Biotechnol Lett (2011) 33:1367–1374
DOI 10.1007/s10529-011-0576-x

ORIGINAL RESEARCH PAPER

Enhanced thermotolerance for ethanol fermentation of *Saccharomyces cerevisiae* strain by overexpression of the gene coding for trehalose-6-phosphate synthase

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Received: 28 November 2010 / Accepted: 22 February 2011 / Published online: 6 March 2011

Abstract The effect of overexpression of the trehalose-6-phosphate (T6P) synthase gene (*TPS1*) on ethanol fermentation of *Saccharomyces cerevisiae* has been studied at 30 and 38°C. The activity of T6P synthase and the accumulation of trehalose during ethanol fermentation were significantly improved by overexpression of *TPS1*, and especially at 38°C. Ethanol produced by transformants with and without *TPS1* gene overexpression at 38°C was approx. 60 and 37 g/l, respectively. The fermentation efficiency of transformants with *TPS1* gene overexpression at 38°C was similar to that at 30°C. The critical growth temperature was increased from 36 to 42°C by *TPS1* gene overexpression. These results indicated that overexpression of the *TPS1* gene had a beneficial effect on the fermentation capacity of the title yeast strain at high temperatures.

Keywords Ethanol fermentation · *Saccharomyces cerevisiae* · Thermotolerance · *TPS1* gene · Trehalose-6-phosphate synthase

Introduction

Saccharomyces cerevisiae is used for industrial ethanol production. However, the temperature must be maintained at about 30°C which necessitates considerable cooling. On the other hand, the simultaneous saccharification and fermentation process, which has been employed by most major ethanol producers, requires higher temperatures to achieve efficient ethanol conversion. Hence, improvement of the thermotolerance of these yeast strains would be beneficial for low-cost bioethanol production. Physical and chemical mutagenesis (Rajoka et al. 2005), adaptation (Balakumar et al. 2001), protoplast fusion (Ezeronye et al. 2001) as well as gene shuffling (Shi et al. 2009) have been employed to increase the thermotolerance of yeast.

The relationship between thermotolerance and trehalose has been studied (Singer and Lindquist 1998), and trehalose is invariably accumulated after heat shock (Estruch 2000; Mahmud et al. 2010). However, studies on the effect of trehalose accumulation and the constitutive synthesis of trehalose on the thermotolerance of yeast during ethanol fermentation remain limited (Soto et al. 1999).

Electronic supplementary material The online version of this article (doi:10.1007/s10529-011-0576-x) contains supplementary material, which is available to authorized users.

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Trehalose-6-phosphate (T6P) synthase converts glucose 6-phosphate and UDP-glucose to trehalose 6-phosphate. Since the trehalose content and the activity of T6P synthase show a positive correlation (Cansado et al. 1998; Hottiger et al. 1987), in this study the influence of T6P synthase gene (*TPS1*) overexpression on the thermotolerance of the *S. cerevisiae* strain during ethanol fermentation has been investigated. It was envisaged that this might provide a much simpler means of improving the thermotolerance of industrial yeast for ethanol production. Guo et al. (2010) reported that by overexpressing *TPS1* and *TPS*2 (encoding trehalose-6-phosphate phosphatase), the osmotic stress tolerance, growth rate, and ethanol fermentation ability of the yeast strain were improved; however, the effect on thermotolerance was not studied.

Materials and methods

Yeast strains

The *S. cerevisiae* strains used in this study are shown in Table 1. Strain 10151 were used as host strains for transformation and strains EP-1 and 14801 were used for preparing chromosomal DNAs.

Construction of pI-RED1-*TPS1* plasmid with *TPS1* overexpression cassette

Figure 1 shows a flow chart for the construction of pI-RED1-*TPS1* plasmid with the *TPS1* overexpression cassette. The *TPS1* gene was amplified from the chromosomal DNA of strain EP-1 by PCR using the primers TPS1F and TPS1R (Supplementary Table 1). KOD-Plus polymerase (Toyobo, Osaka, Japan) was used according to the manufacturer's instructions. The constructed plasmid was designated pI-RED1-*TPS1* and used for transformations.

