Induction of Freeze-sensitive Mutants from a Freeze-tolerant Yeast, *Torulaspora delbrueckii*

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Freeze-sensitive strains of yeast were induced from a freeze-tolerant yeast *Torulaspora delbrueckii* by incubation with ethylmethane sulfonate as a mutagen. A maximum ratio of mutation was attained by the incubation at 30°C for 75 min. One-hundred and fifty strains of freeze-sensitive yeast were selected by plating-culture for the first screening. The freeze-tolerance ratio of each strain was examined based on the fermentative activity before and after freezing in liquid medium and dough. Strain 60B3 showed the highest freeze-sensitivity in a pre-fermented dough (pre-fermented at 30°C for 2 h, and frozen at -20°C for 7 days) among eight strains finally selected.

A frozen-dough process has been developed in baking industry for the benefit of supplying oven-fresh bakery products to consumers and of saving labor time for bakers. However, ordinary bakers' yeast is susceptible to freeze-injury during storage of frozen doughs which were fermented for a certain time before freezing.1 These pre-fermented frozen doughs give a low quality of bread due to the poor gassing power of freeze-injured yeast. Therefore, the use of freeze-tolerant bakers' yeast that retains a sufficient leavening power of dough even after pre-fermentation and freeze-storage for some time is preferred for the frozen-dough process to produce bread of good quality. Some strains of freeze-tolerant yeast suitable for the frozen-dough process so far reported are specified as *Saccharomyces rosei* (now classified as *Torulaspora delbrueckii*),2 *Saccharomyces cerevisiae*,3,4 and *Kluyveromyces thermotolerans*.5 We screened, for practical purposes, freeze-tolerant yeast strains from natural sources, and some of yeast strains classified as *Torulaspora delbrueckii* were found to have a high fermentative activity in frozen doughs.6,6

On the other hand, the mechanism of freeze-injury and freeze-tolerance of yeast has been attracted attention in viewpoints of the preservation of microorganisms at low temperatures and the improvement of bakers' yeast good for the frozen-dough process. The growth and viability of yeast cells are injured by freezing, resulting in poor leavening activity. The freeze-injury of physiological functions of yeast arises from various causes; the denaturation of functional proteins by low temperature, the leakage of intracellular materials from yeast cells due to the breakage of yeast plasma membrane by ice-crystal formation, the change of osmotic pressure caused by condensation of cellular materials, etc.7 Therefore, to understand the mechanism of freeze-tolerance of yeast, it is important to compare biological and physiological properties of freeze-sensitive yeast with those of a freeze-tolerant one of the same genus and species. It was observed that 2% glucose, 0.5% yeast extract, and 0.5% polyepitene, pH 5.0. Cells were harvested by centrifugation at 3000 rpm, washed twice with cold distilled water, and suspended in 10 ml of 0.1 M phosphate buffer, pH 7.0, containing 3% ethylmethane sulfonate (EMS). The cell suspension, adjusted to 4 x 107 cells per ml, was incubated at 30°C for 90 min with gentle shaking, and cells were harvested by filtration at 15-min intervals of incubation and washed with 5% sodium thiocarbonate and distilled water. Cells were diluted with an appropriate amount of sterilized water, and cultivated on a YPG agar plate for 48-72 h. The colonies appearing on the plates were counted, and the survival ratio was calculated from the number of cells before incubation. The survival ratio of yeast by incubation was 45% at 30 min, 20% at 60 min, and less than 2% at 90 min.

The screening of freeze-sensitive mutants was done in four successive steps: First, 15 ml of an agar medium containing 0.57% urea, 0.2% ammonium sulfate, 6.3% sucrose, and 0.3% glucose, pH 5.6, was stacked upon two replicates YPG plates containing 20-30 colonies per plate from incubation with EMS for every 30, 45, 75, and 90 min. One of the two plates was incubated immediately at 30°C for 3-4 h, and CO2 production around colonies in the stacked medium was investigated. The other plate was kept at -20°C for one week, then thawed and incubated at 30°C for 3-4 h. Colonies with little or poor CO2 production on the plate after freezing but with a high CO2 production before freezing were picked up from the master plate as freeze-sensitive mutants tentatively. A maximum ratio of freeze-sensitive strains was attained after incubation for 75 min at the survival ratio of 10%. One-hundred and fifty strains of freeze-sensitive mutant were obtained by the first screening, which were tested for liquid fermentation for the second screening.

