# Screening of Freeze-Tolerant Yeasts and Their Bread Dough Fermentative Properties

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Freeze-tolerant yeasts were screened from natural sources and their characteristics for dough fermentative ability were studied. One-hundred and 44 strains of fermentable yeasts were isolated from various sources using Koji extract as a selection medium. Four strains of yeast, $D_{2-4}$, $E_2$, $F_6$ and $A_2$, which had relatively high freeze-tolerance and dough fermentative ability, were selected. Their freeze-tolerance rate ranged from 70 to 92%, which reflected the high fermentative ability of prefermented frozen doughs after storing at $-30°C$ for several days. The $D_{2-4}$ strain also showed the highest fermentative ability in the sweet doughs, but in sponge doughs, $CO_2$ production in the later stage of fermentation tended to decrease due to the lack of maltose fermentation ability. The quality of bread from frozen dough with the $D_{2-4}$ strain was superior to that prepared by *Saccharomyces cerevisiae*, which was used as a control strain.

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## Introduction

Recent use of the frozen-dough method in the baking industry has been increasing in spite of the reduced dough fermentative ability of the yeast after frozen doughs are thawed. As reported by many workers,[1,2] dough fermentation before freezing is the most important factor affecting the stability of yeast. Many studies have shown that dough fermentation before freezing causes extreme freeze-damage to bakers' yeast, and the quality of bread prepared from prefermented frozen dough is inferior to that of bread from unfrozen dough.[3-5] However, doughs with full fermentation are known to produce bread with a better loaf volume, aroma, taste and texture than those without fermentation. Therefore, the use of freeze-tolerant yeast which does not undergo much freeze-damage by fermentation before freezing is necessary in the baking industry. Several freeze-tolerant yeasts suitable for practical use in baking, *e.g.*, *Saccharomyces rosei* (*Torulaspora delbrueckii*),[6] *S. cerevisiae* FRI-413[7] and FRI-802[8], and *Kluyveromyces thermotolerans* FRI-501[9] have been reported. The screening of yeasts from natural sources which have acceptable freeze-tolerance and good baking qualities remains important.

In the present paper, we report the isolation of some strains of freeze-tolerant yeast from natural sources, their dough fermentative ability and their baking quality.

## Materials and methods

*Yeast strains.* Two strains of yeast were employed throughout this work as controls: *Saccharomyces cerevisiae* 2001 (a strain of commercial bakers' yeast from Oriental Yeast Co., Ltd.) as the non-freeze-tolerant strain, and *S. rosei* IFO 1129 (now classified as *Torulaspora delbrueckii*) as the freeze-tolerant strain.

*Cultivation of the yeasts.* All the yeasts were preserved on potato agar slant medium containing 500 ml of 20% potato extract, 30 g of pressed bakers' yeast, 15 g of glucose, 15 g of sucrose, and 15 g of agar in a total volume of 1 *l*. One loopful of yeast was inoculated into 5 ml of YPG medium which contained 2% glucose, 0.5% yeast extract and 0.5% polypeptone, and was cultivated with shaking at 30°C for 24 hr. The culture was transferred to 100 ml of the same medium, and cultivated aerobically in a 500-ml shaking flask

at 30°C for 24 hr. The yeast cells were harvested by centrifugation at 3,000 rpm for 10 min, washed twice with distilled water, and kept at 4°C until use.

*Isolation of yeasts from various sources.* Koji extract (Brix 12°) containing 100 ppm of chloramphenicol was used as a medium for accumulating and selecting the fermentable yeasts from natural sources. A small amount of various samples for screening was added to 5 ml of this medium, and cultivation was carried out with reciprocal shaking at 30°C for 72–96 hr. Yeast colonies were obtained by streaking the culture broth on YPG agar medium and incubating at 30°C for 48–72 hr. The yeast colonies which had a different appearance on the culture plates were picked up, and pure culturing was repeated three times by plating them on the same medium. The isolated yeast strains were cultivated with shaking in 100 ml of YPG medium at 30°C for 24 hr, harvested by centrifugation at 3,000 rpm, and washed twice with distilled water. The harvested cells were used for the preparation of frozen dough to examine their freeze-tolerance according to the method described below.

