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# Enhanced freeze tolerance of baker's yeast by overexpressed trehalose-6-phosphate synthase gene (*TPS1*) and deleted trehalase genes in frozen dough

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**Abstract** Several recombinant strains with overexpressed trehalose-6-phosphate synthase gene (*TPS1*) and/or deleted trehalase genes were obtained to elucidate the relationships between *TPS1*, trehalase genes, content of intracellular trehalose and freeze tolerance of baker's yeast, as well as improve the fermentation properties of lean dough after freezing. In this study, strain $TL301_{TPS1}$ overexpressing *TPS1* showed 62.92 % higher trehalose-6-phosphate synthase (Tps1) activity and enhanced the content of intracellular trehalose than the parental strain. Deleting *ATH1* exerted a significant effect on trehalase activities and the degradation amount of intracellular trehalose during the first 30 min of prefermentation. This finding indicates that acid trehalase (Ath1) plays a role in intracellular trehalose degradation. *NTH2* encodes a functional neutral trehalase (Nth2) that was significantly involved in intracellular trehalose degradation in the absence of the *NTH1* and/or *ATH1* gene. The survival ratio, freeze-tolerance ratio and relative fermentation ability of strain $TL301_{TPS1}$ were approximately twice as high as those of the parental strain (BY6-9α). The increase in freeze tolerance of strain $TL301_{TPS1}$ was accompanied by relatively low trehalase activity, high Tps1 activity and high residual content of intracellular trehalose. Our results suggest that overexpressing *TPS1* and deleting trehalase genes are sufficient to improve the freeze tolerance of baker's yeast in frozen dough. The present study provides guidance for the commercial baking industry as well as the research on the intracellular trehalose mobilization and freeze tolerance of baker's yeast.

**Keywords** Baker's yeast · Freeze tolerance · *TPS1* · *ATH1* · *NTH2* · Fermentation · Dough

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## Introduction

Frozen dough is of increasing importance in the baking sector [33]. Its application provides consumers with fresh bread of proper texture and permits the large-scale production and distribution of dough in frozen form independent of the subsequent baking process [10, 28]. However, traditional baker's yeast is damaged during the frozen storage of dough after prefermentation, which results in substantial decreases in the bread-making potential of frozen dough [2, 10]. Thus, developments of baker's yeast strains with high freeze tolerance are highly desirable.

The disaccharide trehalose [α-D-glucopyranosyl(1-1)-α-D-glucopyranoside] is a nonreducing disaccharide of glucose present in many organisms, including bacteria, fungi, insects, and plants [13]. In yeast, *Saccharomyces cerevisiae*, trehalose is synthesized by an enzyme complex which is encoded by the genes *TSL1*, *TPS1*, *TPS2* and *TPS3*, respectively [7]. Trehalose can accumulate up to 15 % of the cell dry mass depending on the growth conditions and

environmental stress. One of the functions of trehalose is to act as a stress protectant of proteins and biological membranes against adverse conditions. This function suggests that trehalose is a crucial physiological factor that affects freeze tolerance and provides tolerance to baking-associated stress [18, 28, 36]. Hence, many improvements intended to increase the freeze tolerance of yeast have focused on the accumulation of intracellular trehalose [12, 26]. Another function of trehalose is to act as a storage carbohydrate in the *S. cerevisiae*. This function is supported by the rapid loss of intracellular trehalose upon the resumption of growth of starved cells during the initiation of fermentation [13, 32, 33]. The rapid drop in intracellular trehalose is due to the activation of trehalase, which seems to be essential to recover normal cell functions when the stress condition disappears.

In baker's yeast, two kinds of trehalase activities have been described: the neutral trehalase (Nth1 and Nth2) activity encoded by the *NTH1* and *NTH2* genes and the acid trehalase encoded by the *ATH1* [4, 6, 13]. Nth1 is localized in the cytosol and possesses maximal activity at neutral pH. The principal function of Nth1 is to hydrolyze intracellular trehalose, although its participation in extracellular trehalose assimilation has also been described [14]. The *NTH2* gene shares a high degree of sequence homology (77 %) with the *NTH1* gene, but no trehalase activity has been associated with the former thus far [35]. Recent studies suggest that *NTH2* is involved in trehalose mobilization in the *tps1Δnth1Δ* yeast strain in the presence of extracellular trehalose. It also participates in trehalose accumulation in the *ath1Δ* and *nth1Δath1Δ* yeast strains in response to saline stress [5, 13]. Ath1 has been classically considered vacuolar [1, 8], it is implicated in the degradation of extracellular trehalose as a carbon source for growth [3, 14, 21]. However, new data suggest the possibility of dual localization of the Ath1 both in the vacuole and in the periplasmic space [11, 14, 22], its participation in intracellular trehalose accumulation during growth in a medium with trehalose, and lack of glucose in *TPS1* mutants or under severe saline stress [6, 13].

Preliminary results obtained in our laboratory show that the mutants with one or both *ATH1* and *NTH1* disrupted by the introduction of plasmid DNA sequences could increase the content of intracellular trehalose and the freeze tolerance in the mid-exponential phase [17]. In the current study, we constructed baker's yeast strains with overexpressed *TPS1* and/or deleted trehalase genes to (1) shed light on the relationships between *TPS1*, trehalase genes, the content of intracellular trehalose and the freeze tolerance of baker's yeast and (2) develop an improved strain with lower trehalase activity, higher Tps1 activity and higher freeze tolerance in frozen dough. The activity of trehalase, the activity of Tps1 and the intracellular trehalose mobilization of the mutants were examined during prefermentation in the glucose low sugar model liquid dough (LSMLD). The survival ratio, the freeze-tolerance ratio and the relative fermentation ability were also measured.

## Materials and methods

### Strains, plasmids and growth conditions

Strains and plasmids used in the study are summarized in Table 1. Baker's yeast strain BY6-9α was obtained from the Yeast Collection Center of the Tianjin Key Laboratory of Industrial Microbiology. Yeast genomes and plasmid DNA were obtained utilizing a yeast genomic DNA extraction kit (Solarbio, Beijing, China) and plasmid DNA extraction kit (Solarbio, Beijing, China), respectively.