Each strain obtained by the first screening was inoculated into duplicate test tubes containing 5 ml of YPG medium and a Durham tube, and incubated statically at 30°C. The CO2 production was examined after 24 h for the first tube, while the second one was immediately frozen at -20°C for one week, followed by thawing and incubating at 30°C for 24 h. Eighty-two strains which had little or a poor CO2-producing activity after freezing in the medium were selected. The freeze-tolerance ratio as an index of freeze-sensitivity was estimated to screen more freeze-sensitive strains from them for the third screening.

Wet yeast cells (660 mg) of a 24-h culture on YPG medium were inoculated into 25 ml of a modified ASF medium with two Meissl flasks81 consisted of 35 g of sucrose, 2 g of urea, 1 g of ammonium sulfate, 0.8 g of magnesium sulfate, 1.6 mg of thiamine hydrochloride, 1.6 mg of pyridoxine, 16 mg of nicotinic acid, and 50 ml of 1/15 M potassium phosphate buffer (pH 6.0) in a total volume of 350 ml. The weight of CO2 evolved at 30°C for 2 h [F10(2h)] and 5 h [F10(5h)] was measured for one flask. The other flask was incubated for 2 h, frozen at -20°C for 4 days, followed by thawing at 30°C for 10 min and CO2 produced by successive incubation for 3 h was measured [F10(F)]. The freeze-
tolerance ratio (FTR/liquid) of yeast was measured by the following equation:

\[ \text{FTR/liquid} = \frac{F10(F)}{F10(5\text{h}) - F10(2\text{h})} \times 100(\%) \]

Forty strains that had low freeze-tolerance ratios were selected as freeze-sensitive yeasts, and tested for dough fermentation for the fourth screening.

A regular dough consisted of 100 g of flour (14% m.b., 12% protein), 4 g of sucrose, 2 g of sodium chloride, 2 g of wet yeast (70% m.b.) of a 24-h culture on YPG medium, and 62 ml of distilled water was prepared by the method of the Japan Yeast Industry Association. The gassing power (CO₂ production) of three kinds of dough (each 50 g per piece), i.e., an unfrozen dough, a frozen dough at \(-20^\circ\text{C}\) for 7 days without fermentation, and a frozen dough at \(-20^\circ\text{C}\) for 7 days after it was pre-fermented at 30°C for 2 h was measured at 30°C by a Fermograph (AF-1000, ATTO Co., Ltd.). The freeze-tolerance ratio (FTR/dough) of yeast in dough fermentation was calculated by the following equation:

\[ \text{FTR/dough} = \frac{\text{CO}_2(\text{ml}/3\text{h}) \text{ of pre-fermented frozen dough}}{\text{CO}_2(\text{ml}/5\text{h}) - \text{CO}_2(\text{ml}/2\text{h}) \text{ of unfrozen dough}} \times 100(\%) \]

Eight strains of freeze-sensitive yeast were finally selected based on the freeze-tolerance ratio calculated from both liquid and dough fermentations. The fermentative activities and freeze-tolerance ratios of these strains are shown in Table. Some of the strains had a fermentative activity almost equivalent to that of the parent strain, D2-4, in liquid medium and dough, while their freeze-tolerance ratios were apparently lower than that of the parent strain. We finally selected 60B3 as a most freeze-sensitive strains from these strains because of its relatively high fermentative activity in dough and low freeze-tolerance ratio in both liquid medium and dough. The survival ratio of D2-4 and 60B3 cells in late logarithmic growth phase after storage at \(-20^\circ\text{C}\) for 7 days in liquid medium was 90 and 28%, respectively (data not shown). As is shown in Fig., the gassing power of 60B3 in both unfermented and pre-fermented frozen doughs was clearly decreased as compared to that of the parent D2-4 strain. The mechanism of freeze-tolerance of yeast is an interest research problem to be solved. Comparative studies on biological and physiological properties of the freeze-sensitive and -tolerant yeasts will help us understand the mechanism of freeze-tolerance of yeast.

### References