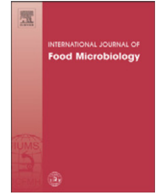
International Journal of Food Microbiology 152 (2012) 40–43

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro



Short communication

Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough

Yu Sasano [a,1], Yutaka Haitani [b,1], Iwao Ohtsu [a], Jun Shima [b], Hiroshi Takagi [a,*]

[a] Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan
[b] Research Division of Microbial Sciences, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

ARTICLE INFO

Article history:
Received 6 August 2011
Received in revised form 3 October 2011
Accepted 9 October 2011
Available online 14 October 2011

Keywords:
Industrial baker's yeast
Proline
High-sucrose stress
Stress tolerance
Bread dough
Fermentation ability

ABSTRACT

During bread-making processes, yeast cells are exposed to various baking-associated stresses. High-sucrose concentrations exert severe osmotic stress that seriously damages cellular components by generation of reactive oxygen species (ROS). Previously, we found that the accumulation of proline conferred freeze–thaw stress tolerance and the baker's yeast strain that accumulated proline retained higher-level fermentation abilities in frozen doughs than the wild-type strain. In this study, we constructed self-cloning diploid baker's yeast strains that accumulate proline. These resultant strains showed higher cell viability and lower intracellular oxidation levels than that observed in the wild-type strain under high-sucrose stress condition. Proline accumulation also enhanced the fermentation ability in high-sucrose-containing dough. These results demonstrate the usefulness of proline-accumulating baker's yeast for sweet dough baking.

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1. Introduction

During the bread-making process, baker's yeast (mostly strains of *Saccharomyces cerevisiae*) is exposed to many environmental stresses such as air-drying, freeze–thaw, and high-sucrose concentrations (Attfield, 1997). Yeast cells used for bread making must adapt to different sucrose concentrations during dough-fermentation processes (Tanaka et al., 2006). In particular, sweet dough (high-sugar dough) contains up to approximately 30% sucrose per weight of flour. Such high-sucrose concentrations exert severe osmotic stress that seriously damages cellular components (Verstrepen et al., 2004) and inhibit the optimal fermentation ability of yeast. To avoid lethal injury, baker's yeast cells need to acquire osmotolerance, but the development of osmotolerant baker's yeast strains will require knowledge of the molecular mechanism involved in high-sucrose stress tolerance, for example, by the induction of stress proteins, the accumulation of stress protectants, and the changes in membrane composition (Shima & Takagi, 2009).

When high osmotic pressure is sensed, *S. cerevisiae* cells accumulate glycerol and trehalose (Cronwright et al., 2002; De Virgilio et al., 1994; Hino et al., 1990; Hirasawa et al., 2006; Shima et al., 1999). Microarray analysis and genome-wide screening using a deletion strain collection revealed that the metabolism of glycerol and trehalose, both of which are known as osmoprotectants, is important for high-sucrose stress tolerance (Ando et al., 2006; Tanaka-Tsuno et al., 2007). In response to osmotic stress, proline is accumulated in many plant and bacterial cells as an osmoprotectant (Csonka, 1981; Verbruggen & Hermans, 2008). During various stresses, yeast cells induce glycerol or trehalose synthesis, but the intracellular proline level is not increased under various stress conditions (Kaino & Takagi, 2008). Proline has many functions *in vitro*, such as protein and membrane stabilization, lowering the T_m of DNA, and scavenging of reactive oxygen species (ROS), but the mechanisms of these functions *in vivo* are poorly understood (Takagi, 2008). Previously, we constructed *S. cerevisiae* cells that accumulate proline, and the engineered strains successfully showed enhanced tolerance to many stresses, including freezing, desiccation, oxidation, and ethanol (Matsuura & Takagi, 2005; Morita et al., 2002; Takagi et al., 1997; Takagi et al., 2000; Takagi et al., 2005; Terao et al., 2003) With regard to high osmotic pressure, we found that the proline oxidase-deficient strain, which had a significantly higher proline level, was clearly more osmotolerant than were other strains in the presence of 1 M NaCl (Takagi et al., 1997). Recently, we found that proline-accumulating baker's yeast retained higher-level fermentation ability in the frozen dough than that of the wild-type strain (Kaino et al., 2008). Based on these results, we conclude that it is possible that proline accumulation confers tolerance to high-sucrose stress on baker's yeast. For the application of recombinant yeasts for commercial use, a self-cloning yeast that has no foreign genes or DNA sequences except for yeast DNA, might be more acceptable for consumers than a genetically modified yeast.

