic medium [12] with 200 g sucrose/l and 10 g ammonium sulfate/l (see the Supplementary 1). The yeast transformants were grown in YNB medium containing 20 g glucose/l and 10 g ammonium sulfate/l.

Plasmids

The 18S rDNA integration vector pMIRSC11 containing the constitutive promoter *PGK1* and the effective terminator *CYC1*, in *Saccharomyces* sp. W0 was constructed in the previous study [13] (see the Supplementary 2). The plasmid pMD19-T simple was purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd.

Isolation of DNA, Restriction Digestions, and Transformation

DNA manipulations were carried out using the standard methods [14]. Bacterial plasmid DNA was purified using Perfectprep plasmid minikits (Eppendorf). Yeast genomic DNA for amplification of the *TPS1* gene in *S. fibuligera* A11 was isolated as described by Chi et al. [15]. Restriction endonuclease digestions and DNA ligations were performed according to the manufacture's recommendations. *Escherichia coli* was transformed with plasmid DNA according to Sambrook et al. [14]. *Escherichia coli* transformants were plated out onto LB medium containing ampicillin (100 μg/ml). *Saccharomyces* sp. W12d was transformed with the pMIRSC11 carrying the *TPS1* gene and the pMIRSC11 without carrying the *TPS1* gene.

Isolation of Uracil Mutants

The uracil mutant of *Saccharomyces* sp. W0 was isolated as described by Wang et al. [16]. After *Saccharomyces* sp. W0 was cultivated in the medium containing 0.075 % of 5-FOA at 25 °C for 4 days, the uracil mutants appeared were selected. One of the mutants was named *Saccharomyces* sp. W12d.

Integration of the *TPS1* Gene into Chromosomal DNA of *Saccharomyces* sp. W0

The *TPS1* gene was amplified using the genomic DNA of *S. fibuligera* A11 as template and the primers [the forward primer: tps1up 5′-CTGCAGATGGTTAACGGAAACGTT CTTG-3′ (the underlined bases encode *Pst*I restriction site) and the reverse primer: tps1down 5′-CTCGAGTCATAA CTCCATCTTAACTTG-3′ (the underlined bases encode *Xho*I restriction site)] designed according to the cloned *TPS1* gene from *S. fibuligera* A11 (the accession number: DQ364059) and multiple cloning sites of the expression vector pMIRSC11 (see the Supplementary 2). The PCR reaction and the conditions for the PCR amplification were used according to the methods described by Wang et al. [13]. The PCR products were cloned into pMD19-T simple vector and transformed into the competent cells of *E. coli* DH5α. The recombinant plasmid pMD19S-*TPS1* was extracted and was digested with *Pst*I and *Xho*I and the digests carrying the *TPS1* gene were ligated into pMIRSC11 digested with the same restriction enzymes. The resulting plasmid was designated as pMIRSC11-*TPS1* (see the Supplementary 2). The recombinant plasmids and pMIRSC11 without the *TPS1* gene were digested with *Not*I. The fragments carrying the *TPS1* gene and without carrying the *TPS1* gene were purified and transformed into the competent cells of the uracil mutant *Saccharomyces* sp. W12d, respectively, and different positive transformants were selected from the YNB medium. The different positive transformants were grown in the YPD medium at 28 °C for 3 days. The trehalose content and Tps1 activity in the different positive transformants were determined as described below. Finally, it was found that the transformant Z8 among them had the highest content of trehalose. Therefore, the transformant Z8 carrying the heterologous DNA fragment with the *TPS1* gene was used in the subsequent investigations and the transformant (the negative control) carrying the heterologous DNA fragment without the *TPS1* gene was named the transformant Z1.

Confirmation of Integration of the Target Gene into Genomic DNA of *Saccharomyces* sp. W0

To get the evidence that the DNA fragments carrying the *TPS1* gene had been integrated into the genomic DNA of *Saccharomyces* sp. W0, the genomic DNAs from the corresponding transformant Z8, the uracil mutant *Saccharomyces* sp. W12d and *Saccharomyces* sp. W0 were extracted and used as templates for PCR, respectively. The forward primer used for this checking was tps1up and the reverse primer was tps1down (sequences described earlier). PCR amplification was performed as described earlier. The sizes of the PCR products were estimated using the automated gel documentation and analysis system (Gene-Genius, USA).