Construction of a DNA fragment with the *TPS1* overexpression cassette by fusion PCR

Fusion PCR was used to amplify the *TPS1* overexpression cassette with *URA3* as the marker (Fig. 2). The P_{URA3}-*URA3*-P_{ADH1}-*TPS1*-T_{ADH1} fragment and the downstream fragment of the *URA3* gene were amplified respectively by PCR1 and PCR2. The final fusion fragment (4.6 kb) was amplified by PCR3 and then used for transformations. An approximately 500 bp fragment of P_{URA3}-*URA3* containing the 5′-part of the *URA3* gene sequence was amplified from strain 14801 for construction of the *URA3*-recovered strain of 10151.

Table 1 *Saccharomyces cerevisiae* strains and plasmids used in this study

	Relevant genotype	Reference
Strain		
NBRC10151	*MAT*a *his3-532 trp1-289 ura3-1 ura3-2 ade2*	NITE Biological Resource Center, Japan
NBRC10481	*MAT*a *his6*	
EP1	*MAT*a/α	Our lab
10151-pI-RED1	*MAT*a *his3-532 trp1-289 ura3-1 ura3-2 ade2 YAP1::(URA3-YAP1)* (pI-RED1)	This work
10151-pI-RED1-*TPS1*	*MAT*a *his3-532 trp1-289 ura3-1 ura3-2 ade2 YAP1::(URA3-TPS1-YAP1)* (pI-RED1-TPS1)	This work
10151-*URA3*	*MAT*a *his3-532 trp1-289 ade2 ura3::URA3*	This work
10151-*URA3-TPS1*	*MAT*a *his3-532 trp1-289 ade2 ura3::(URA3-TPS1)*	This work
Plasmid		
pAUR123	*ADH1p-ADH1t*	Our lab
pI-RED1	*URA3, YAP1*	Toyobo, Japan
pI-RED1-*TPS1*	*ADH1p-TPS1-ADH1t, URA3, YAP1*	This work