*Preparation of doughs and measurement of fermentative ability.* Commercial bread-making flour (Kameria, Nisshin Flour Mills Co., Ltd.) was used for preparing the doughs. The ingredients of each dough and their preparation followed the method of the Japan Yeast Industry Association.[10] Straight bread dough contained 100 g of flour, 5 g of sucrose, 2 g of salt, 2 g of yeast (wet weight) and 62 ml of water. Sponge dough contained 100 g of flour, 0.5 g of salt, 2 g of yeast, and 65 ml of water. Sweet dough contained 100 g of flour, 30 g of sucrose, 0.5 g of salt, 3 g of yeast and 52 ml of water. Each dough was mixed to its optimum development by a bread mixer (Osaka Gas 08-002), the dough temperature being kept at 30°C throughout mixing. The fermentative ability of each dough (30 g) was measured by a Fermograph AF-1000 (ATTO Co., Ltd.), which can measure automatically the gas production in fermenting dough with time.[11] The dough expansion ability of sweet dough was examined with a bottomless graduated cylinder according to the method of the Japan Yeast Industry Association.[10]

*Preparation of frozen dough.* Straight bread doughs prepared by the formulation just described were prefermented at 30°C for a given period of time,

then divided into 30 g pieces, rounded, protected in wrapping film, and kept at −30°C. After storing for 3 to 14 days in a freezer, each dough was thawed at 30°C for 2 hr and remolded, and its $CO_2$ production measured by the Fermograph.

*Examination of the freeze-tolerance of yeasts.* After 2 hr of prefermentation at 30°C, each dough (30 g) was punched and stored at −30°C for 1 week. The doughs were then thawed at 30°C for 2 hr, and their fermentative ability was measured by the Fermograph. The yeast strains which released more than 100 ml of $CO_2$ per 30 g of dough for 5 hr were selected as freeze-tolerant yeasts, based on the $CO_2$ production by *S. cerevisiae* of about 200 ml of $CO_2$ per 30 g of dough for 5 hr. The freeze-tolerance rate of each yeast was measured[12] in 25 ml of Atkin's medium[13] (described later), in which maltose was replaced by 10% sucrose, in a Meisel flask, wet yeast cells (200 mg dry weight) being added and fermented at 30°C for 3 hr. The amount of $CO_2$ released was measured [F10 (3hr)]. In another flask, fermentation was carried out for 1 hr [F10 (1 hr)], and immediately followed by freezing at −30°C. After storing for 1 week at −30°C, the flask was immersed in a water bath at 30°C for 10 min, and the $CO_2$ production was measured for 2 hr [F10 (F)]. The freeze-tolerance rate was calculated by the following equation:

$$\text{Freeze-tolerance rate } (\%) = \text{F10 (F)}/\{\text{F10 (3 hr)} - \text{F10 (1 hr)}\} \times 100.$$

*Estimation of maltose fermentative ability.* Atkin's medium was used for measuring the maltose fermentative ability of the yeasts.[13] The medium contained 5 g of maltose, 0.3 g of glucose, 150 ml of 1/15 M phosphate buffer (pH 5.6), 100 ml of nutrient solution which contained 5.7 g of urea, 2.9 g of ammonium sulfate, 2.3 g of magnesium sulfate, 4.6 mg of thiamine hydrochloride, 4.6 mg of pyridoxine hydrochloride and 46 mg of niacin in a total volume of 1 *l*. Twenty ml of this medium was taken into a Meisel flask, 0.4 g of wet yeast was added, and the flask was incubated at 30°C. The amount of $CO_2$ released in 2 hr was measured from the lost weight of each flask.