Our objectives in this study were i) to construct self-cloning diploid baker's yeast strains that accumulate proline, ii) to evaluate the cell

* Corresponding author. Tel.: +81 743 72 5420; fax: +81 743 72 5429.
E-mail address: hiro@bs.naist.jp (H. Takagi).
[1] The first two authors contributed equally to the manuscript.

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doi:10.1016/j.ijfoodmicro.2011.10.004

viability and the intracellular ROS level of these strains under high-sucrose stress condition, and iii) to measure the fermentation ability of these strains in high-sucrose-containing dough. We report here that baker's yeast strains with proline accumulation showed high-sucrose stress tolerance and increased the leavening ability in sweet dough.

2. Materials and methods

2.1. Strains and media

We used the diploid Japanese baker's yeast strain of *S. cerevisiae*, derived from 3346 (*MAT*a) and 3347 (*MAT*α), as the wild-type strain, WT. To construct the diploid baker's strains that accumulate proline, we transformed the haploid strain 3346-ura3 and 3347-ura3 (Sasano et al., 2010) by integrating linearized pRS406-I150TPRO1 (Kaino et al., 2008) to replace the *PRO1* gene by the *PRO1-I150T* allele. The *PRO1* gene encodes γ-glutamyl kinase (GK), and the GK activity is subjected to feedback inhibition by proline (Sekine et al., 2007). The I150T-mutant GK is much less sensitive to proline-feedback inhibition than is the wild-type GK, allowing cells to accumulate proline. The replacement was confirmed by uracil auxotrophic selection, DNA sequencing, and proline accumulation, indicating that the resultant strains were bona fide self-cloning yeasts. By mating these resulting two haploid strains, we were able to construct the diploid strain PRO1-I150T by the method described previously (Kaino et al., 2008). For more proline accumulation, the *PUT1* gene, which encodes proline oxidase was disrupted in PRO1-I150T by the method described previously (Kaino et al., 2008). The resulting diploid strain was named PRO1-I150T/Δ*put1*.

The media used for growth of yeast cells were nutrient rich medium YPD (2% glucose, 2% Bacto peptone [Difco Laboratories, Detroit, MI], and 1% Bacto yeast extract), cane molasses medium (5.88% NEO MOLASSEST [EM laboratory, Shizuoka, Japan], 0.193% Urea, and 0.046% KH_2PO_4), and liquid fermentation medium. The liquid fermentation medium was a model medium of the flour-free liquid dough (Panadero et al., 2005). The composition of the liquid fermentation medium was the same as that described in previous reports (Tanaka et al., 2006; Tanaka-Tsuno et al., 2007), except that the sucrose concentration was 27.8% and the maltose concentration was 2.78%.

2.2. Measurement of intracellular proline content

Yeast cells were cultured in YPD medium. After overnight cultivation, 1 mL of the cultured sample was washed and inoculated into 20 mL of cane molasses medium. After cultivation in cane molasses medium for 48 h at 30 °C with rotary shaking, cells were washed twice and intracellular amino acids were extracted by boiling water. After centrifugation (5 min at 15,000 × g), each supernatant was subsequently quantified with an amino acid analyzer (model L-8500A; Hitachi, Tokyo, Japan). Proline content was expressed as a percentage of dry weight.

2.3. High-sucrose stress tolerance test

Yeast cells grown in cane molasses medium for 48 h were inoculated in 20 mL of liquid fermentation medium at the initial $OD_{600} = 10$ and the samples were cultivated for 2 h at 30 °C with 140 rpm rotary shaking. The incubation period was the same as fermentation test. At intervals of 1 and 2 h, aliquots of cell culture were removed and spread onto YPD solid media to examine the colony-forming unit. The number of colonies was expressed as a percentage of the number of colonies before high-sucrose stress treatment.

2.4. Measurement of intracellular oxidative level

Yeast cells grown in cane molasses medium for 48 h were collected, washed twice and inoculated into the high-sucrose-containing liquid fermentation medium. At intervals of 1 and 2 h, aliquots of cell culture were removed and the intracellular oxidation levels were measured. To measure the intracellular oxidation level, we used an oxidant-sensitive fluorescent probe 2′,7′-dichlorofluorescein (Molecular Probes, Eugene, OR) following the method described previously (Nishimura et al., 2010).

2.5. Fermentation test

Stationary-phase cells cultivated in cane molasses medium for 48 h were washed twice with distilled water, and excess water was removed using a porous plate (Nikkato, Osaka, Japan). The filter was placed at 4 °C for 1 h. The formula of dough was 50 g of bread-making flour, 15 g of sucrose, 0.25 g NaCl, 3 g of yeast, and 27.5 mL of water. The ingredients were mixed for 3 min with a Swanson type mixer (National Mfg. Co., Ltd., Sterling, IL). The mixed dough was divided into pieces (40 g each) and kept in screw cap bottles. The fermentation ability was assayed by measuring CO_2 gas production in the dough using a Fermograph II (Atto, Tokyo, Japan) (Nishida et al., 2004). The relative fermentation ability was expressed as a percentage of total CO_2 gas production for 2 h compared with WT.