Strains were cultivated using 2% YPD medium with 40 mg adenine/l at 28°C

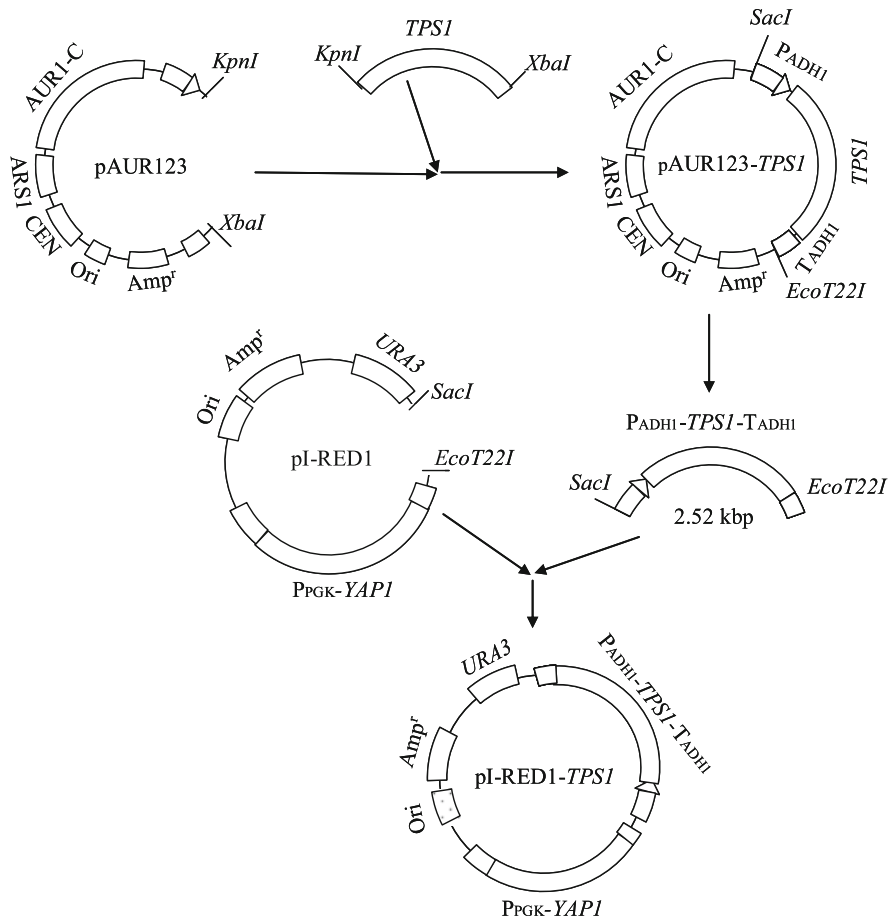

Fig. 1 Construction of plasmid pI-RED1-*TPS1* with *TPS1* high-expression cassette. The amplified TPS1 fragment was digested with *Kpn*I and *Xba*I and inserted into plasmid pAUR123, which had *ADH1* promoter and terminator, and had been linearized with *Kpn*I and *Xba*I. The constructed plasmid was designated pAUR123-*TPS1*. P_{ADH1}-*TPS1*-T_{ADH1} fragment was obtained by digesting pAUR123-*TPS1* with *Sac*I and *Eco*T22I and inserted into *Sac*I and *Eco*T22I-digested plasmid pI-RED1 (*URA3*, *YAP1*)

Transformation of plasmid and DNA fragment to the host strain

Yeast transformation was performed by the lithium acetate method (Gietz et al. 2002). Plasmids pI-RED1-*TPS1* and pI-RED1 were linearized with *Aor*51HI (one digestion site in the *YAP1* gene) and transformed to the *YAP1* gene region by homology transformation in the host strain 10151 to produce *TPS1* overexpression strain 10151-pI-RED1-*TPS1* and *URA3*-recovered strain 10151-pI-RED1. Fragments P_{URA3}-*URA3*-P_{ADH1}-*TPS1*-T_{ADH1}-$URA3_{downstream}$ and P_{URA3}-*URA3* were transformed to *URA3* gene region by homology transformation (Fig. 2) in the host strain 10151 to produce *TPS1* overexpression strain 10151-*URA3*-*TPS1* and *URA3*-recovered strain 10151-*URA3*. Transformants 10151-pI-RED1 and 10151-*URA3* were used to eliminate the possible effect of the *URA3* gene on the evaluation of overexpression of the *TPS1* gene. The transformants were selected using *URA3* as the selection marker on an MM plate (1.7 g YNB/l, 5 g $(NH_4)_2SO_4$/l, 20 g glucose/l, 20 g agar/l) with the addition of 40 mg adenine/l, 40 mg tryptophan/l and 40 mg histidine/l. Chromosomal DNA of the host strain and all transformants was extracted by using a *Gen Toru Kun* kit (Takara Shuzo Co., Ltd., Shiga, Japan) and used as a DNA template for PCR check. Primers A123MCS-F and A123MCS-R amplifying fragments between P_{ADH1} and T_{ADH1} were used to check the introduced *TPS1* gene in transformant 10151-pI-RED1-*TPS1*. Primers URA3Up-F and URA3Down-R were used to check the introduced *TPS1* gene in transformant 10151-*URA3*-*TPS1*.

Ethanol fermentation of host strain and transformants at 30 and 38°C

Ethanol fermentation of host and transformants were carried out at 30 and 38°C using 5% YPD as precultivation medium and 15% YPD as fermenting