The *Escherichia coli* DH5α strain for constructing plasmids was grown at 37 °C in Luria–Bertani medium (10 g $l^{-1}$ tryptone, 5 g $l^{-1}$ yeast extract and 10 g $l^{-1}$ NaCl) supplemented with ampicillin (100 mg $l^{-1}$). The yeast strain was grown at 30 °C in YPD medium (20 g $l^{-1}$ glucose, 20 g $l^{-1}$ peptone and 10 g $l^{-1}$ yeast extract). Approximately 800 mg $l^{-1}$ of G418 was added to the YPD plates for the yeast culture to select Geneticin (G418)-resistant transformants. The YPD plate was supplemented with 500 mg $l^{-1}$ Zeocin (Promega, Madison, USA) to select Zeocin-resistant yeast strains. Then, the YEPG medium (10 g $l^{-1}$ yeast extract, 20 g $l^{-1}$ peptone, and 20 g $l^{-1}$ galactose) was used to induce the expression of *Cre* in the yeast transformants.

Yeast cells were grown in Erlenmeyer flasks (200 ml cultures in 500 ml flasks) in a rotary shaker (180 rpm) at 30 °C for 48 h utilizing liquid YPD medium. After centrifugation (5,000 rpm, 5 min, 4 °C), the yeast cake was washed twice with distilled water (4 °C) and compressed for the assay of the activity of trehalase, the activity of Tps1, the freeze tolerance and the intracellular trehalose mobilization in the glucose LSMLD medium.

### Plasmid construction and yeast transformation

PCR primers used in the current work are listed in Table 2. Plasmid pUC-AABK, which was used to delete the *ATH1* gene, was constructed as followed. First, the AA segment was amplified with the primers AA-up and AA-down from genomes of the parental strain (BY6-9α), while the AB segment was amplified with the primers AB-up and AB-down. After amplification, the AA and AB segments were purified by an Omega PCR purification kit and, respectively, *Sph*I–*Bam*HI and *Bam*HI–*Kpn*I double-digested. These segments were then inserted into the *Sph*I–*Kpn*I-digested plasmid pUC19, creating the plasmid pUC-AAB.

**Table 1** Strains and plasmids used in this study

| *S. cerevisiae* yeast strain | Relevant features | Source or reference |
|---|---|---|
| BY6-9α (the parental strain) | *MATα* (haploid derived from BY6 strain) | Yeast Collection Center of the Tianjin Key Laboratory of Industrial Microbiology |
| BY6-9α + TPS1 | *MATa* Yep-TPS1 | This study |
| TL101 | BY6-9α *MATα nth1Δ:: loxP-KanMX-loxP* | This study |
| TL1001 | BY6-9α *MATα nth1Δ:: LoxP* | This study |
| TL102 | BY6-9α *MATα nth2Δ:: loxP-KanMX-loxP* | This study |
| TL1002 | BY6-9α *MATα nth2Δ:: loxP* | This study |
| TL103 | BY6-9α *MATα ath1Δ:: loxP* | This study |
| TL201 | BY6-9α *MATα nth1Δ:: LoxP nth2Δ:: loxP-KanMX-loxP* | This study |
| TL2001 | BY6-9α *MATα nth1Δ:: LoxP nth2Δ:: LoxP* | This study |
| TL202 | BY6-9α *MATα ath1Δ:: loxP-KanMX-loxP nth1Δ:: LoxP* | This study |
| TL203 | BY6-9α *MATα ath1Δ:: loxP-KanMX-loxP nth2Δ:: LoxP* | This study |
| TL2003 | BY6-9α *MATα ath1Δ:: LoxP nth2Δ:: LoxP* | This study |
| TL301 | BY6-9α *MATα nth1Δ:: LoxP nth2Δ:: LoxP ath1Δ:: loxP-KanMX-loxP* | This study |
| TL3001 | BY6-9α *MATα nth1Δ:: LoxP nth2Δ:: LoxP ath1Δ:: LoxP* | This study |
| $TL301_{TPS1}$ | BY6-9α *MATα nth1Δ:: KanMX-PGK1P-TPS1-PGK1 nth2Δ:: LoxP ath1Δ:: LoxP* | This study |
| Plasmid | | |
| pGAPZa | Zeocin, $Ap^r$ | Tianjin Key Laboratory of Industrial Microbiology |
| pUC-ABK | A-*loxP-KanMX-loxP*-B | Tianjin Key Laboratory of Industrial Microbiology |
| Yep-TPS1 | *CUP1-KanMX-PGK1P-TPS1-PGK1T* | Tianjin Key Laboratory of Industrial Microbiology |
| pUC-AKPTB | TA-*Kan-PGK1P-TPS1-PGK1T*-TB | Tianjin Key Laboratory of Industrial Microbiology |
| pUC-AAB | AA–AB | This study |
| pUC-NAB | NA–NB | This study |
| pUC-AABK | AA-*loxP-KanMX-loxP*-AB | This study |
| pUC-NABK | NA-*loxP-KanMX-loxP*-NB | This study |

The *KanMX* marker gene, which was utilized in the plasmid as a resistance mark towards G418, was then amplified utilizing the primers *Kan*-up and *Kan*-down from the vector pUG6. The *KanMX* marker gene sequence was subcloned into the *Bam*HI site of pUC-AAB to obtain a plasmid called pUC-AABK.

The pUC-NABK plasmid, which was used to delete *NTH2*, was created and described as plasmid pUC-AABK. The NA segment was amplified by PCR utilizing BY6-9α genomic DNA as templates with the NA-up and NA-down primers, and the NB segment was similarly amplified employing the NB-up and NB-down primers. The PCR products obtained from these primers were *Hin*dIII–*Bam*HI and *Bam*HI–*Kpn*I double-digested, respectively, and then inserted into the *Hin*dIII–*Kpn*I-digested plasmid pUC19, creating the plasmid pUC-NAB. The *KanMX* marker gene sequence was then subcloned into the *Bam*HI site of pUC-NAB to obtain a plasmid called pUC-NABK.