3. Results and discussion

In order to confirm proline accumulation, we measured the intracellular proline contents of strains WT, PRO1-I150T, and PRO1-I150T/Δ*put1*. As shown in Fig. 1, the proline content in PRO1-I150T and PRO1-I150T/Δ*put1* was increased approximately 20-fold and 50-fold, respectively, compared to that in the control strain WT (Fig. 1), although the content of other amino acids were not significantly changed (data not shown). This result showed that PRO1-I150T and PRO1-I150T/Δ*put1* accumulated intracellular proline after cultivation in cane molasses medium.

To examine the effect of proline accumulation on high-sucrose stress tolerance, we tested the cell viability by counting the colony-forming unit after inoculation into the liquid fermentation medium (Fig. 2). At 1 h after inoculation, there was no significant difference among the three strains. Interestingly, at 2 h after inoculation, the two proline-accumulating strains (PRO1-I150T and PRO1-I150T/Δ*put1*) showed higher cell viability than that of WT, suggesting that

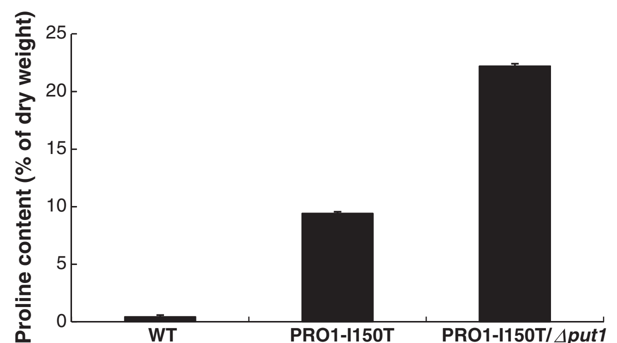

Fig. 1. Intracellular proline contents. Cells were precultured at 30 °C for 48 h in cane molasses medium. Approximately 10^8 cells were then washed twice and intracellular amino acids were extracted by boiling water. The intracellular proline content was measured with an amino acid analyzer. The values are the means and standard deviations of results from three independent experiments.

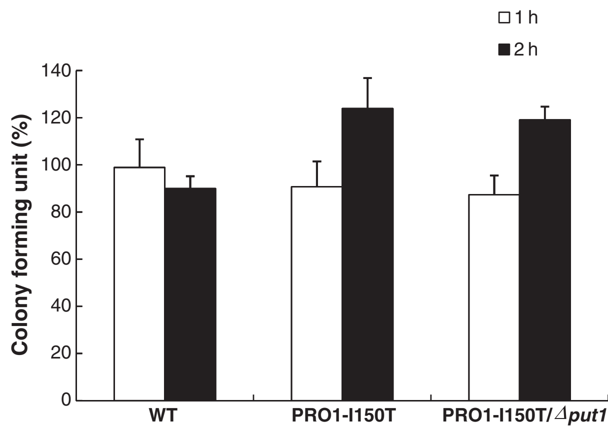

Fig. 2. Cell viability of baker's yeast strains under high-sucrose stress condition. Cells cultured in cane molasses medium were washed and inoculated into high-sucrose-containing liquid fermentation medium at the $OD_{600}=10$. The number of colonies after exposure to high sucrose concentrations for 1 h or 2 h was expressed as a percentage of the number of colonies before high-sucrose stress treatment. The values are the means and standard deviations of results from three independent experiments. Significant difference of PRO1-I150T and PRO1-I150T/*Δput1* from WT at 2 h was confirmed by Student's *t* test, $P<0.05$ and $P<0.01$, respectively.

proline accumulation confers tolerance to high-sucrose stress on yeast cells.

Landolfo et al. reported that during fermentation of high-sugar-containing medium, reactive oxygen species (ROS) accumulation caused oxidative damage to wine yeast cells (Landolfo et al., 2008), probably due to the denaturation of antioxidant proteins or the dysfunction of mitochondrial membranes. We examined whether ROS was generated under high-sucrose stress condition in baker's yeast cells (Fig. 3). When yeast cells were inoculated into the high-sucrose-containing liquid fermentation medium described above, the ROS level increased approximately twofold in all of the strains tested under high-sucrose stress condition, indicating that, as in the case of wine yeast, ROS accumulation occurs after exposure to high sugar concentrations in baker's yeast. At 2 h, proline-accumulating strains showed intracellular ROS levels that were much lower than that of WT, whereas at 1 h there were no significant differences in ROS levels among strains. Taken into consideration with the result shown in Fig. 2, a high concentration of sucrose was suggested to induce cell death via ROS accumulation. Furthermore, it appears that proline accumulation confers tolerance to high-sucrose stress on yeast cells by reducing the ROS level. We also found that the specific activity of superoxide dismutase was significantly higher in PRO1-I150T/*Δput1* than that of WT (approximately 1.7-fold), suggesting that intracellular proline protects antioxidant enzymes from high osmotic pressure.