Assay of Tps1 Activity and Protein Estimation

The transformant Z8, the transformant Z1, and *Saccharomyces* sp. W0 were aerobically cultivated in the YNB medium at 28 °C for 18 h. The yeast cells in 50.0 ml of the cultures were harvested by centrifugation and washed three times with deionized water. The washed cells were resuspended in 1.0 ml of the buffer ($Na_2HPO_4{\cdot}7H_20$ 16.19 g, $NaH_2PO_4{\cdot}H_20$ 5.5 g, KCl 0.75, $MgSO_4{\cdot}7H_20$ 0.246 g, mercaptoethanol 2.7 ml, distilled water 1,000 ml, pH 7.0). The cell suspensions were mixed with 1.0 g of glass beads (0.25–0.30 mm diameter). The mixtures were shaken in a Merckenschlager cell homogenizer (Braun-Melsungen, Germany) with CO_2 cooling for 7 min. Then, the mixtures were centrifuged at 4 °C and 14,000×*g* for 20 min. The supernatants obtained were used as the Tps1 preparations. In general, Tps1 activity was assayed at 37 °C by a colorimetric method as described by Hottiger et al. [17]. Briefly, all the sugars except trehalose-6-phosphate in the supernatants were removed by acid hydrolysis and trehalose-6-phosphate in the hydrolysate was reacted with the Anthrone reagent. Protein was measured by Bradford reagent as per technical bulletin provided by the manufacturer, against BSA standards. Unit of enzyme activity (U) was expressed as μmole of trehalose-6-phosphate synthesized per minute under the assay conditions. The specific enzyme activity was expressed as units per mg of protein.

Trehalose Extraction, Assay, and Measurement of Cell Dry Weight

Trehalose in the yeast cells was extracted with 0.5 M trichloroacetic acid and trehalose content in the extract was assayed by the anthrone method [18]. Cell dry weight was determined according to the methods described by Chi et al. [2]. Briefly, all the yeast cells in 5.0 ml of the culture were collected by centrifugation and washed using sterile saline water. The washed cells were dried at 80 °C until cell weight was constant.

Ethanol Shock Treatment

Ethanol shock treatment at 18 ml of absolute ethanol/100 ml of suspension and cell density of 3×10^7 cells/ml was carried out according to the methods described by Chi et al. [11]. Ethanol tolerance expressed as percentage survivors was determined by comparing the colony count of the ethanol-shocked cells with that of a non-shocked control. The transformant Z8 and *Saccharomyces* sp. W0 were aerobically cultivated in the YPD medium at 28 °C for 18 h. The same amount of the cell suspension was transferred to 50.0 ml of YPD media which contained 0 ml of ethanol per 100 ml of medium, 3.0 ml of absolute ethanol per 100 ml of medium, and 6.0 of absolute ethanol per 100 ml of medium, respectively, and the final $OD_{600\ nm}$ values of the initial cultures were 0.1. The cultures were continuously incubated by shaking at 50 rpm and 28 °C and $OD_{600\ nm}$ of the cultures was determined at interval of 8 h during the cell growth.

Ethanol Fermentation

After the yeast cells were aerobically grown at 28 °C in the synthetic medium with 20 g/l glucose and 10 g/l ammonium sulfate [12] for 18 h; around 15 ml of the culture (3×10^8 cells/ml) was collected and centrifuged at 4,000×*g* and 4 °C for 5 min. The collected cells (the total cell numbers were 45×10^8 cells) were transferred to each 300-ml bottle with 150 ml of the synthetic medium containing 200 g/l sucrose and 10 g/l ammonium sulfate and the final concentration of the yeast cells was 3×10^7 cells/ml of the fermentation medium. The bottles were fitted with rubber bungs perforated by a needle and incubated statically at 30 °C. The loss of weight by CO_2 liberation during the fermentation was monitored daily until fermentation ceased. As fermentation proceeded, additional sucrose was complemented to keep a suitable concentration of sucrose in the medium. The trehalose content and specific Tps1 activity were assayed as described above. The ethanol concentration in the fermented medium was determined by using gas chromatography (HP5890II, Hewlett-Packard, USA); the chromatography column was a fused silica AC-20 capillary column (30 m × 0.25 mm i.d., 0.25 m film thickness) and column temperature was 60 °C; detector: FID; detector temperature was 150 °C; injector temperature: 150 °C; carrier gas: helium, 1.0 ml/min; injection volume: 1.0 ml; the concentration of standard ethanol was 1.0 ml/ml.