Yeast transformation was carried out utilizing a lithium acetate procedure described previously [31]. The selection of strains with deleted trehalase genes was performed in YPD medium supplemented with 800 mg $l^{-1}$ G418. PCR was applied to verify the recombinant strains with accurate site integration. Several different pairs of primers were designed in Table 2.

Construction of recombinant baker's yeast

The A-*loxP-KanMX-loxP*-B deletion cassette was amplified from the plasmid pUC-ABK (Table 1). The DNA fragment was transformed and integrated into the chromosome at the *NTH1* locus of BY6-9α by homologous recombination to construct *NTH1* gene deletion strain, TL101. The transformants were screened on G418 selective plates after transformation and verified by PCR employing primer pairs *NTH1*-up and *Kan*-up-BAT. *Cre*

**Table 2** PCR primers used in this study

| Primer name | Sequence (5′ → 3′) | Restriction site |
|---|---|---|
| AA-up | ACACGCATGCCTCTTCTTTGCCCCTCAAAA | *Sph*I |
| AA-down | CGGGATCCGCATTGAACCAAAGCGATCT | *Bam*HI |
| AB-up | GGGGGATCCGATGATAACAAAGGAGCT | *Bam*HI |
| AB-down | GGGGTACCTAGAAAACTGTTGTGGGCT | *Kpn*I |
| NA-up | GCCCAAGCTTAATGAGTAGAAATTCACGCA | *Hind*III |
| NA-down | GCGGATCCTATTTGAGTATATGCAGGTC | *Bam*HI |
| NB-up | CGCGGATCCATAACCATGAGATTGTGCCT | *Bam*HI |
| NB-down | CGGGGTACCGTAATGATAGTGTTCCCGAT | *Kpn*I |
| *Kan*-up | CGGGATCCCAGCTGAAGCTTCGTACGC | *Bam*HI |
| *Kan*-down | CCGGGATCCGCATAGGCCACTAGTGGATCTG | *Bam*HI |
| TA-up | CCGGAATTCCGTCTTCTTCCATTGTCTT | |
| TB-down | ACATGCATGCCTAGGTTATCTATGCTGTCT | |
| *ATH1*-up | TCTGGATGGCAAACAGTTCGTG | |
| *Kan*-up-BAT | CCTTTTATATTT CTCTACAGGGGCG | |
| *NTH2*-up | AAGCACGTTAGAATCGCATCTC | |
| *NTH1*-up | ATCATCATCTGTAATCGCTTCACC | |

The restriction enzyme sites are underlined

recombinase was expressed and *KanMX* was excised after introducing the plasmid pGAPZa into TL101. Loss of pGAPZa changed the genotype of the mutant into *nth1*Δ*::LoxP*, which was designated as TL1001.

The AA-*loxP-KanMX-loxP*-AB and NA-*loxP-KanMX-loxP*-NB deletion cassettes were amplified from the plasmids pUC-AABK and pUC-NABK, respectively. The AA-*loxP-KanMX-loxP*-AB deletion cassette was transformed and integrated into strains BY6-9α and TL1001 by homologous recombination to construct *ATH1* gene deletion strains, TL103 and TL202. The transformants were verified by PCR utilizing primer pairs *ATH1*-up and *Kan*-up-BAT as described above. The *NTH2* gene deletion strains, TL102 and TL201, were constructed by transforming NA-*loxP-KanMX-loxP*-NB disruption cassette into BY6-9α and TL1001 employing the same method described above. The strains were verified utilizing the primers *NTH2*-up and *Kan*-up-BAT. The TL1002 (*nth2Δ::LoxP*), TL2001 (*nth1Δ::LoxP nth2Δ::LoxP*), TL203 (*ath1Δ:: loxP-KanMX-loxP nth2Δ::LoxP*), TL301(*nth1Δ::LoxP nth2Δ::LoxP ath1Δ:: loxP-KanMX-loxP*), and TL3001 (*nth1Δ::LoxP nth2Δ::LoxP ath1-Δ::LoxP*) were constructed as described above.

The plasmid Yep-TPS1 was introduced into BY6-9α, construct *TPS1* gene overexpression strains, BY6-9α + TPS1, using the lithium acetate procedure. The TA-*Kan-PGK1P-TPS1-PGK1T*-TB deletion cassette was amplified from the plasmid pUC-AKPTB with the TA-up and TB-down primers. $TL301_{TPS1}$ (*nth1Δ:: KanMX-PGK1P-TPS1-PGK1 nth2Δ::LoxP ath1Δ:: LoxP*) was constructed as described above.

## Assay of the intracellular trehalose content

The compressed baker's yeast obtained from liquid YPD medium was the starting yeast cells in glucose LSMLD fermentation. Cells incubated in a glucose LSMLD medium (40 g $l^{-1}$ glucose, 2.5 g $l^{-1}$ $(NH_4)_2SO_4$, 5 g $l^{-1}$ urea, 16 g $l^{-1}$ $KH_2PO_4$, 5 g $l^{-1}$ $Na_2HPO_4$, 0.6 g $l^{-1}$ $MgSO_4$, 0.0225 g $l^{-1}$ nicotinic acid, 0.005 g $l^{-1}$ Ca-pantothenate, 0.0025 g $l^{-1}$ thiamine, 0.00125 g $l^{-1}$ pyridoxine, 0.001 g $l^{-1}$ riboflavin and 0.0005 g $l^{-1}$ folic acid) at 15 min intervals for 60 min were collected and washed twice with distilled water to obtain fresh yeast cells. The fresh yeast cells were dried overnight at 85 °C to calculate the weight of the dry yeast cells. Trehalose was extracted from 0.1 g of fresh yeast cells with 4 ml of 0.5 mol $l^{-1}$ cold trichloroacetic acid and the extracts employed for measuring by the Anthrone method as described previously [16, 30, 34].

