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Breeding of Freeze-Tolerant Baker's Yeast by the Regulation of Trehalose Metabolism

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Abstract
Accumulation of trehalose, which is a disaccharide composed of 2 molecules of glucose, is generally considered to be a critical determinant in improving the stress tolerance of the baker's yeast *Saccharomyces cerevisiae*. To retain this accumulation in yeast cells, we constructed diploid homozygous trehalase (trehalose-hydrolyzing enzyme)-deficient mutants by the gene disruption method form commercial baker's yeast. During fermentation, degradation of intracellular trehalose was inhibited in the trehalase-deficient mutants. Their freeze tolerance was maintained at a higher level than that of the parent strain. Due to the improved freeze tolerance exhibited by the trehalase-deficient mutants, these strains may be suitable for use in the baking process of frozen dough.

Discipline: Food microbiology
Additional key words: bread

Introduction

In the baking industry, frozen dough technology has recently been applied due to its advantages in supplying oven-fresh bakery products to consumers, as well as improving labor conditions for bakers[10]. Standard commercial baker's yeast is generally susceptible to damage during frozen storage and does not retain a sufficient leavening ability after frozen storage. Freeze tolerance is an important characteristic for yeast used in frozen doughs, because post-thaw leavening activity is essential prior to baking. The ability to tolerate freeze stress depends on many factors, including the growth phase and rate, nutritional status, rate of freezing, and the intracellular amount of cryoprotective compounds[12,18,21]. Although freeze-tolerant yeast strains have also been obtained by conventional mutation procedures[14,16,25], bread baked with such strains has less taste and flavor compared with bread baked with parent strains. The objective of this study was to develop freeze-tolerant baker's yeast from commercial strains using DNA recombinant techniques, without loss of the yeast's other beneficial properties.

Because disaccharide trehalose is considered to be a critical determinant of stress tolerance in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*)[5,7,9,21], we constructed trehalase-deficient mutants and investigated their freeze tolerance. Two enzymes are capable of hydrolyzing trehalose: a neutral cytosolic trehalase (designated as Nth1p) and an acidic trehalase (designated as Ath1p)[13]. Both Nth1p and Ath1p have been purified from *S. cerevisiae*, and the corresponding genes have been cloned and sequenced[1,6,11].

In order to determine the effect of trehalase gene disruption during the baking process, we constructed trehalase-deficient diploid strains from commercial baker's yeast. We showed that these mutants accumulated higher levels of trehalose than their parent strain under optimal culture conditions for high trehalose contents, and that trehalose accumulation was correlated with a higher freeze tolerance in frozen dough baking. We also showed that commercial application of the mutant strains could be obtained by gene disruption.

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Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Description
T7	Prototroph (*MATa NTH1 ATH1*)
T18	Prototroph (*MATα NTH1 ATH1*)
T7dN	*MATa nth1::URA3 ATH1*, derived from T7
T18dN	*MATα nth1::URA3 ATH1*, derived from T18
T7dA	*MATa NTH1 ath1::URA3*, derived from T7
T18dA	*MATα NTH1 ath1::URA3*, derived from T18
T7dNA	*MATa nth1::URA3 ath1::URA3*, derived from T7dA
T18dNA	*MATa nth1::URA3 ath1::URA3*, derived from T18dA
T118	*MATa/α NTH1/NTH1 ATH1/ATH1*, obtained by mating T7 and T18
T154	*MATa/α nth1/nth1 ATH1/ATH1*, obtained by mating T7dN and T18dN
A318	*MATa/α NTH1/NTH1 ath1/ath1*, obtained by mating T7dA and T18dA
AT418	*MATa/α nth1/nth1 ath1/ath1*, obtained by mating T7dNA and T18dNA

Materials and methods

1) Yeast strains and culture conditions

The strains of baker's yeast used in this study are listed in Table 1.

The medium used in this study was described in the previous report[24].

Yeast cells used in the baking and freeze tolerance tests (see below) were grown in molasses medium using a continuously fed batch culture (simulating industrial yeast production) in a 30-L fermentation jar (Oriental Bioserves, Japan) at 30°C[19]. Diploid strains were constructed by mating strains of opposite mating types in YPD medium.

2) Plasmid construction and disruption of trehalase genes

We constructed the trehalase-deficient strains using one-step gene disruption, which involved double recombination events at the homologous site[20]. Transformation was carried out using the LiCl methods described by Schiestl and Gietz[23]. The gene disruption method of trehalase genes was described in the previous paper[24].

3) DNA isolation, Southern blot analysis, and molecular biology methods

Yeast DNA was isolated in a manner essentially similar to that described by Hereford et al.[8]. Southern hybridization was carried out using a Hybond-N nylon membrane (Amersham Pharmacia) and an ECL direct nucleic acid labeling and detection system (Amersham Pharmacia) according to the manufacturer's instructions. Standard molecular biology techniques were adopted as described by Sambrook et al.[22].