*Preparation of bread.* Straight dough bread from fresh and frozen dough was made from 400 g of flour, 20 g of sucrose, 8 g of salt, 248 ml of water, and 8 g of wet yeast, which had been cultivated aerobically in YPG medium at 30°C for 24 hr as

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previously described. Each fermented dough was divided into four pieces (165 g/piece) to make white bread. The baking conditions were a mixing time of 15 min, fermentation time of 150 min. proofing time of 70 min for fresh dough, baking time of 20–25 min, and baking temperature of 220°C. The weight and volume of each loaf was measured immediately after baking, the loaf volume being determined by the method of rapeseed displacement. Sensory evaluation scores were obtained for the aroma, taste, grain, texture and color of the bread.

### Results and discussion

*Isolation of the freeze-tolerant yeasts*

The yeasts were isolated from such sources as soil, sap, flowers, fruits and fermented foods, using Koji extract as a selection medium. As shown in Table 1, 144 strains of fermentable yeasts were isolated, and their freeze-tolerance was examined by measuring the dough fermentative ability after thawing a frozen dough which had been prefermented for 2 hr. Four strains, $A_2$ from soil, $D_{2-4}$ from grain, $E_2$ from flowers, and $F_6$ from fruit, were found to have relatively strong freeze-tolerant characteristics as well as a high fermentative ability. The freeze-tolerance rate of the selected strains as an index of freeze-tolerance in frozen dough was compared with that of *Saccharomyces cerevisiae* 2001 and *Saccharomyces rosei* IFO 1129 from laboratory collections, which are known as non-freeze- and freeze-tolerant yeast, respectively. As shown in Table 2, the four selected strains had a high freeze-tolerance rate ranging from 70 to 92%, while *S. cerevisiae* had a lower rate as was expected. $E_2$ strain showed the

Table 1. Isolation of freeze-tolerant yeasts from various sources

| Source | Number of isolated yeast strains | Number of freeze-tolerant yeasts (strain) |
|---|---|---|
| Soil | 31 | 1 ($A_2$) |
| Water | 14 | 0 |
| Sap | 5 | 0 |
| Grains | 9 | 1 ($D_{2-4}$) |
| Flowers | 61 | 1 ($E_2$) |
| Fruits | 17 | 1 ($F_6$) |
| Foods | 7 | 0 |
| Total | 144 | 4 |

highest freeze-tolerance rate, which was comparable to that of *S. rosei*, the control freeze-tolerant yeast.

*Effect of prefermentation time and frozen-storage period on the dough fermentative ability*

It has been reported that bakers' yeast is liable to undergo freeze-damage by the prolonged prefermentation time and frozen-storage period, resulting in a poor fermentative ability and long proof time for frozen dough.[14)15)] Figure 1 shows that the fermentative ability of frozen dough made by *S. cerevisiae* decreased considerably with the increase of prefermentation time and frozen-storage period. The fermentative ability of frozen dough by *S. cerevisiae*, which was prefermented for 2 hr, after storing for 1 week at −30°C was reduced to a quarter of the value for fresh dough.

On the other hand, the fermentative ability of the selected strains and *S. rosei* was less affected by the prefermentation time and frozen-storage period than was *S. cerevisiae*. In the frozen dough made

Table 2. Freeze-tolerance rate of various yeasts

| Yeast strain | F10 (3h) (ml) | F10 (F) (ml) | Freeze-tolerance rate (%)* |
|---|---|---|---|
| *S. cerevisiae* | 260 | 80 | 36 |
| *S. rosei* | 180 | 137 | 91 |
| $A_2$ | 190 | 138 | 86 |
| $D_{2-4}$ | 240 | 180 | 90 |
| $E_2$ | 160 | 120 | 92 |
| $F_6$ | 230 | 130 | 70 |