## Determination of the survival ratio of yeast after freezing and thawing

Compressed baker's yeast was incubated in a glucose LSMLD medium at 30 °C for 60 min. Then 100 μl aliquots were shifted to −20 °C at 15 min intervals. After frozen storage for 21 days, cell samples were thawed at 30 °C for 10 min. An aliquot of the suspension was then spread on YPD plates after appropriate dilution, and the plates were incubated at 30 °C for 48 h. The survival ratio was calculated from the number of living cells before and after freezing.

### Freeze-tolerance ratio and relative fermentation ability of yeast in lean dough

The ingredients of the dough were 280 g of flour, 150 ml of distilled water, 4 g of salt, and 6 g of compressed baker's yeast according to the Chinese National Standards for yeast utilized in food processing. The dough was evenly and rapidly stirred for 5 min at (30 ± 0.2) °C and placed in a fermentograph box (Type JM451, Sweden). The dough samples were prefermented at 30 °C for 60 min and then stored at −20 °C for 21 days. After storage, the dough samples were thawed at 30 °C for 10 min, and $CO_2$ production (A) was determined at 30 °C for 60 min in the fermentograph box. As a control experiment, prefermented dough without freezing was further fermented at 30 °C for 60 min, and $CO_2$ production (B) was determined under the same conditions. The freeze-tolerance ratio (%) was calculated as A/B × 100. The relative fermentation ability was expressed as a percentage of $CO_2$ production (A) over 60 min compared with that of the parental strain (BY6-9α).

### Assay of neutral and acid trehalase activity

The activities of acid trehalase on intact yeast cells and neutral trehalase in crude extracts were measured utilizing the Matthieu Jules method as described in [14]. The liberated glucose was analyzed by HPLC employing an Aminex HPX-87H column with 5 mmol $l^{-1}$ $H_2SO_4$ as the mobile phase at a flow rate of 0.6 ml $min^{-1}$ at 65 °C. One unit of trehalase activity was defined as the amount of trehalase producing 1.0 μM glucose per min under assay conditions. The specific trehalase activity was expressed as the units per gram dry cell weight (CDW).

### Assay of Tps1 activity

Tps1 activity was measured as described by Hottiger [9]. The trehalose-6-phosphate formed during the reaction was quantitatively determined using the Anthrone method [16]. One unit of Tps1 activity was defined as the amount of Tps1 producing 1.0 μM 6-phosphate-trehalose per min under assay conditions. The specific Tps1 activity was expressed as the units per gram CDW.

### Statistical analyses

Statistical significance analyses were performed by SPSS 20.0. Paired-Samples T Test was used in this study. Differences with a $P$ value of <0.05 were considered statistically significant.

## Results

### Overexpressed *TPS1* gene results in enhanced intracellular trehalose content

In baker's yeast, trehalose is synthesized in a two-step process. First, trehalose-6-phosphate (T-6-P) is formed from α-glucose-6-phosphate and uridine diphosphate glucose by Tps1. Then, T-6-P is dephosphorylated to trehalose by T-6-P phosphatase (Tps2) [37]. Therefore, Tps1 activities of BY6-9α + TPS1, $TL301_{TPS1}$ and the parental strain (BY6-9α) were tested, and the intracellular trehalose contents before and after prefermentation in the glucose LSMLD medium were assayed. The results in Table 3 showed that the specific Tps1 activity of strains BY6-9α + TPS1 and $TL301_{TPS1}$ was approximately 30.3 and 62.9 %, respectively, higher than the parental strain (BY6-9α) ($P < 0.05$), resulting in 6.8 and 9.5 % enhancements ($P < 0.05$) in intracellular trehalose content at 30 °C for 48 h utilizing liquid YPD medium (see in "Materials and methods", the initial content of intracellular trehalose of yeast cells in a glucose LSMLD medium at 0 min remained the same as that in liquid YPD medium after fermentation for 48 h), respectively (Fig. 1c). The content of intracellular trehalose in strain $TL301_{TPS1}$ was significantly higher than that of TL301 (*nth1Δnth2Δath1Δ*) at 15-min interval for 60 min of prefermentation in the glucose LSMLD medium. Similar effects were observed between strains BY6-9α + TPS1 and the parental strain (BY6-9α). These results suggest that overexpression of the *TPS1* gene could significantly enhance the content of intracellular trehalose in baker's yeast before and after prefermentation.

### Ath1 is involved in intracellular trehalose degradation at the initial fermentation period

Ath1 is implicated in extracellular trehalose utilization [3, 14, 21] and participates in intracellular trehalose accumulation during saline stress [6]. We compared the degradation amount of intracellular trehalose, the neutral trehalase activity and the acid trehalase activity of the parental strain (BY6-9α), strain TL103 (*ath1Δ*) and strain TL102 (*nth2Δ*) to investigate the possible involvement of acid trehalase in intracellular trehalose degradation during the first 30 min of prefermentation in the glucose LSMLD medium. The intracellular trehalose content of strains TL103 (*ath1Δ*) and TL102 (*nth2Δ*) quickly degraded, showing degradation curves similar to that of the parental strain (BY6-9α) (Fig. 1a). The neutral trehalase activities of strains BY6-9α and TL102 (*nth2Δ*) were 7.6 and 22.0 % lower than that of strain TL103 (*ath1Δ*) ($P < 0.05$), respectively, whereas the acid trehalase activities of strains BY6-9α and TL102

