

Loading of Exogenous Trehalose into Baker's Yeast and Its Influence on Freeze Tolerance in Frozen Dough

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Abstract : Intracellular trehalose level of baker's yeast is known to be a critical determinant of stress tolerance, and it has a close correlation with freeze tolerance which is an essential quality of the yeast for frozen dough process in bread-making. This study was undertaken to obtain freeze-tolerant yeasts applicable to frozen dough process by loading exogenous trehalose into baker's yeast or cultured yeast strains. When commercial baker's yeast or laboratory cultured yeast strains with different freeze sensitivity were incubated in 1 M trehalose solution at 4~20°C for 7 days, the intracellular trehalose level of yeast increased rapidly within a short time of incubation, and the maximum amount of trehalose in yeast reached to 2 to 5-fold initial trehalose level before incubation. Some of the baker's yeast strains into which a large amount of trehalose was introduced by the incubation showed an apparent increase in freeze tolerance. This result suggested that a simple loading method of exogenous trehalose into baker's yeast would improve the freeze tolerance of yeast and help to transform freeze-sensitive yeast to freeze-tolerant yeast which is appropriate for frozen dough process.

Key words : baker's yeast, trehalose, freeze tolerance

Introduction

Frozen dough process has been accepted in commercial baking due to several advantages such as improvements of baking process in terms of labor time and cost for manufacturers and easy supply of freshly baked products to consumers.^{1, 2)} Baker's yeast for this purpose has to be freeze-tolerant during a long-term storage of frozen dough without losing its gassing power after thawing.^{3, 4)} Freeze-tolerant yeasts which are suitable for frozen dough have been developed by various approaches such as isolation from nature⁵⁻⁸⁾ mutation⁹⁻¹¹⁾ and molecular genetics.^{12, 13)} The mechanism of freeze tolerance of yeast has also been in-

vestigated by many researchers, and trehalose, a non-reducing storage carbohydrate in yeast, is known to play an important role as a stress protectant of yeast from damages by various environmental factors such as desiccation, freezing and heat shock.¹⁴⁾ Baker's yeast is generally manufactured with molasses medium under strictly aerobic culture conditions in its final culture stage, but the content of intracellular trehalose and its freeze sensitivity varies with yeast strains due to their genetic characteristics and culture conditions. Recent studies have indicated that trehalose is one of the critical determinants of freeze tolerance of yeast,^{15, 16)} and that the intracellular trehalose content correlates closely to freeze tolerance of

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baker's yeast, and all the freeze-tolerant and osmo-tolerant yeast have higher level of intracellular trehalose.^{12, 17, 18)} As the frozen dough process has recently been accepted in baking industry due to several advantages, some strains of freeze-tolerant baker's yeast have been available in the market, but the development of yeast strains with higher freeze tolerance and superior characteristics for frozen dough process is still required for bread-making.

It is reported that the addition of exogenous glycerol to *Saccharomyces* yeast has led to an equilibration of the polyol across the yeast cell membrane, resulting in an improved leavening ability of frozen sweet dough.^{19, 20)} These reports gave us a suggestion that the addition of exogenous trehalose to baker's yeast might lead to an enhancement of the intracellular level of trehalose, and result in the improvement of freeze tolerance of yeast for frozen dough process. In the previous work,²¹⁾ we investigated the incubation conditions for loading exogenous trehalose into commercial baker's yeast in order to enhance intracellular level of trehalose, by which the improvement of freeze tolerance of yeast was anticipated. It was found that when a strain of baker's yeast was incubated in 0.5~1 M trehalose solution at various temperatures between 4~30°C, the freeze tolerance of yeast increased with incubation time in parallel with increasing amount of intracellular trehalose.²¹⁾ The result showed a close correlation between intracellular level of trehalose and freeze tolerance of yeast, and indicated that the loading of exogenous trehalose into yeast to enhance intracellular trehalose would be an effective and convenient means to obtain freeze-tolerant yeast from freeze-sensitive yeast which is not appropriate for the frozen dough process.

This study was carried out to investigate the effect of loading of exogenous trehalose into several

strains of commercial baker's yeast in the market and laboratory yeast strains of different freeze sensitivity on the change in intracellular trehalose level, freeze tolerance and dough fermentation activity. Laboratory yeast strains were isolated from commercial baker's yeast strains by pure culture, and they were grown under the same culture conditions, followed by examination for the freeze tolerance after loading with exogenous trehalose.