* F10 (F)/{F10 (3h)−F10 (1h)} ×100. Freeze-tolerance rate was determined by the method described in the materials and methods section.
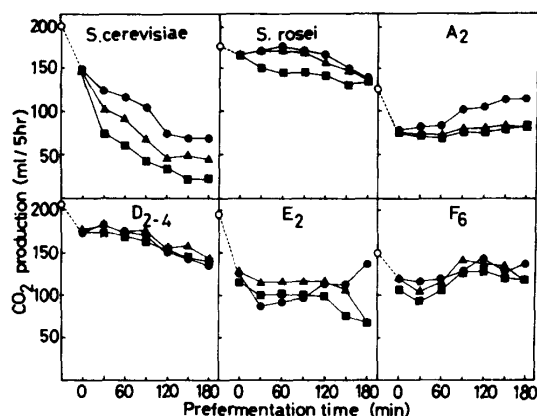
Fig. 1. Effect of prefermentation time and freeze-storage period on the dough fermentative ability of various yeasts

Freeze-storaeg period: ● 3 days, ▲ 7 days, ■ 14 days. ○ initial fermentative ability of unfrozen dough.



Fig. 2. Gassing power of fresh and frozen doughs made from various yeasts

(A) Fresh dough, (B) frozen dough. Each dough was prefermented at 30°C for 2 hr before freezing in (B). ● S. cerevisiae, ○ S. rosei, ■ A₂, ◐ D₂₋₄, ▲ E₂, △ F₆.

with $A_2$ and $F_6$ strains, the fermentative abilities seemed to increase slightly with an increase of prefermentation time. The same phenomenon has been reported with such freeze-tolerant strains as S. cerevisiae FRI-054 and S. rosei.[15] $D_{2-4}$ strain showed relatively high fermentative ability in unfrozen fresh dough and frozen dough, and little difference in the dough fermentative ability was observed throughout a frozen-storage period from 3 to 14 days.

*Fermentation curves for fresh and frozen doughs*

The gassing power of fresh and frozen doughs prepared from the four selected yeast strains, S. cerevisiae and S. rosei was investigated. As shown in Fig. 2(A), $D_{2-4}$ strain maintained a relatively high fermentative ability in fresh dough for about 3 hr of fermentation, while that of S. cerevisiae seemed to decrease gradually after fermentation for 2 hr. $F_6$ and $A_2$ strains had poorer fermentative ability than $E_2$ strain and S. rosei. The fermentative ability of these yeast strains in frozen doughs stored at −30°C for 1 week, which were prefermented for 2 hr, is shown in Fig. 2(B). The fermentative ability of all the strains tested was decreased as compared with the result shown in Fig. 2(A). S. cerevisiae was affected greatly by freezing, suggesting that this yeast is very susceptible to damage by freezing. It has been reported that activated bakers' yeast after initial fermentation is more susceptible to freeze-damage than
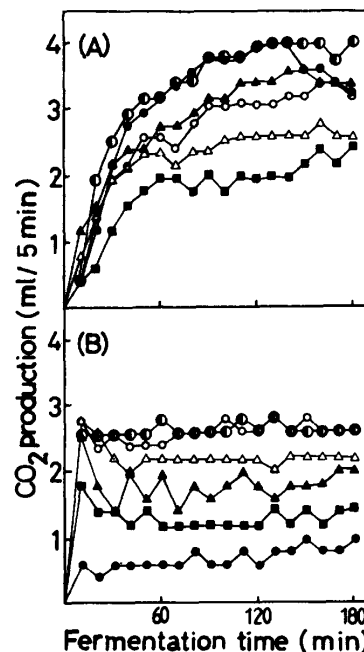
non-activated yeast, and that ethanol as the main fermentative product by yeast may cause damage to the yeast during frozen storage.[14] As shown in Fig. 2(A), both S. cerevisiae and $D_{2-4}$ strain gave the maximum gassing rate in the early stage of fermentation, which suggests that similar amounts of ethanol may have been accumulated in the fermented dough made by these yeast strains. Therefore, the distinct difference of the degree of freeze-damage between S. cerevisiae and $D_{2-4}$ strain as shown in Fig. 2(B) may partly be due to the difference in susceptibility of the two yeast strains to ethanol, although this deduction needs to be investigated further in detail. From these results, $D_{2-4}$ strain was found to be a potent freeze-tolerant strain among the selected strains.