**Table 3** The degradation amounts of intracellular trehalose, the acid trehalase activities and the neutral trehalase activities of eight strains when cultivated in glucose LSMLD medium

| Strain | Degradation amount of intracellular trehalose [mg (g CDW)$^{-1}$][a] | neutral trehalase activity [U (g CDW)$^{-1}$] | Tps1 activity [U (g CDW)$^{-1}$] | Ath1 activity [U (g CDW)$^{-1}$] |
|---|---|---|---|---|
| BY6-9α | 77.18 ± 1.06 | 13.24 ± 0.91 | 0.89 ± 0.09 | 2.66 ± 0.05 |
| TL103(*ath1Δ*) | 78.74 ± 2.09 | 14.33 ± 0.85 | [b] | 2.23 ± 0.03 |
| TL102(*nth2Δ*) | 77.40 ± 1.21 | 11.33 ± 0.61 | [b] | 2.72 ± 0.12 |
| TL203(*nth2Δath1Δ*) | 73.57 ± 0.95 | 9.73 ± 0.79 | [b] | 2.46 ± 0.02 |
| TL101(*nth1Δ*) | 63.64 ± 0.82 | 9.58 ± 0.56 | [b] | 2.65 ± 0.03 |
| TL202(*nth1Δath1Δ*) | 58.31 ± 1.59 | 10.81 ± 1.03 | [b] | 2.16 ± 0.11 |
| TL201(*nth1Δnth2Δ*) | 57.17 ± 1.73 | 9.19 ± 0.57 | [b] | 2.61 ± 0.06 |
| TL301(*nth1Δnth2Δath1Δ*) | 49.08 ± 2.35 | 8.19 ± 0.51 | [b] | 2.22 ± 0.08 |
| BY6-9α + TPS1 | 83.22 ± 1.28 | 13.39 ± 0.83 | 1.16 ± 0.08 | 2.61 ± 0.08 |
| $TL301_{TPS1}$ | 50.37 ± 0.71 | 7.87 ± 0.76 | 1.45 ± 0.11 | 2.18 ± 0.11 |

All the results shown are mean values of three independent experiments including standard deviations

[a] The degradation amount of intracellular trehalose was calculated from the content of intracellular treholose in glucose LSMLD during the first 30 min

[b] Not determined

(*nth2Δ*) were 19.3 and 22.0 % higher than that of strain TL103 (*ath1Δ*) ($P < 0.05$), respectively (Table 3). These results reveal that Ath1 is involved in intracellular trehalose degradation in strains BY6-9α and TL102 (*nth2Δ*) at the initial fermentation period.

The function of Ath1 in strains BY6-9α and TL102 (*nth2Δ*) prompted us to investigate whether Ath1 participates in intracellular trehalose degradation in strains TL101 (*nth1Δ*) and TL201 (*nth1Δnth2Δ*). The degradation of intracellular trehalose in strain TL202 (*nth1Δath1Δ*) decreased to 58.31 ± 1.59 mg (g CDW)$^{-1}$, a decrease ratio of 8.4 %, compared with strain TL101 (*nth1Δ*) ($P < 0.05$) (Table 3), during the first 30 min of fermentation. However, the neutral trehalase activity of TL202 (*nth1Δath1Δ*) was 12.8 % higher than that of strain TL101 (*nth1Δ*) ($P < 0.05$). It was also found that the acid trehalase activity of TL101 (*nth1Δ*) was 22.7 % higher than that of strain TL202 (*nth1Δath1Δ*) ($P < 0.05$). These results reveal that Ath1 is involved in intracellular trehalose degradation in strain TL101 (*nth1Δ*). The neutral trehalase activity in strain TL202 (*nth1Δath1Δ*) was 17.6 % higher than that in strain TL201 (*nth1Δnth2Δ*) ($P < 0.05$) (Table 3). However, no significant difference ($P > 0.05$) was observed between the degradation amount of intracellular trehalose in strain TL202 (*nth1Δath1Δ*) and that in strain TL201 (*nth1Δnth2Δ*). We also observed that the acid trehalase activity in strain TL202 (*nth1Δath1Δ*) was 17.2 % lower than that in strain TL201 (*nth1Δnth2Δ*) ($P < 0.05$). These results suggest that Ath1 is involved in intracellular trehalose degradation in strain TL201 (*nth1Δnth2Δ*), which is not consistent to the previously described [20].

### Nth2 is involved in intracellular trehalose degradation in *NTH1* and/or *ATH1* gene deletion mutants

Deletion of *NTH2* in the parental strain and the *ath1Δ* and *nth1Δath1Δ* mutants (TL102, TL203 and TL301), respectively, led to 14.4, 32.1 and 24.2 % decreases in neutral trehalase activity ($P < 0.05$) (Table 3). The decreased activity in strains TL102 (*nth2Δ*), TL203 (*nth2Δath1Δ*) and TL301 (*nth1Δnth2Δath1Δ*) could be attributed to the product of *NTH2*, Nth2. A high level of neutral trehalase activity, which significantly corresponds to Nth2, was also shown by strains TL202 (*nth1Δath1Δ*) and TL101 (*nth1Δ*). These finding suggest that *NTH2* encodes a functional neutral trehalase in the stationary phase in glucose medium.

We compared the degradation amounts of intracellular trehalose in strains TL102 (*nth2Δ*), TL202 (*nth1Δath1Δ*) and the parental strain (BY6-9α) to obtain an evidence of the direct effect of *NTH2* deletion alone in intracellular trehalose degradation. The degradation amount of intracellular trehalose in strain TL102 (*nth2Δ*) was indistinguishable from that of the parental strain (BY6-9α), whereas apparently slower degradation was displayed by strain TL202 (*nth1Δath1Δ*) (Table 3). These results indicate that the deletion of the *NTH2* gene is not significantly effective in intracellular trehalose degradation in the presence of the *NTH1* and *ATH1* genes. We then measured the degradation amounts of intracellular trehalose in other strains to obtain more evidence of the role of *NTH2* in trehalose degradation. As shown in Table 3, the degradation amount of intracellular trehalose in strain TL203 (*nth2Δath1Δ*) was 6.6 % lower than that of strain TL103 (*ath1Δ*) ($P < 0.05$). The degradation amount