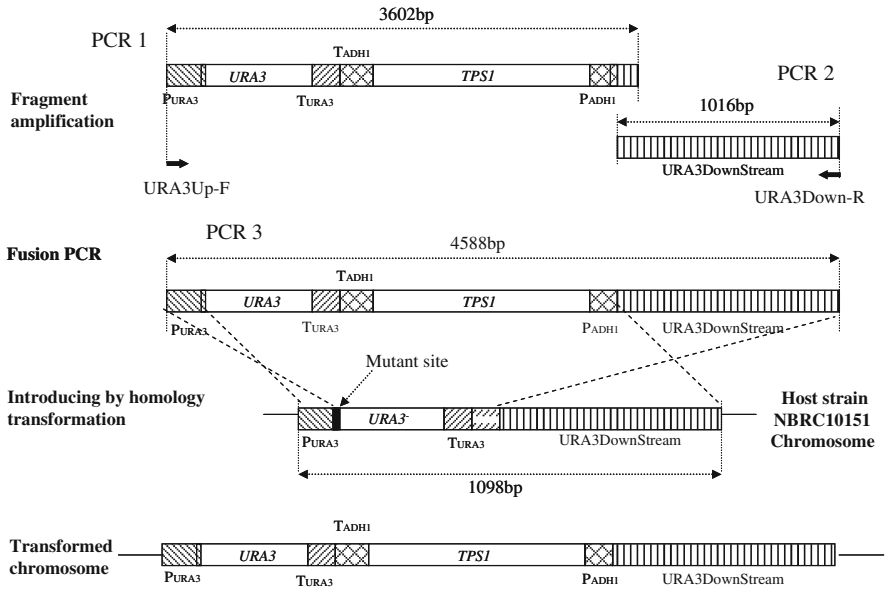

Fig. 2 Preparation of DNA fragment with *TPS1* high-expression cassette for homology transformation. PCR1 was carried out using primers URA3Up-F and URA3Down-P_{ADH1}-R (Supplementary Table 1) to amplify the P_{URA3}-*URA3*-P_{ADH1}-*TPS1*-T_{ADH1} fragment (3.6 kb) with the plasmid pI-RED1-*TPS1* as template. PCR2 was carried out using primers URA3Down-F and URA3Down-R to amplify the downstream fragment (1 kb) of the *URA3* gene with the chromosomal DNA of strain 10151 as the template. The PCR products from PCR1 and PCR2 were purified using Ultraclean PCR clean-up kit (MO BIO Laboratories, Inc., Carlsbad, CA). Primers URA3Up-F and URA3Down-R were used to amplify a 4.6 kb fusion fragment by mixing the PCR1 and PCR2 products as templates

medium. The fermentation was performed for 48 h and the ethanol concentration, cell number, T6P synthase activity and trehalose concentration were analyzed to evaluate the influence of the overexpression of *TPS1* gene.

Growth of transformants at 36–42°C

Growth of strains 10151-*URA3* and 10151-*URA3*-*TPS1* was compared at 36, 38, 40, and 42°C under aerobic conditions using a TVS062CA biophotorecorder (Advantec, Tokyo, Japan) with 2% YPD medium. Growth was started with an initial cell number of 1×10^6 cells/ml, which was approximately 1/10 of that used in the ethanol fermentation experiments.

Analytical methods

Ethanol in the supernatant of centrifuged broth (2,500×*g* at 4°C for 10 min) was assayed by GC with 2-propanol as internal standard (Tang et al. 2006). The total number of cells and the number of viable cells were calculated by the Methylene Blue staining method using a hemocytometer. The activity of T6P synthase and the concentration of trehalose were determined as described previously (Hottiger et al. 1987). One unit of T6P synthase was determined as that responsible for the synthesis of 1 μmol trehalose 6-phosphate in 1 min at 35°C. HPLC equipped with a Shodex Sugar SC1821 column and an RI detector was used for trehalose determination.

Results and discussion

Plasmid and DNA fragment construction and transformation

Plasmid pI-RED1-*TPS1* was constructed according to Fig. 1 and checked by restriction endonuclease digestion (data not shown). P_{URA3}-*URA3*-P_{ADH1}-*TPS1*-T_{ADH1}-$URA3_{downstream}$ fragment (4.6 kb) was obtained by fusion PCR as described in Fig. 2. Transformants 10151-pI-RED1-*TPS1*, 10151-pI-RED1, 10151-*URA3*-*TPS1*, and 10151-*URA3* were