Materials and Methods

Chemicals

Trehalose with a purity of 99.5% was obtained from Hayashibara and Ajinomoto Co. Ltd. Trehalase from porcine kidney was purchased from Sigma Chemical Co., for the identification of trehalose. Yeast extract and polypeptone were purchased from Nakarai Tesque Co. Ltd., and all other chemicals were of analytical grade.

Commercial baker's yeast and laboratory yeast strains

Freeze-sensitive and freeze-tolerant types of baker's yeast were from the following companies: Oriental Yeast (Or), Kaneka Corporation (Ka), Asahi Chemical Industry (As), Kyowa Hakko Industry (Ky), and Toyo Jozou (To). Laboratory yeast strains were isolated from these commercial yeast strains by repeated plate-culture using YPG agar medium containing 0.5% yeast extract, 1% polypeptone, 5% glucose, 0.1% KH₂PO₄, 0.1% MgSO₄ · 7 H₂O, and 2% agar, pH 5.5. Laboratory yeast strains used were numbered as 2001 (Or), 2004 (To), 2012 (Ky), 2014 (Ka), and 2015 (As), and were stocked at 4°C until use.

Cultivation of laboratory yeast strains

A loopful of yeast from slant culture was inoculated into 10 ml of YPG medium in a test tube and cultivated by shaking at 30°C for 24 h. The

culture was transferred to 200 ml of the same medium with a 500 ml-Erlenmeyer flask and cultivated at 30°C on a rotary shaker at 200 rpm for 24 h. Yeast cells were harvested by centrifugation at 3000 rpm for 10 min, washed twice with cold 0.85% saline solution, and collected yeast cells were spread on a water-absorbent clay plate to remove excess water. Yeast cake with a moisture content of 68–70% was obtained and stored at 4°C and used within 24 h.

Dough preparation and estimation of fermentation activity

Dough was prepared according to the evaluation method of baker's yeast reported by Japan Yeast Industry Association.²²⁾ The dough formula was 200 g of bread-making flour (Camelia, 14% moisture basis), 10 g of sucrose, 4 g of NaCl, 4 g of compressed baker's yeast or cultured yeast cake, and 124 g of water. Dough was kneaded with a dough mixer (Pro-KM-230, Aikosha) at 30°C for 10~12 min until a maximum development of gluten was attained. The mixed dough was divided into 50 g per piece and the fermentation activity of duplicate doughs was determined at 30°C with Fermograph AF-1000 (Atto) by measuring the volume of CO₂ (ml) evolved at every 10 min for 5 h.

Assay of freeze tolerance of yeast in frozen dough

Freshly prepared dough (50 g, duplicate) was prefermented at 30°C for 2 h, then molded into a flat shape (1 cm in height, 10 cm in diameter), wrapped with a plastic film, and stored at -20°C.

After storage for 7 days, the frozen dough was thawed at 30°C for 30 min, and the dough fermentation activity was measured for 3 h with Fermograph (F'3 h). The volume of CO₂ evolved for 2 h (F 2 h) and 5 h (F 5 h) before freezing was assayed, and the freeze tolerance ratio (FTR) of pre-

fermented frozen dough was calculated by the following equation :

$$\text{FTR (\%)} = \text{F}'3 \text{ h} \div (\text{F} 5 \text{ h} - \text{F} 2 \text{ h}) \times 100$$

Loading of exogenous trehalose into yeast cells and assay of intracellular trehalose

Five grams of compressed baker's yeast or cultured yeast cake of laboratory strains was suspended into 50 ml of 1 M trehalose solution, and incubated at 4~20°C for 7 days by slow shaking. The yeast suspension (5 ml) was taken out at intervals and centrifuged at 3000 rpm for 10 min, and cells were washed twice with 5 ml of cold 0.5 M KCl solution, followed by distilled water. Washed cells were suspended into 10 ml of 12.5% trichloroacetic acid (TCA) solution, kept at 30°C for 1 h by slow shaking, followed by centrifugation at 3000 rpm for 10 min. The amount of trehalose in the supernatant TCA extract was routinely determined as follows. To 5 ml of an ice-cold anthrone reagent (0.2% anthrone solution in 70% sulfuric acid) in a test tube, was added slowly 1 ml of the TCA extract, mixed, and heated in a boiling water bath for 3 min. After cooling the tube in tap water to room temperature, the absorbance of color-developed solution was measured at 620 nm by Hitachi 200-10 spectrophotometer. The amount of trehalose was determined by a calibration curve of authentic trehalose assayed by the same method.