Results

18S rDNA Integration of the *TPS1* Gene into Chromosomes of *Saccharomyces* sp. W0

Many results obtained in our laboratory have shown that *Saccharomyces* sp. W0 used in this study can produce high concentration of ethanol (more than 16.0 % v/v) [2, 11]. Our results also showed that *S. fibuligera* A11 could accumulate more than 25 g trehalose per 100 g of cell dry weight in its cells [2]. In order to enhance ethanol tolerance and construct a stably recombinant *Saccharomyces* sp. W0 in which the *TPS1* gene is integrated into its chromosomal DNA, the *TPS1* gene cloned from *S. fibuligera* A11 was ligated into the 18S rDNA integration vector pMIRSC11 (see the Supplementary 2). The linear DNA fragments carrying the *TPS1* gene and without carrying the *TPS1* gene were transformed into the competent cells of the uracil mutant of *Saccharomyces* sp. W0 and the different positive transformants obtained were grown in the YNB medium at 28 °C for 3 days. The trehalose contents in the transformants and *Saccharomyces* sp. W0 were determined as described in "Materials and Methods" section. The results in Fig. 1 showed that the transformant Z8 carrying the heterologous DNA fragment with the *TPS1* gene among them had the highest content of trehalose (6.23 g trehalose/100 g of cell dry weight) while the wild type *Saccharomyces* sp. W0 and the transformant Z1 (the negative control) that carried the heterologous DNA fragment without the *TPS1* gene only contained 4.05 g and 4.10 trehalose/100 g of cell dry weight, respectively. After Tps1 activity in the cells of the transformant Z8, the transformant Z1 and *Saccharomyces* sp. W0 was determined, respectively, the specific Tps1 activity (1.31 ± 0.13 U/mg) in the cells of the transformant Z8 was also found to be higher than that (0.75 ± 0.08 U/mg) in the cells of *Saccharomyces* sp. W0 and that (0.74 ± 0.07 U/mg) in the cells of the transformant Z1 (Table 1). After ANOVA analysis, there were significant differences ($P = 0.012$) between trehalose contents and between Tps1 activities of the transformant Z8 and *Saccharomyces* sp. W0 or the transformant Z1. However, there were no differences ($P > 0.12$) between trehalose contents and Tps1 activities of *Saccharomyces* sp. W0 and the transformant Z1. Therefore, only the transformant Z8 and *Saccharomyces* sp. W0 were used in the subsequent investigation.

As shown in the Supplementary 3, the results indicated that the linear DNA fragments (1,459 bp) encompassing the *TPS1* gene indeed had been integrated into the genomic DNA of the transformant Z8. However, no such PCR products were amplified from the genomic DNAs of *Saccharomyces* sp. W0 and the uracil mutant of *Saccharomyces* sp. W0 without carrying the cloned *TPS1* gene (the Supplementary 3).

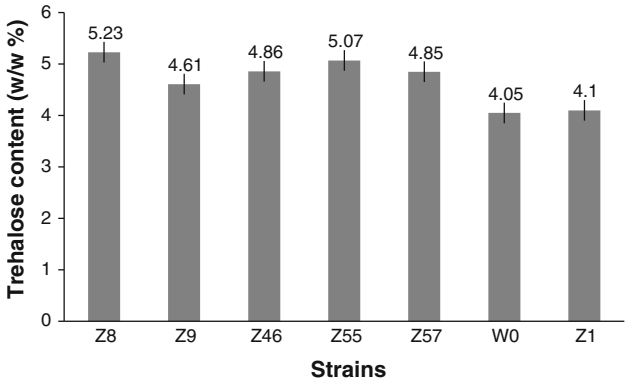

Fig. 1 Trehalose content of different transformants and *Saccharomyces* sp. W0. The different positive transformants and *Saccharomyces* sp. W0 were grown in the YPD medium at 28 °C for 3 days. Then, the trehalose contents were determined. Data are the mean standard derivates ($n = 3$)