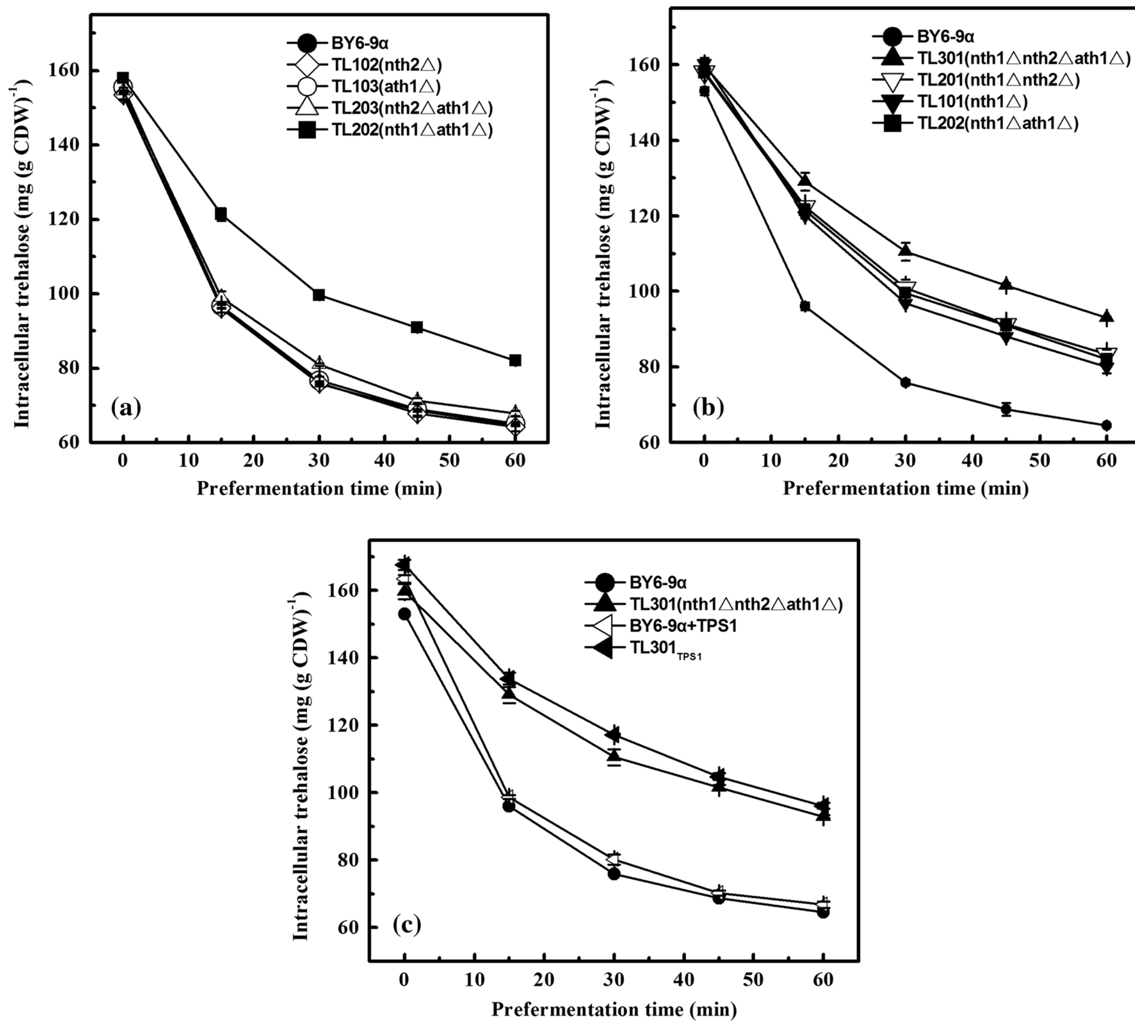


**Fig. 1** Content of intracellular trehalose in ten yeast strains during shaking cultivation in glucose LSMLD. CDW means cells dry weight. Data are averages from three independent experiments and *error bars* represent ± SD. BY6-9α was used as the control strain for the other nine yeast strains

of intracellular trehalose of strain TL201 (*nth1Δnth2Δ*) was 10.2 % lower than that of strain TL101 (*nth1Δ*) ($P < 0.05$); a 15.8 % decrease was also found between strains TL301 (*nth1Δnth2Δath1Δ*) and TL202 (*nth1Δath1Δ*) ($P < 0.05$). Significant differences in the degradation amounts of intracellular trehalose between the two strains then disappeared after prefermentation for 30 min (Fig. 1). These results indicate that the Nth2 is significantly involved in intracellular trehalose degradation in the *NTH1* and/or *ATH1* gene deletion mutants at the initial fermentation period.

## Residual intracellular trehalose content is positively correlated with the survival ratio of yeast after prefermentation and freezing

Intracellular accumulation of trehalose is believed to improve yeast's tolerance to freezing [12, 26]. Interestingly, a number of reports have shown that the trehalose degradation is necessary in the initial fermentation period because trehalose can block enzymatic activities [25] and/or obstruct chaperone access to partially denatured proteins to restore functionality [29]. We assessed the intracellular trehalose contents and survival ratios of overexpressed *TPS1* and/or deleted trehalase gene mutants and the parental strain (BY6-9α) by the method described in the previous section to investigate the effect of trehalose degradation on freeze stress resistance. As results shown in Fig. 1, the residual intracellular trehalose contents of strains TL101 (*nth1Δ*), TL202 (*nth1Δath1Δ*), TL201 (*nth1Δnth2Δ*), TL301 (*nth1Δnth2Δath1Δ*) and $TL301_{TPS1}$ were 80.10, 81.98, 83.46, 92.95 and 96.15 mg (g CDW)$^{-1}$, respectively. These contents represent increases of 24.0, 26.9, 29.2, 43.9, and 48.9 %, respectively, compared with that of the