Identification of intracellular trehalose

Intracellular trehalose in the TCA extract from yeast was identified by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). TCA extract was concentrated under reduced pressure after excess TCA was removed by extraction with diethyl ether, and the neutralized concentrate was applied to HPLC with a column of Shodex Axpak WA-624 and Shodex RID-2 A as a detector. The elution was carried out at

50°C with acetonitrile : distilled water (3 : 1, v/v) at a flow rate of 1 ml/min. Trehalose was also identified by TLC with Silicagel 60 F-245 plates (Merk) using n-butanol : acetone : 0.1 M phosphate buffer (pH 5) (4 : 5 : 1, v/v/v) as a developing solvent. Sugars were detected with a spraying reagent consisted of equal volume of 0.2% naphthoresorcine-ethanol solution and 20% sulfuric acid. The trehalose in TCA extract was also identified as glucose after hydrolyzed by a specific enzyme trehalase. The reaction mixture contained 20 μ l of 200 mM citrate buffer (pH 5.7), 22 μ l of trehalase (3.2 U/ml), and 20 μ l of TCA extract from yeast, and was incubated at 37°C for 1 h. The reaction was terminated by heating in boiling water for 1 min, and an aliquot of the reaction mixture was applied to TLC and HPLC. Authentic trehalose was hydrolyzed under the same reaction conditions as described above.

Results and Discussion

Change in intracellular trehalose level of baker's yeast and laboratory yeast strains by loading exogenous trehalose

Five strains of baker's yeast from three companies and five laboratory strains with different freeze sensitivity in frozen dough were incubated in 1 M trehalose solution at 4°C, 10°C and 20°C for 7 days, and the change of the intracellular trehalose content during incubation was investigated. As a reference, each yeast strain was incubated in 0.85% sodium chloride solution instead of trehalose solution. As shown in Fig. 1, the initial trehalose content of five industrial strains before incubation ranged from 60 mg to 130 mg/g dry weight, but when they were incubated in 1 M trehalose solution, intracellular trehalose was rapidly increased within 1 day, and afterward increased slowly until 7 days incubation. The maximum amounts of trehalose loaded into 4 strains at 20°C for 7 days at-

tained to 230~260 mg/g dry weight, about 2 to 4 fold-increase against initial amount of intracellular trehalose. The incorporation rate of trehalose into yeast cells seemed to be affected by incubation temperature in these strains, low temperature tended to delay the incorporation of trehalose. On the other hand, as shown in Fig. 2, initial trehalose level of laboratory yeast strains was low as compared to five industrial strains, ranging between 15~20 mg/g dry weight, but the intracellular trehalose level increased to a maximum in 1 day when yeast strains were incubated in trehalose solution. The maximum level of trehalose after 7 days incubation reached to 2~5-fold increase against their initial levels. It was found that the rate of trehalose incorporation into laboratory yeast strains also seemed to be accelerated by higher incubation temperature. These results indicate that the loading of exogenous trehalose under the incubation conditions as described in Fig. 1 and Fig. 2 will ensure the increase in intracellular trehalose level of baker's yeast and cultured yeast.

Identification of intracellular trehalose in TCA extract from yeast

The TCA extract from baker's yeast and cultured yeast strains after they were incubated in trehalose solution contained a large amount of an anthrone-positive compound as shown in Fig. 1 and Fig. 2, which was determined tentatively as trehalose for routine assays. Therefore, we had to identify the compound to be trehalose by other analytical methods. Figure 3 shows an elution profile by high-performance liquid chromatography of sugars in the TCA extract from yeast before and after incubation in 1 M trehalose and 0.85% NaCl solution. The retention time of the compound found in TCA extract coincided with authentic trehalose, and no other compound except trehalose was detected. It was also obvious that the amount