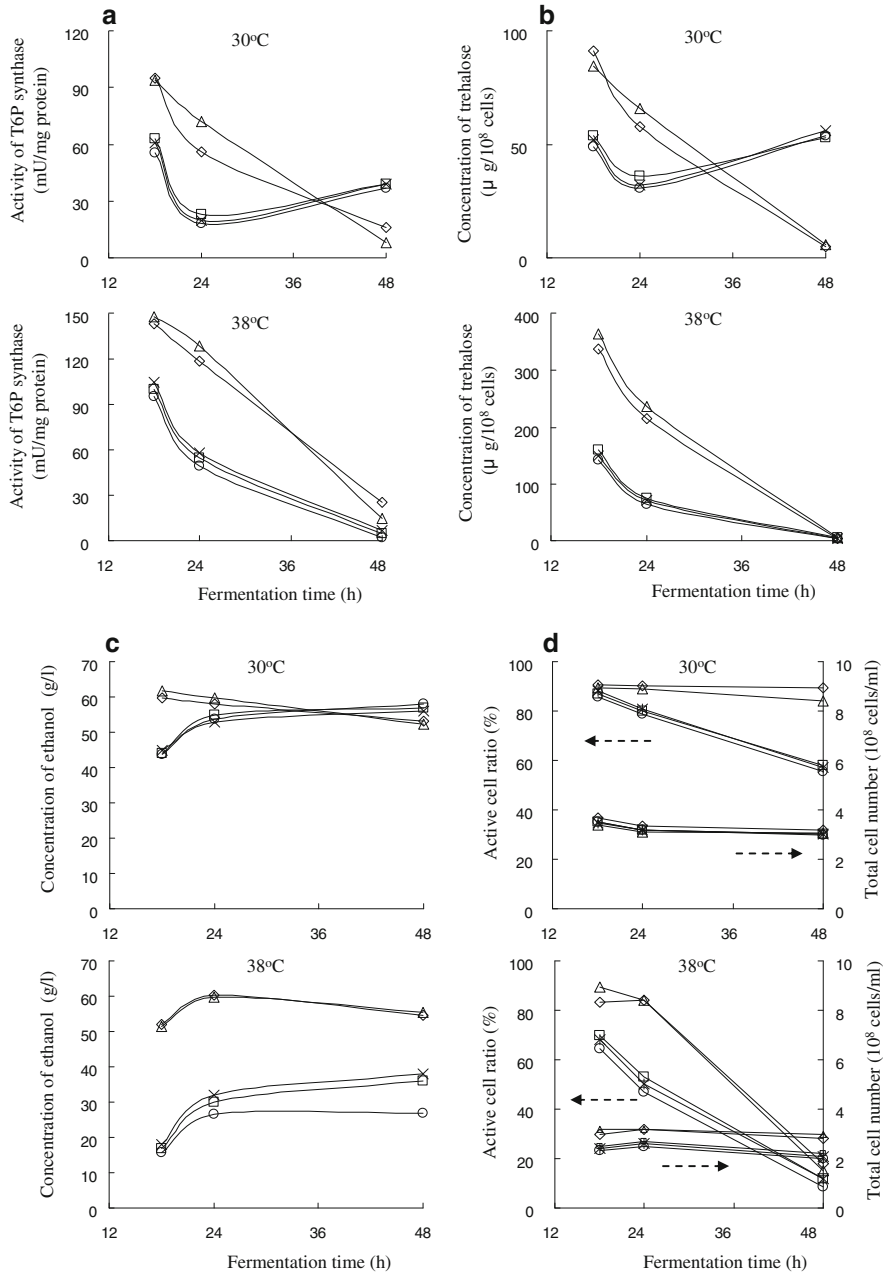

Fig. 3 Comparison of the activity of trehalose-6-phosphate synthase (**a**), the concentration of trehalose (**b**), the concentration of ethanol (**c**), and the total cell number and the ratio of active cells (**d**) of host strain 10151 (*circles*), *URA3*-recovered strains 10151-pI-RED1 (*squares*) and 10151-*URA3* (*stars*), and *TPS1* overexpression strains 10151-pI-RED1-*TPS1* (*triangles*) and 10151-*URA3-TPS1* (*diamonds*), during ethanol fermentation at 30 and 38°C using 15% YPD medium. Cells grown on a 2% YPD plate were transferred to 100 ml of a pre-cultivation medium (5% YPD) in a 500 ml flask. Pre-cultivation was performed aerobically at 30°C for 16 h with shaking at 160 rpm using a rotary shaker. A 10 ml inoculum of pre-cultivation broth was added to 90 ml of fermentation medium (15% YPD) in a 200 ml flask. Flasks were immersed in water baths thermostatted at 30 or 38°C and the cultivation media were stirred using magnetic stirrers. Aliquots of approximately 10 ml of the broth were sampled for analysis. All parameters were determined in duplicate. The absolute difference between duplicate measurements of ethanol concentration was within 1% and the absolute differences between duplicate measurements of cell number, trehalose-6-phosphate synthase activity, and trehalose concentration were within 3%. Data are means of two replications

successfully obtained by transforming plasmid pI-RED1-*TPS1*, plasmid pI-RED1, DNA fragment P_{URA3}-*URA3*-P_{ADH1}-*TPS1*-T_{ADH1}-$URA3_{downstream}$, and DNA fragment P_{URA3}-*URA3*, respectively, with the host strain 10151. A PCR check was performed to confirm the successful introduction (data not shown).