*Fermentative ability of yeast for sweet dough and sponge dough*

Figure 3 shows the dough expansion ability of various yeasts in sweet doughs containing 30% sucrose, based on the flour by cylinder method. $D_{2-4}$ strain had the highest dough expansion ability in sweet dough, followed by $E_2$, $F_6$ and $A_2$
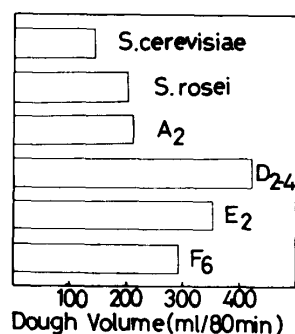
26 (118)

Fig. 3. Dough expansion ability for various yeasts in sweet doughs

● *S. cerevisiae*, ○ *S. rosei*, ■ A$_2$, ◐ D$_{2-4}$, ▲ E$_2$, △ F$_6$.

strains, while *S. rosei*, which is known as an osmophilic strain, showed a lower dough fermentative ability, and *S. cerevisiae* the lowest. These results indicate that the selected yeast strains were all osmotolerant (sugar-tolerant) and that they may be applicable for preparing sweet-dough bread.

The fermentative ability of these strains in sponge dough was examined by the Fermograph (Fig. 4). It has been reported that an initial fermentation of sponge dough by bakers' yeast occurs by the degradation of glucose, fructose and sucrose in the flour. After the consumption of these fermentable sugars, yeast adapted to maltose fermentation utilizes the maltose derived from damaged starch by flour amylases, which results in the second peak of maltose fermentation.[16] As shown in Fig. 4-a, the Fermogram of *S. cerevisiae* gave two fermentation peaks, the first one arising from the fermentation of glucose and sucrose, and the second one from that of maltose. D$_{2-4}$ and E$_2$ strains (Fig. 4-d, e), on the other hand, seemed to have little fermentative ability for maltose like *S. rosei* (Fig. 4-b), since the CO$_2$ production in the later period of fermentation apparently decreased without a second fermentation peak appearing. In A$_2$ and F$_6$ strains (Fig. 4-c, f), however, CO$_2$ production was continued for 5 hr, indicating that these strains may have had maltose fermentation activity. These results corresponded to the result for the liquid fermentation ability of maltose by yeast shown in Fig. 5. A$_2$ and F$_6$ strains showed a relatively higher maltose fermentative ability than D$_{2-4}$ and E$_2$ strains.

*Quality of bread prepared by the freeze-tolerant yeasts*

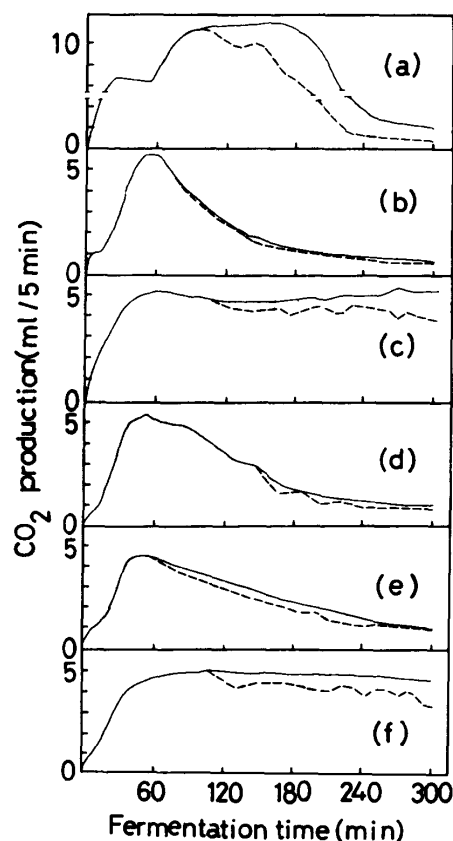A baking test was carried out in order to as-