Table 1 Tps1 activity of the transformant Z8, the transformant Z1, and *Saccharomyces* sp. W0

Yeast strains	TPS1 activity (units/mg of protein)
W0	0.75 ± 0.08
Z1	0.72 ± 0.07
Z8	1.31 ± 0.13

The yeast cells were aerobically cultivated in the YNB medium at 28 °C for 18 h. Then, Tps1 activity was determined. Data are the mean standard derivates ($n = 3$)

Ethanol Tolerance

The transformant Z8 and *Saccharomyces* sp. W0 were aerobically grown in YPD medium for 18 h and their cells were washed by centrifugation. The washed cells (3×10^7 cells/ml) were treated in 18.0 ml of absolute ethanol per 100 ml of the solution and cell survival was determined as described in "Materials and Methods" section. The results in Fig. 2 indicated that more cell survival of the yeast transformant Z8 was observed than that of *Saccharomyces* sp. W0 within the first 3 h of the treatment. For example, only 12.09 % of the cells of *Saccharomyces* sp. W0 survived within the first 3 h of the treatment, while the transformant Z8 kept 25.14 % survivors under the same conditions. After ANOVA analysis, there were significant differences ($P = 0.023$) between cell survivors of the two organisms. This meant that ethanol tolerance of the transformant Z8 was indeed enhanced compared to that of *Saccharomyces* sp. W0.

Cell Growth at Different Concentrations of Ethanol

When the transformant Z8 and *Saccharomyces* sp. W0 were grown in the YPD media contained 3.0 ml of absolute

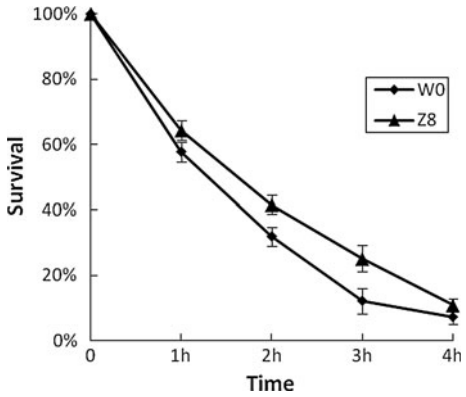

Fig. 2 Ethanol tolerance of the transformant Z8 and *Saccharomyces* sp. W0 during high ethanol shock treatment; the ethanol concentration was 18 % (v/v); the final cell density was 3×10^7 cells/ml; ethanol tolerance was expressed as percentage survivors. Data are the mean standard derivates ($n = 3$)

ethanol per 100 ml of the medium and 6.0 of absolute ethanol per 100 ml of the medium, respectively, the results in Fig. 3 revealed that growth rate of the transformant Z8 was higher than that of *Saccharomyces* sp. W0. However, when the transformant Z8 and *Saccharomyces* sp. W0 were grown in the YPD media containing 0 ml of ethanol per 100 ml of the cell suspension, the results in Fig. 3 indicated that growth rate of the transformant Z8 was similar to that of *Saccharomyces* sp. W0. This again demonstrated that ethanol tolerance of the transformant Z8 was indeed enhanced compared to that of *Saccharomyces* sp. W0 as the trehalose content and Tps1 activity in the transformant Z8 were higher than those in *Saccharomyces* sp. W0 (Fig. 1; Table 1).

Ethanol Fermentation

During ethanol fermentation by *Saccharomyces* spp., 1 mol of glucose is converted to 2 mol of CO_2 and 2 mol of CH_3–CH_2OH and CO_2 will be released from the fermentation system. The molecular weight (44) of CO_2 is almost the same as that (46) of CH_3–CH_2OH. Therefore, the lost weight (CO_2) was used to estimate the weight of ethanol produced in the closed fermentation system. This was the basis of carbon dioxide quantification in this study and CO_2 evolved was monitored by the decrease in weight of the whole culture [13]. The results in Fig. 4 showed that the lost weight per gram of cell dry weight in the culture of the transformant Z8 was higher than that per gram of cell dry weight in the culture of *Saccharomyces* sp. W0. At the end of the fermentation, the final ethanol concentration (16.4 ± 0.3 ml of ethanol/100 ml of medium) in the culture of the transformant Z8 was also higher than that (14.2 ± 0.25 ml of ethanol/100 ml of medium) in the culture of *Saccharomyces* sp. W0 (Table 2). The data in