4) Assay for trehalose, protein, and trehalase activity

Yeast cells were collected by centrifugation (1,000 × g) for 10 min and washed 3 times with cold (4°C) distilled water. After extraction with 0.5 M trichloroacetic acid from the yeast cells, the trehalose content in the extracts was determined by the anthrone method[3]. Crude lysate of yeast cells was prepared by disruption using glass beads. The protein concentration of the crude lysate was determined by the Bradford method[2]. The activity of neutral and acid trehalase was assayed from crude extracts of cells as described by Mittenbuhler et al.[15].

5) Baking

Baking and evaluation of bread were carried out by the method applied by Japan Yeast Industry Association.

Results and discussion

1) Construction of trehalase-deficient mutants by gene disruption from commercial baker's yeast

We used the haploid strains, which were selected from commercial baker's yeast, for the trehalase gene disruption and formation of diploid strains. Since the haploid parent strains did not have any selectable makers, the spontaneous *ura3* mutants were obtained by 5-

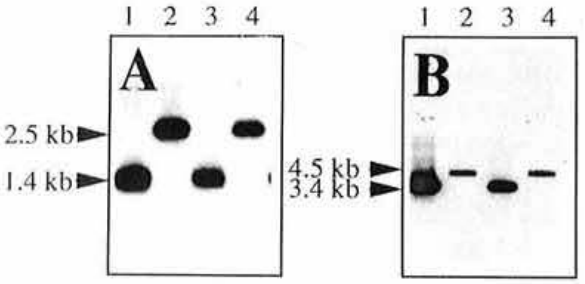

Fig. 1. Southern blot analysis of gene-disruption for the production of *nth1* mutants (A) and *ath1* mutants (B)

Total yeast genomic DNA was digested with *Eco*RI, electrophoresed on 1% agarose gels, transferred to a nylon membrane, and hybridized with (A) a 1.4-kb-*Eco*RI-*Kpn*I fragment containing *NTH1* ORF, and (B) a 1.1-kb fragment containing *ATH1* ORF.

A: lane 1, T7; lane 2, T7dN; lane 3, T18; lane 4, T18dN.

B: lane 1, T7; lane 2, T7dA; lane 3, T18; lane 4, T18dA.

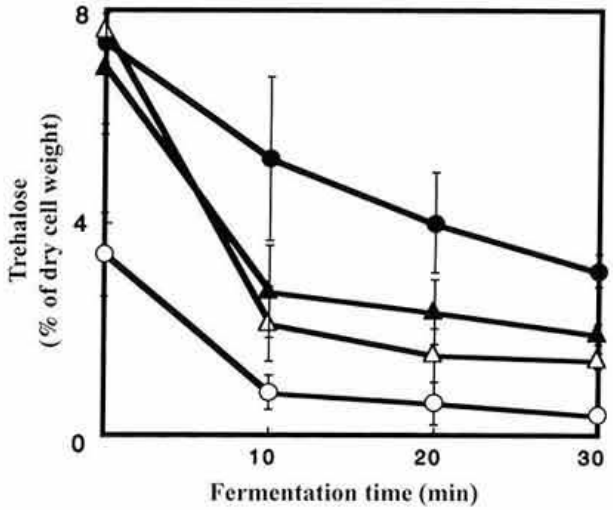

Fig. 2. Changes in intracellular trehalose content during fermentation in LF medium at 30°C

The results represent the mean ±S.D. of 3 independent experiments.
○ : T118, ● : T154, △ : A318, ▲ : AT418.

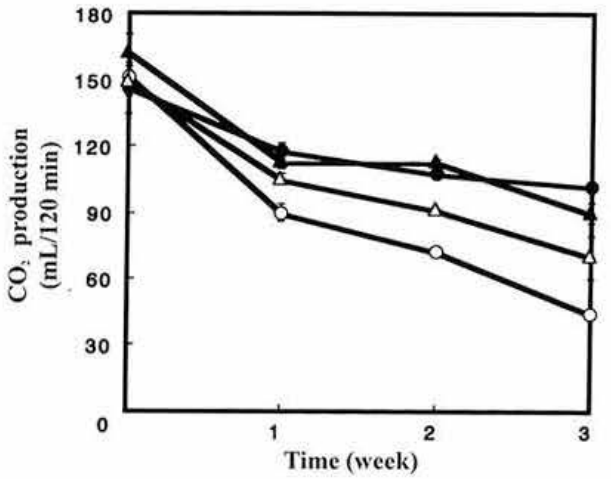

Fig. 3. Gassing power in frozen doughs stored for different periods of time at –20°C