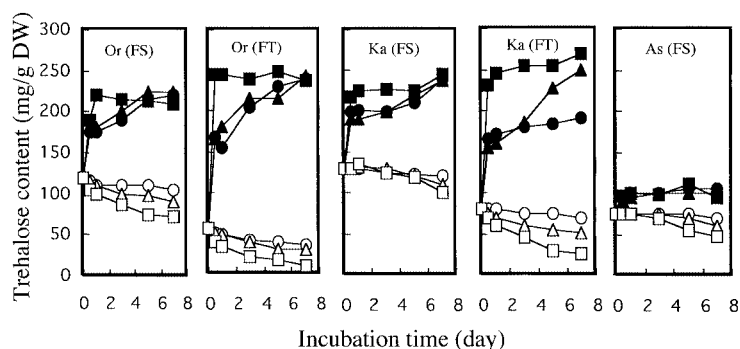


Fig. 1 Changes in Intracellular Trehalose Level of Baker's Yeast Strains Incubated in Trehalose Solution. Each strain of compressed baker's yeast (5 g) was suspended into 50 ml of 1 M trehalose solution and incubated at 4°C (●), 10°C (▲), and 20°C (■) for 7 days by slow shaking. As a control, each strain was incubated in 0.85% NaCl solution at 4°C (○), 10°C (△), and 20°C (□) for 7 days. Or, Ka and As were the names of yeast makers shown in the Materials and Methods. FS : freeze-sensitive strain, FT : freeze-tolerant strain.

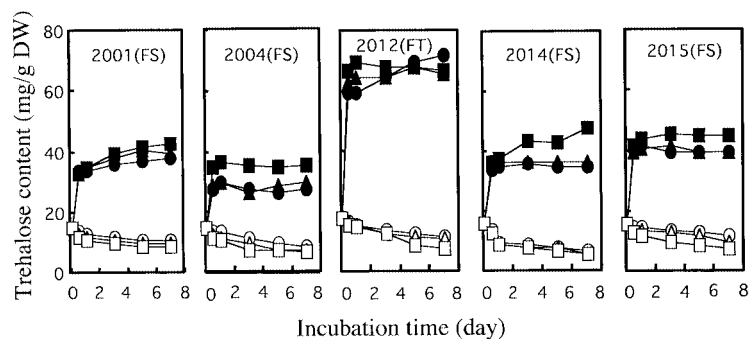


Fig. 2 Changes in Intracellular Trehalose Level of Cultured Yeast Strains Incubated in Trehalose Solution. Each strain of cultured yeast (5 g) was suspended into 50 ml of 1 M trehalose solution and incubated at 4°C (●), 10°C (▲), and 20°C (■) for 7 days by slow shaking. As a control, each strain was incubated in 0.85% NaCl solution at 4°C (○), 10°C (△), and 20°C (□) for 7 days. Numbers 2001, 2004, 2012, 2014, and 2015 were the stock numbers of laboratory yeasts isolated from commercial baker's yeast strains (see Materials and Methods). FS : freeze-sensitive strain, FT : freeze-tolerant strain.

of trehalose from yeast incubated in trehalose solution was higher than that of yeast incubated without trehalose. Figure. 4 shows a thin-layer chromatogram of authentic sugars and the compound in the TCA extract from yeast before and after treatment with an enzyme trehalase, which hydrolyzes trehalose to glucose. It is shown that the compound was converted to glucose by the enzyme, indicating that the intracellular compound of yeast is only trehalose. Therefore, the anthrone-positive compound in the TCA extract from yeast was identified as trehalose.

Influence of intracellular trehalose level on dough fermentation activity and freeze tolerance of yeast

It was ascertained that the intracellular trehalose of both baker's yeast and cultured yeast strains increased to a maximum level by incubation in trehalose solution at 4~20°C within a few days (Fig. 1, Fig. 2). Then, we investigated the influence of intracellular trehalose level on dough fermentation activity and freeze tolerance of yeast strains after they were incubated in 1 M trehalose solution at 10°C for 3 days. As shown in Fig. 5 and Fig. 6,

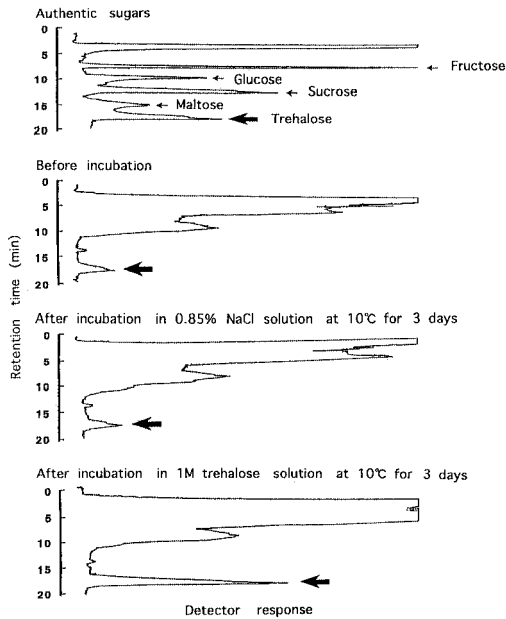


Fig. 3 Identification of Trehalose in TCA Extract from Baker's Yeast by High Performance Liquid Chromatography.