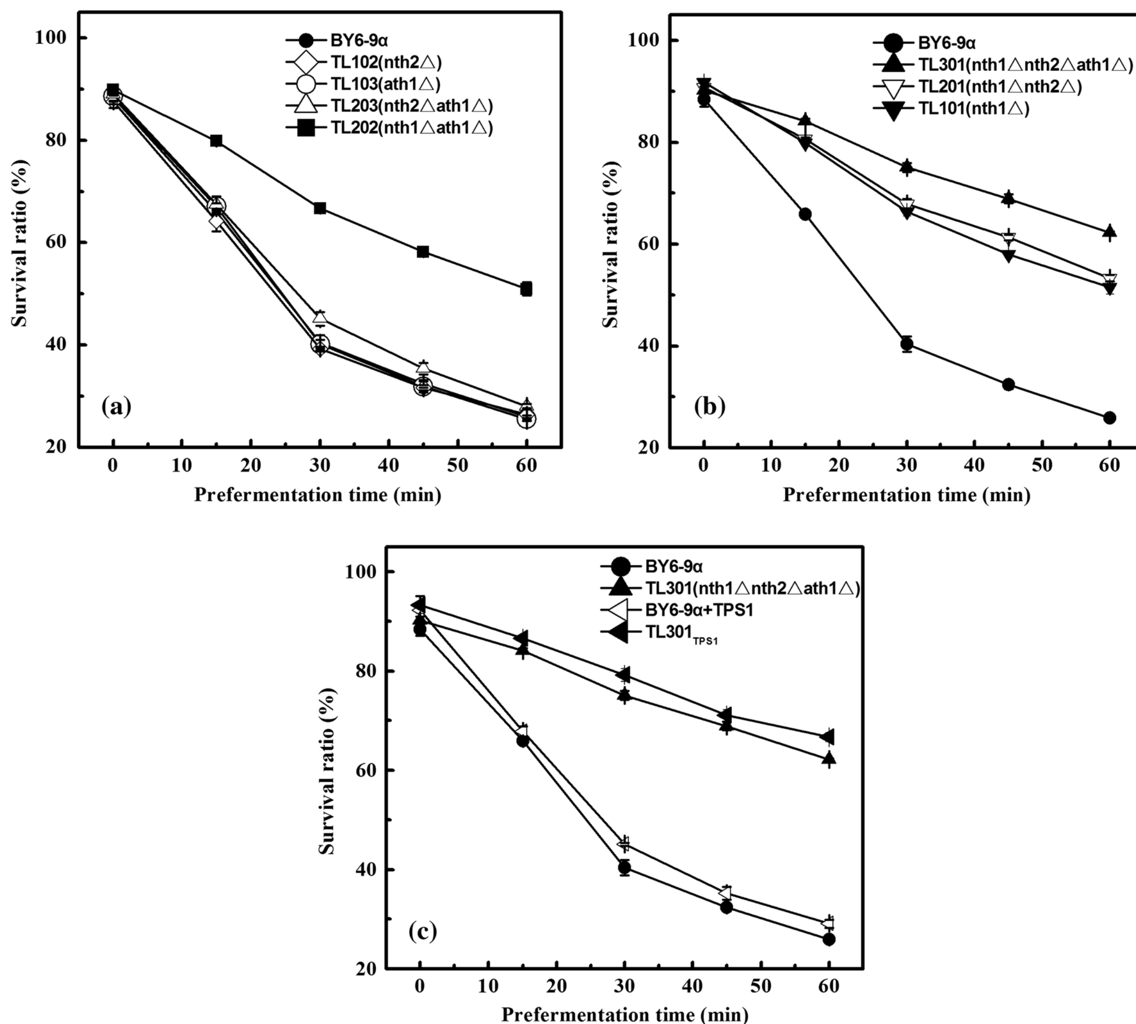


**Fig. 2** Survival ratio of ten yeast strains which were frozen for 21 days at −20 °C after prefermentation for different time periods in glucose LSMLD. Data are averages from three independent experiments and error bars represent ± SD. BY6-9α was used as the control strain for the other nine yeast strains

parental strain ($P < 0.05$) after prefermentation for 60 min. The survival ratios of strains TL101 (*nth1Δ*), TL202 (*nth1Δath1Δ*), TL201 (*nth1Δnth2Δ*), TL301 (*nth1Δnth2Δath1Δ*) and TL301$_{TPS1}$ after freezing treatment (see "Materials and methods") were 98.5, 96.3, 105, 140, and 157 % (Fig. 2) higher, respectively, than that of the parental strain ($P < 0.05$) after prefermentation for 60 min. These results imply that the five mutants with *NTH1* deletion exhibit slower trehalose degradation amounts and higher survival ratios after freezing and thawing compared with the parental strain. The residual intracellular trehalose content is positively correlated with the survival ratio of yeast after prefermentation and freezing. However, higher initial intracellular trehalose contents of yeast (e.g. BY6-9α + TPS1, TL103) did not always correspond to higher survival ratios after prefermentation (Figs. 1, 2).

## Effect of *TPS1* overexpression and/or trehalase genes deletion on the fermentation characteristics of yeast in lean dough after prefermentation and freezing

Freeze tolerance and sufficient leavening ability are necessary characteristics of baker's yeast used in frozen doughs [24, 27]. We explored the freeze-tolerance ratio and the relative fermentation ability of ten yeast strains utilizing the methods described above to investigate the effect of *TPS1* overexpression and/or trehalase genes deletion on the fermentation characteristics of strains in lean dough. As shown in Fig. 3, the freeze-tolerance ratios of strains TL101 (*nth1Δ*), TL202 (*nth1Δath1Δ*), TL201 (*nth1Δnth2Δ*), TL301 (*nth1Δnth2Δath1Δ*) and TL301$_{TPS1}$ were approximately 109, 120, 132, 185, and 204 % higher than that of the parental strain ($P < 0.05$), respectively. The *NTH1* deletion mutants showed remarkably increased