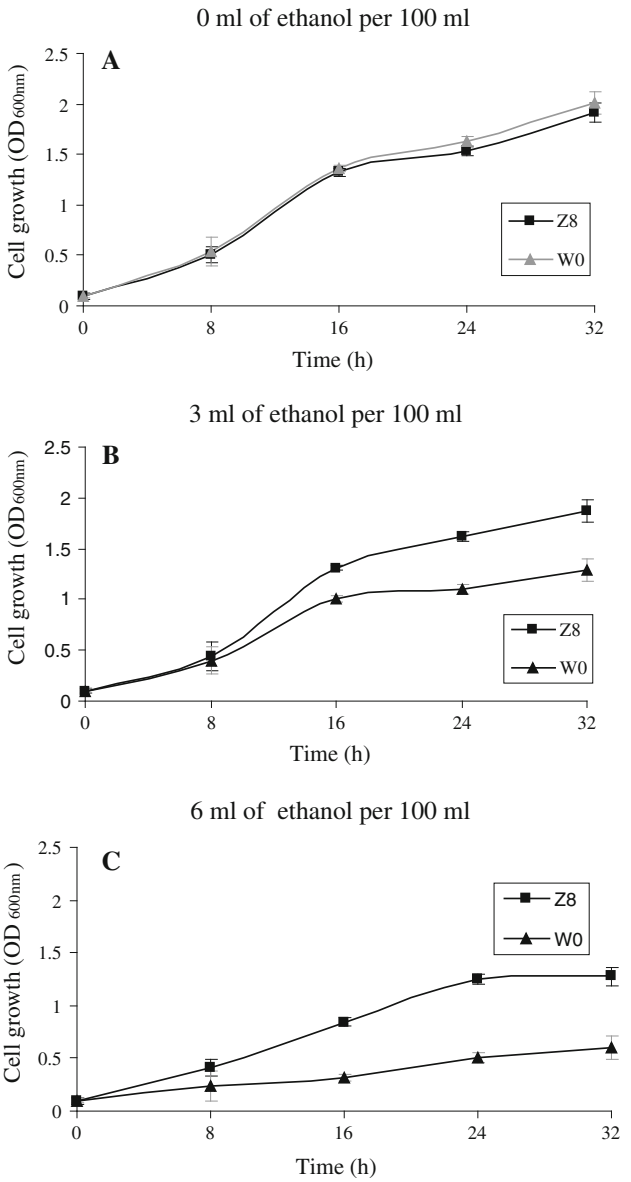

Fig. 3 Cell growth in YPD media with 0 ml of ethanol per 100 ml (**a**), 3 ml of ethanol per 100 ml (**b**), and 6 ml of ethanol per 100 ml (**c**) ethanol. The initial cell density was 0.1 of OD_{600nm}. The cultures were incubated by shaking at 50 rpm and 28 °C and OD_{600nm} of the cultures was determined at interval of 8 h. Data are the mean standard derivates ($n = 3$)

Fig. 4 also showed that more trehalose in the cells of the transformant Z8 was accumulated than that in the cells of *Saccharomyces* sp. W0 and higher specific Tps1 activity in the cells of the transformant Z8 was achieved than that in the cells of *Saccharomyces* sp. W0 during the fermentation. After ANOVA analysis, there were significant differences ($P = 0.015$) between trehalose contents and between Tps1 activities of the two organisms and between ethanol concentrations of the two cultures. The results in Table 2 and Fig. 4 implied that the trehalose may also be responsible for more ethanol production by the transformant Z8.