The TCA extract of freeze-sensitive strain of baker's yeast (Or) shown in Fig. 1 was used for the identification of trehalose.

the dough fermentation activity of baker's yeast and cultured yeast strains was more or less changed by the increase of intracellular trehalose level after incubation, but little correlation between trehalose level and dough fermentation activity of these yeasts was observed, though there were an apparent difference in the fermentation activity between baker's yeast and cultured yeast strains. On the other hand, the freeze tolerance was affected by the intracellular level of trehalose in some baker's yeast strains. The freeze tolerance of freeze-tolerant and freeze-sensitive types of baker's yeast (Or) increased remarkably in parallel with the increase in intracellular trehalose content after incubation for 3 days, especially more than 2.5 fold-increase in freeze tolerance ratio was observed in a freeze-sensitive type of yeast (Fig. 5). A little increase in freeze tolerance was also found in a

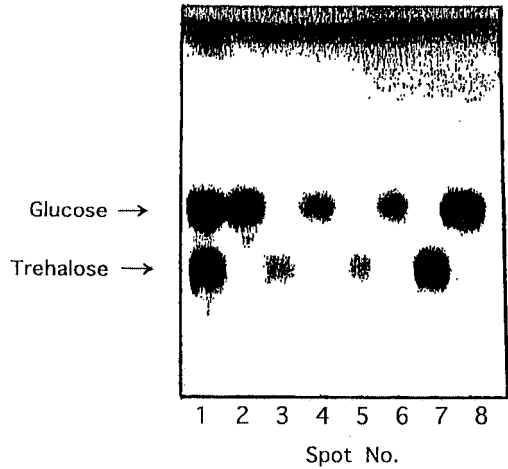


Fig. 4 Identification of Trehalose in TCA Extract from Baker's Yeast by Thin-layer Chromatography.

The TCA extract of freeze-sensitive strain of baker's yeast (Or) shown in Fig. 1 was used for the identification of trehalose.

1. glucose+trehalose, 2. trehalose+trehalase, 3. TCA extract of yeast before incubation, 4. (3)+trehalase, 5. TCA extract of yeast incubated in 0.85% NaCl at 10°C for 3 days, 6. (5)+trehalase, 7. TCA extract of yeast incubated in 1 M trehalose at 10°C for 3 days, 8. (7)+trehalase.

freeze-sensitive type of another baker's yeast (As). However, other strains of baker's yeast (Ka in Fig. 5) and all cultured yeast strains shown in Fig. 6 did not show obvious effect on their freeze tolerance in spite of the increase in intracellular trehalose levels. Therefore, the effect of intracellular trehalose loaded into yeasts on the increase in freeze tolerance in frozen dough seemed to depend on yeast strains, and the present method of loading exogenous trehalose to yeast cells would be applied to some commercial baker's yeasts to enhance their freeze tolerance.

The object of this study is to obtain freeze-tolerant baker's yeast or cultured yeast by loading exogenous trehalose into yeast cells as for convenient method. Previous reports that the gassing power of frozen sweet doughs was improved by loading of baker's yeast with exogenous glyco-

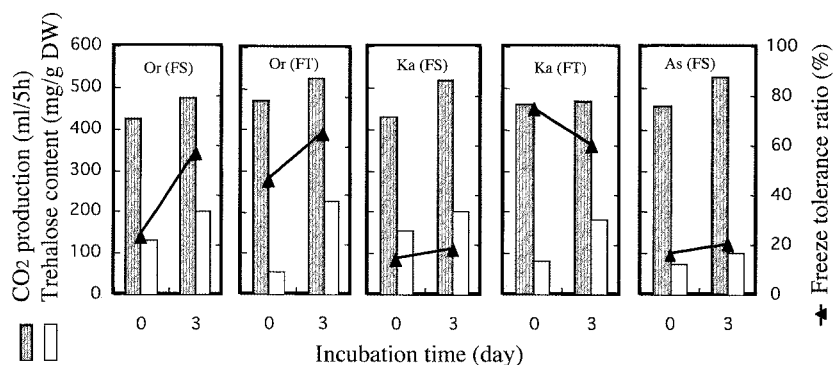


Fig. 5 Changes in Intracellular Trehalose Level, Dough Fermentation Activity and Freeze Tolerance Ratio of Baker's Yeast Strains Incubated in Trehalose Solution.