Comparison of the ethanol fermentation capacities of the host strain and transformants

The host strain 10151 and four transformants each with three strains were grown at 30 and 38°C to evaluate the effect of high expression of the *TPS1* gene on thermotolerance during ethanol fermentation. Since three strains of each type of transformant had the same performance, only the data of one strain of each transformant are shown in Fig. 3.

As shown in Fig. 3a and b, at both 30 and 38°C, high activity of trehalose-6P-synthase resulted in a high concentration of trehalose, which is consistent with the results of previous studies (Cansado et al. 1998; Hottiger et al. 1987). No obvious difference was found in the activity of T6P synthase and the trehalose concentration for strains 10151, 10151-*URA3*, and 10151-pI-RED1, indicating that the expression of *URA3* and *YAP1* genes did not affect the synthesis of trehalose. The similar results for 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1* also suggested that high expression of the *YAP1* gene did not make a significant contribution to trehalose synthesis. The activity of T6P synthase and trehalose concentration at 38°C were higher than those at 30°C for all strains. Higher temperature induced higher expression of the *TPS1* gene, as reported previously (Mahmud et al. 2010; Bell et al. 1992). However, at both 30 and 38°C, the *TPS1* overexpression strains, 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1*, had higher activity of T6P synthase and accumulation of trehalose after 18 and 24 h than the other three strains without *TPS1* overexpression. The constitutive expression of the *TPS1* gene contributed to the difference. The differences between the *TPS1* overexpression strains and other strains were especially notable at 38°C. The concentration of trehalose in strains 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1* was approx. 350 μg/10^8 cells after 18 h and 230 μg/10^8 cells at 24 h, compared to 160 μg/10^8 cells after 18 h and 72 μg/10^8 cells after 24 h for the three strains without *TPS1* overexpression.

At 30°C, both *TPS1* overexpression strains, 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1*, completed their fermentation after 18 h and the ethanol with both strains reached 60 g/l (Fig. 3c). However, the fermentation rates for the other three strains without *TPS1* overexpression were slower and the fermentation continued up to 48 h, whereupon ethanol was approx. 57 g/l. These results suggest that the ethanol fermentation rate was improved by overexpression of the *TPS1* gene. Compared to the results at 30°C, the differences among the strains were more significant at 38°C. The fermentation with both *TPS1* overexpression strains, 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1*, was complete after 24 h and ethanol was 60 g/l, which was the same as that at 30°C. However, the fermentation rates and fermentation efficiencies of the other strains without *TPS1* overexpression were decreased compared to those at 30°C. Strain 10151 ceased fermentation after 24 h and ethanol was only 27 g/l. The other two strains with *URA3* recovered continued their fermentation up to 48 h giving ethanol at approx. 38 g/l. The results indicated that *TPS1* overexpression significantly improved the ethanol fermentation rate and ethanol fermentation efficiency of strain 10151, and essentially similar results were obtained at 30 and 38°C. *URA3* recovery also contributed to the improvement to some extent; however, it was almost negligible compared to the effect of *TPS1* overexpression.

Differences in total cell numbers were not apparent for all of the strains at 30°C, though the ratios of viable cells for strains 10151-pI-RED1-*TPS1* and 10151-*URA3*-*TPS1* were a little higher than those for the other three strains after 18 and 24 h, whereupon the fermentation was in fact complete (Fig. 3d). However, the differences were notable at 38°C. The total cell number and the ratio of viable cells for both *TPS1* overexpression strains were higher than those for the other three strains during the whole fermentation period. The ratio of viable cells for both *TPS1* overexpression strains remained over 85% up to 24 h, which therefore led to much higher fermentation rates and efficiencies, as indicated by the production of ethanol.