Discussion

After the *TPS1* gene was expressed in the high ethanol-producing yeast strain W0, the transformant Z8 obtained had the highest content of trehalose (6.23 g per 100 g of cell dry weight) while *Saccharomyces* sp. W0 only contained 4.05 g trehalose per 100 g of cell dry weight (Fig. 1) and the specific Tps1 activity in the cells of the transformant Z8 was 1.31 ± 0.13 U/mg whereas the Tps1 activity was 0.75 ± 0.08 U/mg in the cells of *Saccharomyces* sp. W0 (Table 1). However, the disruptant (in which the gene encoding acid protease was disrupted) of *S. fibuligera* A11 could accumulate 28.1 g of trehalose per 100 g of cell dry weight within 60 h of the cultivation when it was cultivated in the cassava starch medium while *S. fibuligera* A11 accumulated only 23.6 g of trehalose per 100 g of cell dry weight within 60 h of the cultivation under the same conditions [1]. Therefore, even after the *TPS1* gene cloned from *S. fibuligera* A11 was expressed in *Saccharomyces* sp. W0, the transformant Z8 obtained synthesized much less trehalose than *S. fibuligera* A11. After the *TPS1* gene from *Metarhizium anisopliae* was cloned into *Pichia pastoris* KM71, the activity of the Tps1 produced by the recombinant *P. pastoris* KM71 decreased rapidly as the concentrations of phosphate increased [19].

After the *TPS1* gene was expressed in the high ethanol-producing yeast strain W0, ethanol tolerance of the transformant Z8 was indeed enhanced compared to that of *Saccharomyces* sp. W0 (Figs. 2, 3). Mahmud et al. [9] reported that high trehalose accumulation can make yeast cells resistant to multiple stresses, including ethanol and heat stress induction. In addition, increase in intracellular accumulation of trehalose by deletion of the acid trehalase gene *ATH1* improved ethanol stress tolerance [20, 21]. In recent years, ethanol tolerance in *S. cerevisiae* has also been improved by other approaches. For example, expression of the pol3-01 gene, a proofreading-deficient of DNA polymerase δ, in *S. cerevisiae* W303-1A resulted in three ethanol-tolerant mutants (YFY1, YFY2, and YFY3), which could grow in medium containing 13.0 ml of ethanol per 100 ml. During ethanol shock treatment, viability of the W303-1A cells drastically decreased in the presence of ethanol, while that of YFY strains was maintained by high level [22].

Table 2 The final ethanol concentrations in the fermented media

The yeast strains	Ethanol concentrations (ml/100 ml)
Saccharomyces sp. W0	14.2 ± 0.25
The transformant Z8	16.4 ± 0.3

The ethanol concentration in the fermented cultures was determined using GC. Data are the mean standard derivates ($n = 3$)

The results in Table 2 and Fig. 4 indicated that ethanol production, trehalose biosynthesis and specific Tps1 activity by the transformant Z8 were enhanced compared to those by its parent yeast strain *Saccharomyces* sp. W0, suggesting that trehalose may be responsible for more ethanol production by the transformant Z8. Abe et al. [22] found that when the three ethanol-tolerant mutants (YFY1, YFY2, and YFY3) were grown in the fermenting media with 25 g of glucose per 100 ml, their ethanol productivity was more than 11.0 ml of ethanol per 100 ml, while their wild-type strain W303-1A did not form more than 11.0 ml of ethanol per 100 ml under these conditions. They also found that expression levels of Tps1 and Tsl1, which were responsible for trehalose biosynthesis, were higher in YFY strains than those in its wild-type strain W303-1A,