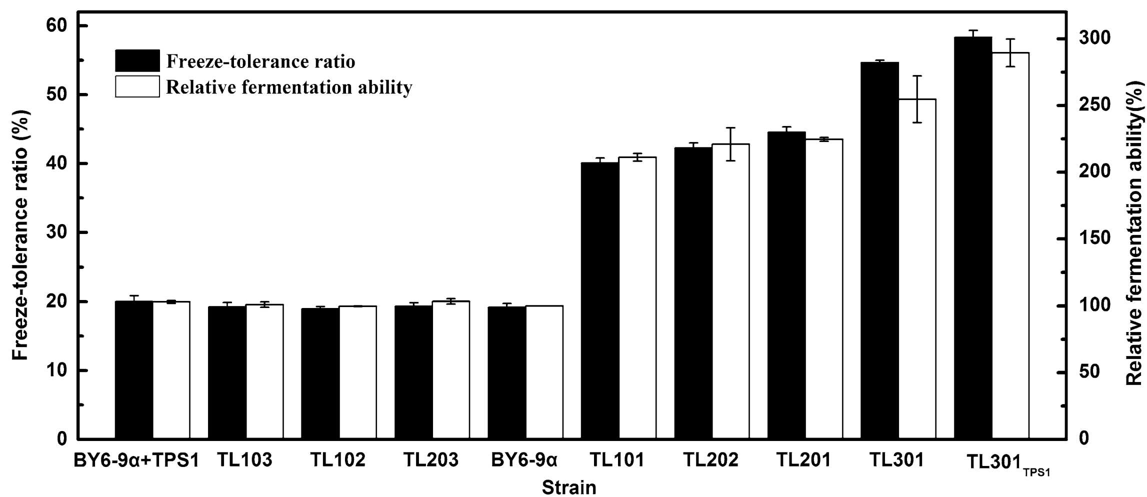


**Fig. 3** Freeze-tolerant ratio and relative fermentation ability of ten yeast strains in lean dough after freezing for 21 days at −20 °C. Data are averages from three independent experiments and error bars represent ± SD. BY6-9α was used as the control strain for the other nine yeast strains

freeze-tolerance ratios, which are associated with higher intracellular trehalose contents during fermentation, and produced more $CO_2$ (data not shown) even after 21 days of frozen storage. Meanwhile, the relative fermentation abilities of strains TL101 (*nth1Δ*), TL202 (*nth1Δath1Δ*), TL201 (*nth1Δnth2Δ*), TL301 (*nth1Δnth2Δath1Δ*) and $TL301_{TPS1}$ were approximately 211, 221, 225, 255, and 289 %, respectively ($P < 0.05$).

## Discussion

Previous studies have reported that the *TPS1* gene is expressed at very low levels in *S. cerevisiae*, and Tps1 plays a key role in the biosynthesis of trehalose [37]. In this study, the transformant $TL301_{TPS1}$ had the highest content of intracellular trehalose, the survival ratio, the freeze-tolerance ratio and the relative fermentation ability, and the specific Tps1 activity in strain $TL301_{TPS1}$ was $1.45 \pm 0.11$ U (g CDW)$^{-1}$, whereas the specific Tps1 activity was $0.89 \pm 0.09$ U (g CDW)$^{-1}$ in the parental strain (BY6-9α). Hence, overexpressed *TPS1* could significantly enhance the content of intracellular trehalose and bring about further increases in Tps1 activity. These results support the conclusion of Rossouw [23], who believed that overexpression of the *TPS1* gene leading to increased trehalose accumulation by yeast strains. Furthermore, the increased Tps1 activity in yeast could have a strong correlation with the freeze tolerance of baker's yeast.

Ath1 is involved in intracellular trehalose degradation, even though the two neutral trehalases, Nth1 and Nth2, are present in the parental strain (BY6-9α), which suggests a new function of acid trehalase other than its previously described role [14, 21, 22]. Although disruption of the *ATH1* gene was previously reported to confer better survival after freezing in a laboratory strain SEY6210 background [15], we did not observe remarkable differences in the degradation patterns of intracellular trehalose and survival ratios of yeast after freezing and thawing between the parental and *ATH1*-deleted (TL103) strains. This phenomenon may be due to differences in the backgrounds of the strains, which can impact the expression and function of *ATH1*.

The decrease of neutral trehalase activity shown by *NTH2* deletion mutants (TL102, TL203 and TL301) suggests that *NTH2* encodes a functional neutral trehalase in the stationary phase in glucose medium, in accordance with the expression level of *NTH2*, which is high in the stationary phase [19]. Similar results were shown by *NTH1* deletion mutants (TL101 and TL202). Meanwhile, Nth2 is significantly involved in intracellular trehalose degradation in the *NTH1* and/or *ATH1* gene deletion mutants at the initial fermentation period, which corresponds to the activity of Nth2 and suggests a new function for Nth2 other than its previously described role in the mobilization of the accumulated trehalose of strain *nth1Δath1Δ* or involves in trehalose mobilization of strain *tps1Δnth1Δ* in the presence of extracellular trehalose [5, 13]. Despite the deletion of triplicate trehalase genes, strain TL301 (*nth1Δnth2Δath1Δ*) showed low levels of acid and neutral trehalase activity, and the content of intracellular trehalose in the strain declined to 92.9 mg (g CDW)$^{-1}$ (Fig. 1). This phenomenon suggests that other unknown mechanisms might be involved in the mobilization of trehalose.

Based on our analyses of the trehalose degradation amounts and trehalase activities in the mutants, a mutual

influence among the three trehalases Nth1, Nth2 and Ath1 is suggested. The nature of these crossed effects is not known, but it may be related to the rapid degradation of intracellular trehalose and the fermentation characteristics of these mutants at the initial fermentation period. These results also imply that trehalose may improve the freeze tolerance of baker's yeast in proper concentration. Further experiments are underway to construct a baker's yeast with proper trehalose concentration and better freeze tolerance by deleting or tuning the expression level of relative genes.

Our investigations also showed that residual intracellular trehalose contents are positively correlated with survival ratios but that higher initial intracellular trehalose contents do not always correspond to higher survival ratios after prefermentation. The strain $TL301_{TPS1}$ retained relatively low trehalase activity, high Tps1 activity, and high residual content of intracellular trehalose before and after prefermentation. The survival ratio, the freeze-tolerance ratio and the relative fermentation ability of strain $TL301_{TPS1}$ were approximately twice as high as those of the parental strain (BY6-9α). These characteristics are consistent with the requirements of baker's yeast for frozen dough in terms of freeze tolerance. This study provides guidance for the commercial baking industry and research on intracellular trehalose mobilization and freeze tolerance of baker's yeast.

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