Based on the above results, *TPS1* overexpression increased the activity of T6P synthase and the synthesis of trehalose, which led to a higher cell concentration with a higher viable cell ratio. The ethanol fermentation rate and the fermentation efficiency at 38°C were hence significantly improved.

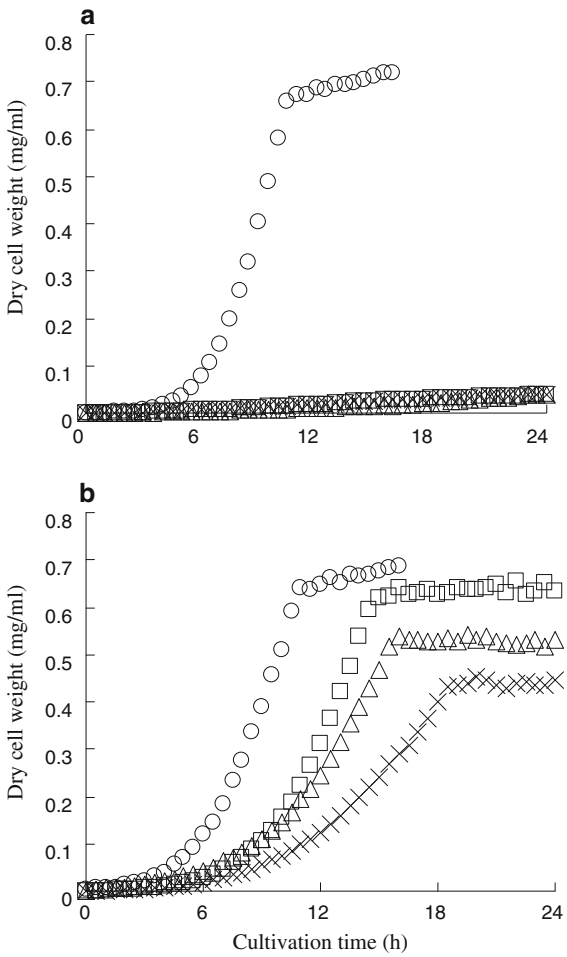

Fig. 4 Growth of transformants 10151-*URA3* (**a**) and 10151-*URA3-TPS1* (**b**) in 2% YPD medium supplemented with adenine at 36°C (*circles*), 38°C (*squares*), 40°C (*triangles*) and 42°C (*stars*). 2% YPD supplemented with 40 mg adenine/l was used as growth medium. Cells pre-cultivated at 30°C using 2% YPD supplemented with uracil and adenine were used for inoculation. 100 µl of diluted pre-cultured broth containing 5×10^6 cells/ml was inoculated into 5 ml aliquots of growth medium in L-type tubes. The cultivation was performed at 50 rpm at different temperatures for 24 h

Comparison of growth of transformants with and without *TPS1* overexpression under high-temperature conditions

As shown in Fig. 4, strain 10151-*URA3* did not grow at 38, 40 or 42°C. However, strain 10151-*URA3-TPS1* grew even at 42°C though the growth rate decreased incrementally with increasing temperature. Overexpression of the *TPS1* gene increased the temperature tolerance of strain 10151 from 36 to at least 42°C. This suggested that the improvement of growth contributed remarkably to the improvement of ethanol fermentation at high temperatures.

In conclusion, strains with overexpression of the *TPS1* gene have been constructed by introducing a P_{ADH1}-*TPS1*-T_{ADH1} cassette into the chromosome of the host strain 10151. Under ethanol fermentation conditions, the activity of T6P synthase and the accumulation of trehalose were significantly improved by overexpression of the *TPS1* gene. The ethanol fermentation performance of transformants with overexpression of the *TPS1* gene at 38°C was similar to that at 30°C, indicating that *TPS1* gene overexpression had a remarkable effect in improving the fermentation capacity of the title yeast strain at high temperatures. Growth improvement is considered to be the crucial factor for the improvement of ethanol fermentation, as the critical temperature of growth was increased from 36 to 42°C by *TPS1* gene overexpression. However, further study is needed to investigate whether this improvement is reproducible on industrial yeast strains.

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