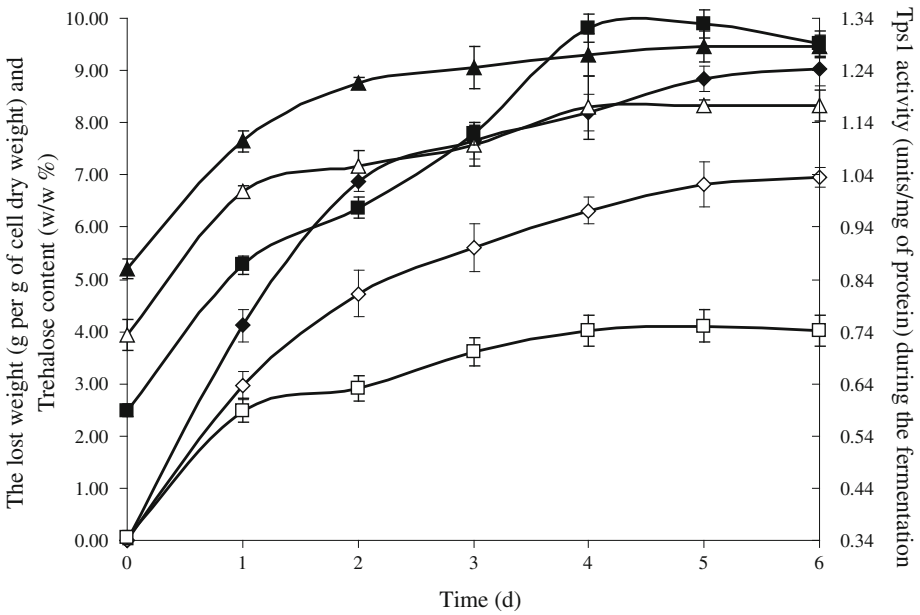

Fig. 4 The time course of the lost weight (*filled diamond* and *open diamond*) (CO_2 release) in the fermentation system, trehalose content (*filled triangle* and *open triangle*) and specific Tps1 activity (*filled square* and *open square*) in the transformant Z8 (*black symbol*) and *Saccharomyces* sp. W0 (*white symbol*), respectively, during the ethanol fermentation. The fermentation was performed in the closed system with 150 ml of the medium incubated at 30 °C. Data are the mean standard derivates ($n = 3$)

resulting in high levels of intracellular trehalose in YFY strains. In contrast, over-expression of *GLT1* gene (encoding glutamate synthase) along with *FPS1* gene (encoding a channel protein responsible for glycerol export) deletion resulted in a 14 % higher ethanol production (ethanol concentration was increased from 10.8 to 11.9 and 12.3 g/100 ml) and a 30 % lower glycerol formation than those in the parental strain under anaerobic fermentation conditions [23].

During ethanol fermentation, yeast cells are also exposed to various stresses including a high concentration of ethanol. An ethanol-tolerant yeast strain would make it possible to reduce fermentation time, leading to an increase in ethanol productivity [24]. As shown above, trehalose could play an important role in ethanol tolerance and ethanol production by *Saccharomyces* sp. W0 (Figs. 2, 3, 4). However, as the mechanism underlying the ethanol tolerance of yeast cells is very complex and involves more than 200 genes [25], other genes related to ethanol tolerance may need to be expressed in *Saccharomyces* sp. W0 in order to further enhance ethanol tolerance of this yeast strain.

Acknowledgments This research was supported by Grant 31070029 from the National Natural Science Foundation of China.