Next, we assayed the high-sucrose tolerance of the proline-accumulating strains in sweet dough. Stationary-phase cells cultivated in cane molasses medium for 48 h were used for sweet dough fermentation. Sweet dough contained approximately 30% sucrose per weight of flour. The fermentation ability was assayed by measuring CO_2 gas production in the dough using a Fermograph. The relative fermentation ability was expressed as a percentage of total CO_2 gas production for 2 h compared with WT (Fig. 4). We found that two proline-accumulating strains showed an approximately 40% increase in the gassing power compared with WT, indicating that proline accumulation enhanced the leavening ability in high-sucrose-containing dough. Our test results also revealed that an appropriate proline level (approximately 9%) in yeast cells is important for its stress-protective effect, because the statistical analysis (Student's *t* test) revealed that there was no significant difference in fermentation ability between PRO1-I150T and PRO1-I150T/*Δput1*.

We showed that intracellular proline accumulation conferred tolerance to high concentrations of sucrose on industrial baker's yeast and enhanced the fermentation ability in sweet dough. These data clearly demonstrate that the proline-accumulating baker's yeast strains are suitable for sweet bread making. Our findings indicate that it is possible to produce bread with greater swelling, to reduce the fermentation period, and to cut the manufacturing cost. It is relatively difficult to breed baker's yeast strains with greater high-sucrose stress tolerance than that of a laboratory strain. The process that involves adding proline or antioxidant externally to the dough remains somewhat laborious for practical application, but producing the baker's yeast strains described here might allow bakery industry to overcome this problem.

Acknowledgments

This work was supported by a grant for the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry to J.S. and H.T.

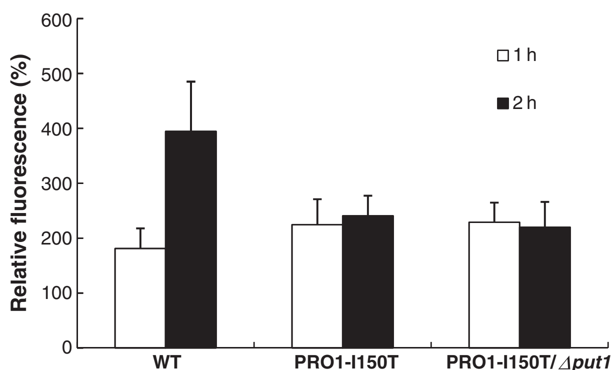

Fig. 3. Intracellular ROS levels of baker's yeast strains under high-sucrose stress condition. Cells cultured in cane molasses medium were washed and inoculated into high-sucrose-containing liquid fermentation medium at the $OD_{600}=10$. The fluorescence intensity before high-sucrose stress treatment of each strain was taken as 100%. The values are the means and standard deviations of results from three independent experiments. Significant difference of PRO1-I150T and PRO1-I150T/*Δput1* from WT at 2 h was confirmed by Student's *t* test, $P<0.1$ and $P<0.05$, respectively.

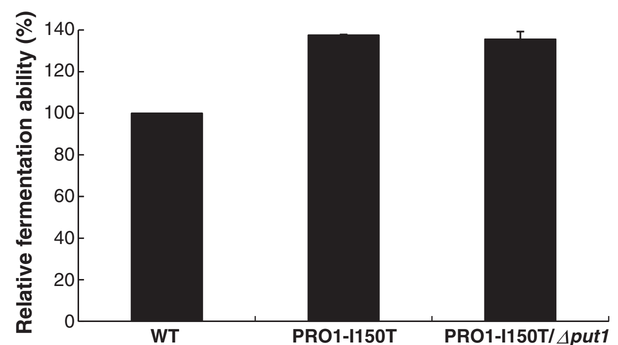

Fig. 4. Fermentation ability of baker's yeast strains under high-sucrose condition. Fermentation ability in sweet dough was monitored by CO_2 gas production using a Fermograph. The total amounts of CO_2 production after 2 h were measured. The gassing power of WT was defined as 100%. The values are the means and standard deviations of results from four independent experiments.

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