Each strain of baker's yeast (5 g) was incubated in 1 M trehalose solution at 10°C for 3 days, and intracellular trehalose level, dough fermentation activity and freeze tolerance ratio were assayed before and after incubation. Or, Ka, and As were the names of yeast makers as shown in the Materials and Methods. FS : freeze-sensitive strain, FT : freeze-tolerant strain.

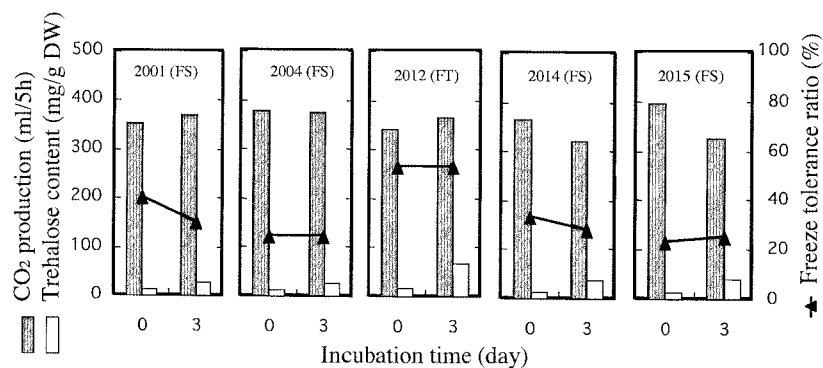


Fig. 6 Changes in Intracellular Trehalose Level, Dough Fermentation Activity and Freeze Tolerance Ratio of Cultured Yeast Strains.

Each strain of cultured yeast (5 g) was incubated in 1 M trehalose solution at 10°C for 3 days, and intracellular trehalose level, dough fermentation activity and freeze tolerance ratio were assayed before and after incubation. Numbers 2001, 2004, 2012, 2014, and 2015 were the stock numbers of laboratory strains isolated from commercial baker's yeast strains (see Materials and Methods). FS : freeze-sensitive strain, FT : freeze-tolerant strain.

erol^{19, 20}) have given us an idea of using exogenous trehalose instead of glycerol to enhance freeze tolerance of yeast in low sugar dough. The result obtained in this work and that of our previous paper²¹) have indicated the possibility of improving freeze tolerance of baker's yeast by incubation in trehalose solution for a short period of time, although the positive effect of exogenous trehalose was observed in only limited strains of baker's yeast. Further study should be done to clarify why

culture yeast strains showed no increase in freeze tolerance in spite of their high intracellular trehalose level by incubation under the conditions described in this study.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 14580168) to HK from the Japan Society for the Promotion of Science from 2002 to 2003.

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パン酵母へのトレハロースの負荷と その冷凍生地における冷凍耐性への影響

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抄録：パン酵母の細胞内トレハロース量は、酵母のストレス耐性を決定する重要な因子であり、冷凍生地製パン法に使用されるパン酵母にとって必須の性質である冷凍耐性との間に密接な関係があることが知られている。本研究は、パン酵母あるいは培養酵母にトレハロースを負荷することによって、冷凍生地法に適した冷凍耐性酵母を取得することを目的として行った。冷凍感受性の異なる種々の市販パン酵母あるいは研究室培養酵母を、4～20℃で1Mのトレハロース溶液中で7日間保持すると、短時間の間に細胞内トレハロース量が急速に増大し、その最大量は保持前の菌体の2～5倍に達した。このように多量のトレハロースを取り込んだパン酵母の中のいくつかで、その冷凍耐性の明らかな増加がみられた。本研究結果は、外来のトレハロースを負荷することによりパン酵母の冷凍耐性を改良し、冷凍に感受性の酵母を冷凍生地法に適した冷凍耐性酵母に改変する簡便な方法を示唆するものである。

キーワード：パン酵母、トレハロース、冷凍耐性

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