References

1. Wang, D. S., Zhao, S. F., Zhao, M. X., et al. (2011). Trehalose accumulation from cassava starch and release by a highly thermosensitive and permeable mutant of *Saccharomycopsis fibuligera*. *Journal of Industrial Microbiology and Biotechnology, 38*, 1545–1552.
2. Chi, Z. M., Liu, J., Ji, J. R., et al. (2003). Enhanced conversion of starch to trehalose by a mutant of *Saccharomycopsis fibuligera* sdu. *Journal of Biotechnology, 102*, 135–141.
3. Eastmond, P. J., & Graham, I. A. (2003). Trehalose metabolism: a regulatory role for trehalose-6-phosphate? *Current Opinion of Plant Biology, 6*, 231–235.
4. Chi, Z., Wang, J. M., Chi, Z. M., et al. (2010). Trehalose accumulation from corn starch by *Saccharomycopsis fibuligera* A11 during 2-l fermentation and trehalose purification. *Journal of Industrial Microbiology and Biotechnology, 37*, 19–25.
5. Chen, L., Chi, Z. M., Chi, Z., et al. (2010). Amylase production by *Saccharomycopsis fibuligera* A11 in solid-state fermentation for hydrolysis of cassava starch. *Applied Biochemistry and Biotechnology, 162*, 252–263.
6. Yu, X. J., Li, H. J., Li, J., et al. (2010). Overexpression of acid protease of *Saccharomycopsis fibuligera* in *Yarrowia lipolytica* and characterization of the recombinant acid protease for skimmed-milk-clotting. *Biotechnology and Bioprocess Engineering, 87*, 669–677.
7. Zhang, Y., Zhang, T., Chi, Z., et al. (2010). Conversion of cassava starch to trehalose by *Saccharomycopsis fibuligera* A11 and purification of trehalose. *Carbohydrate Polymers, 80*, 13–18.
8. Liang, L. K., Wang, X. K., Zhu, K. L., et al. (2006). Trehalose accumulation in a high-trehalose accumulating mutant of *Saccharomycopsis fibuligera* sdu does not respond to stress treatments. *Biochemistry, 71*, 1291–1297.
9. Mahmud, S. A., Hirasawa, T., & Shimizu, H. (2010). Differential importance of trehalose accumulation in *Saccharomyces cerevisiae* in response to various environmental stresses. *Journal of Biosciences ad Bioengineering, 109*, 262–266.
10. Chi, Z. M., & Liu, Z. R. (1993). High-concentration alcoholic production from hydrolysate of raw ground corn by a tetraploid yeast strain. *Biotechnology Letters, 15*, 877–882.
11. Chi, Z. M., Kohlwein, S. D., & Paltauf, F. (1999). Role of phosphatidylinositol (PI) in ethanol production and ethanol tolerance by a high ethanol producing yeast. *Journal Industrial Microbiology and Biotechnology, 22*, 58–63.
12. Chi, Z. M., Tani, Y., & Hayashida, S. (1991). Construction 377 of tetraploid cells by protoplast fusion and heat treatment in ethanol tolerant yeasts. *Annual Reports of ICBiotech, 14*, 135–145.
13. Wang, J. M., Zhang, T., Chi, Z., et al. (2011). 18S rDNA integration of the exo-inulinase gene into chromosomes of the high ethanol producing yeast *Saccharomyces sp*. W0 for direct conversion of inulin to bioethanol. *Biomass and Bioenergy, 35*, 3032–3039.
14. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd ed., pp. 367–370). Beijing: Cold Spring Harbor Laboratory Press. (Chinese translating ed).
15. Chi, Z. M., Ma, C., Wang, P., et al. (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Bioresource Technology, 98*, 534–538.
16. Wang, F., Yue, L. X., Wang, L., et al. (2009). Genetic modification of the marine derived yeast *Yarrowia lipolytica* with high-protein content using a GPI-anchor-fusion expression system. *Biotechnology Progress, 25*, 1297–1303.
17. Hottiger, T., Schmutz, P., & Wiemken, A. (1987). Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. *Journal of Bacteriology, 169*, 5518–5522.
18. Stewart, P. R. (1982). Analytical methods for yeasts. In D. M. Prescott (Ed.), *Methods in cell biology* (Vol. 12, pp. 111–147). London/New York: Academic Press.
19. Cai, Z., Peng, G., Cao, Y., et al. (2009). Trehalose-6-phosphate synthase from *Metarhizium anisopliae*: clone, expression and properties of the recombinant. *Journal of Biosciences and Bioengineering, 107*, 499–505.
20. Kim, J., Alizadeh, P., Harding, T., et al. (1996). Disruption of the yeast *ATH1* gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications. *Applied and Environmental Microbiology, 62*, 1563–1569.
21. Jung, Y. J., & Park, H. D. (2005). Antisense-mediated inhibition of acid trehalase (*ATH1*) gene expression promotes ethanol fermentation and tolerance in *Saccharomyces cerevisiae*. *Biotechnology Letters, 27*, 1855–1859.
22. Abe, H., Fujita, Y., Takaoka, T., et al. (2009). Ethanol-tolerant *Saccharomyces cerevisiae* strains isolated under selective conditions by over-expression of a proofreading-deficient DNA polymerase δ. *Journal of Biosciences and Bioengineering, 108*, 199–204.
23. Kong, Q. X., Gu, J. G., Cao, L. M., et al. (2006). Improved production of ethanol by deleting *FPS1* and over-expressing *GLT1* in *Saccharomyces cerevisiae*. *Biotechnology Letters, 28*, 2033–2038.
24. Jones, R. (1989). Biological principles for the effects of ethanol. *Enzyme and Microbial Technology, 11*, 130–153.
25. Kubota, S., Takeo, I., Kume, K., et al. (2004). Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol. *Biosciences, Biotechnology and Biochemistry, 68*, 968–972.