The doughs were thawed, and then CO_2 production at 30°C for 2 h was measured. The results represent the mean ±S.D. of 3 independent experiments.
○ : T118, ● : T154, △ : A318, ▲ : AT418.

fluoroorotic acid (5-FOA) negative selection[4]. The *ura3* mutants derived from 2 haploid strains (one for mating type **a**, and the other for mating type α were used for transformation. The *Δnth1* mutants and *Δath1* mutants were obtained by transformation of the *ura3* haploid strains with the fragments containing the *nth1::URA3* or *ath1::URA3* allele. The *Δnth1 ura3* mutant was obtained by spontaneous 5-FOA re-selection from *Δnth1* strain for the construction of the *Δnth1 ath1* double mutant, because the *ura3* marker was readily available for the transformation of the baker's yeast strains used here. The *Δnth1 ath1* mutant was constructed by transformation of the *Δnth1 ura3* strain by using the fragment containing the *ath1::URA3* allele. Trehalase gene disruption was confirmed by Southern blot analysis (Fig. 1). In the YPD medium or molasses medium, all these mutants grew as well as their parent strains (data not shown).

2) Intracellular accumulation and degradation of trehalose in the trehalase-deficient mutants

Cells of trehalase-deficient mutants and parent strains were grown using continuously fed batch cultures, which simulated the industrial yeast production process. At various times during the growth, samples were removed and their intracellular trehalose levels measured. In general, during fermentation, the amount of trehalose in baker's yeast decreased very rapidly. When trehalase-deficient mutants underwent fermentation in

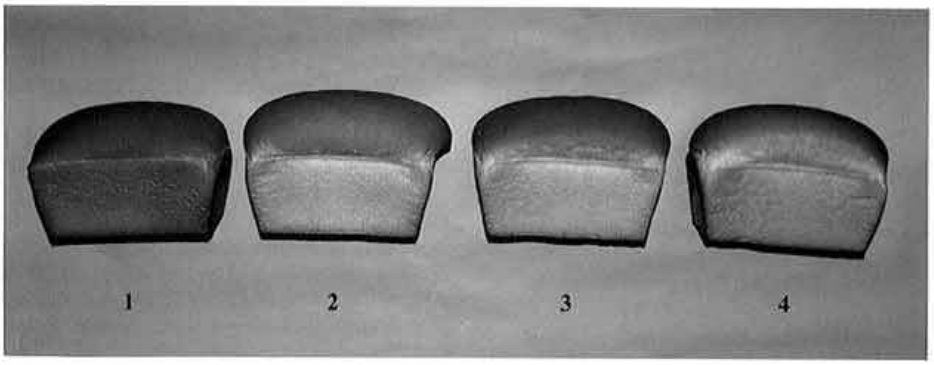

Fig. 4. Freeze dough baking with trehalase-deficient mutants

White bread dough was stored for 1 week at –20°C, molded, proofed, and then baked.
1: White bread baked with T118, 2: White bread baked with T154,
3: White bread baked with A318, 4: White bread baked with AT418.

the liquid fermentation medium (LF) for 10 to 30 min at 30°C, trehalose accumulation in cells differed from that of the parent strains (Fig. 2). In the parent strain T118, the trehalose level decreased below 0.5% after 10 min. In the *Δnth1*, *Δath1*, and *Δnth1 ath1* strains, the trehalose level also decreased initially, but only to between 1.5 to 4% and then remained relatively constant. In the *Δnth1 ath1* strain, no trehalase activity was expected, but the trehalose level slowly decreased, presumably due to the *NTH1* homologue, *NTH2*[17].

3) Freeze tolerance of the trehalase-deficient mutants

Intracellular accumulation of trehalose is considered to enhance yeast's tolerance to freezing. We evaluated this tolerance in white bread dough at various frozen storage times by measuring the rate of CO_2 production in thawed dough that contained trehalase-deficient mutants and their parent strains (Fig. 3). All the mutants showed a higher gassing power than their parent strains. Among the trehalase-deficient mutants, the *Δnth1* strain showed the highest freeze tolerance associated with its high intracellular trehalose level during fermentation, and CO_2 production remained at 100 mL even after 3 weeks of frozen storage.

In the frozen dough baking tests, white bread dough was stored for 1 week at –20°C, molded and proofed, and then baked for 30 min at 200°C. Specific volumes of the dough before and after storage were compared (Fig. 4). The dough containing the trehalase-deficient mutants maintained a higher specific volume than that containing the parent strain. In particular, the *Δnth1* strain showed the highest specific volume. These results suggest that trehalase gene disruption improves the freeze tolerance of baker's yeast in dough.

Bread quality tests showed that the crust color and internal characteristics (grain, crumb color, texture, aroma, and taste) of white bread baked with trehalase-deficient mutants were similar to those of bread baked with the parent strains (data not shown).

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