Fig. 4. Fermentation curve for sponge doughs made from various yeasts

Solid lines indicate the amount of total gas released, and dotted lines indicate that of internal gas retained in the dough. (a) *S. cerevisiae*, (b) *S. rosei*, (c) A$_2$, (d) D$_{2-4}$, (e) E$_2$, (f) F$_6$.
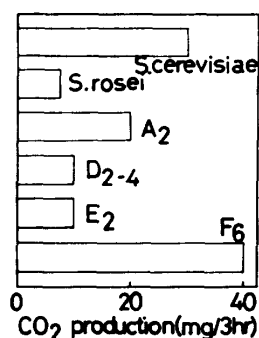


Fig. 5. Maltose fermentative ability of various yeasts in Atkin's liquid medium

certain the quality of bread made from the isolated freeze-tolerant yeasts, in comparison with the control yeast strains with unfrozen fresh dough and frozen dough. As shown in Table 3, the bread prepared by *S. cerevisiae* with fresh dough had the highest specific volume and good quality in ex-

(119)                                                                                                              27

Table 3. Quality of fresh-dough bread from various yeasts

| Yeast strain | Bread volume (ml) | Specific volume (volume/weight) | Quality score* |
|---|---|---|---|
| *S. cerevisiae* | 2,820 | 4.7 | 80.0 |
| *S. rosei* | 2,580 | 4.0 | 79.3 |
| $A_2$ | 2,540 | 3.9 | 75.9 |
| $D_{2-4}$ | 2,950 | 4.5 | 88.0 |
| $E_2$ | 2,920 | 4.1 | 77.1 |
| $F_6$ | 2,600 | 4.1 | 78.6 |

* The samples were rated on the basis of a maximum score of 100 points for the following quality characteristics: external appearance, 30 (volume, color of crust, symmetry of form, crust, evenness); internal appearance, 70 (grain, color, texture, aroma, taste).

Table 4. Quality of frozen-dough bread made from various yeasts

| Yeast strain | Bread volume (ml) | Specific volume (volume/weight) | Quality score | Proof time (min) |
|---|---|---|---|---|
| *S. cerevisiae* | 1,764 | 2.8 | 52.4 | 205 |
| *S. rosei* | 2,400 | 4.0 | 78.6 | 90 |
| $A_2$ | 2,150 | 3.5 | 70.1 | 120 |
| $D_{2-4}$ | 2,420 | 4.1 | 82.9 | 85 |
| $E_2$ | 2,280 | 3.9 | 75.9 | 110 |
| $F_6$ | 2,280 | 3.8 | 73.0 | 102 |

Each dough was prefermented for 120 min prior to freezing, and frozen at $-30°C$ for 1 week. The frozen doughs were thawed at 30°C for 2 hr. The samples were rated by the method described in the footnote to Table 3.

ternal and internal appearance, as judged by a sensory test for such parameters as the color of crust, grain, texture, aroma and taste. The bread prepared from $D_{2-4}$ strain had an almost equal value for specific volume as that of *S. cerevisiae*, and the quality of $D_{2-4}$ was better than that of *S. cerevisiae*.

Table 4 shows the quality of bread prepared from the prefermented frozen doughs with various yeasts, and their proof time. Frozen dough made by *S. cerevisiae* showed a proof time longer than 200 min, and the specific volume and quality score for the bread was the smallest among the yeast strains tested. On the other hand, the proof time for each dough made from the isolated freeze-tolerant strains was shorter than that from *S. cerevisiae*. $D_{2-4}$ strain had the shortest proof time and the largest specific volume of bread with good quality among the yeasts tested. These results indicate that $D_{2-4}$ strain is an efficient freeze-tolerant yeast and that it may be applicable

for practical use in the frozen-dough baking process.

In order to classify the isolated freeze-tolerant yeasts, such properties as the morphological, physiological, cultural and sexual characteristics were investigated, and the yeasts were identified as *Torulaspora* species. These results will be presented in the next paper.

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## 冷凍耐性酵母の検索とそのパン生地発酵特性

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　冷凍生地製パンにおいては，生地解凍後の酵母生存率および発酵能の低下が問題であり，この点は冷凍耐性酵母の使用によって解決される．本研究では，自然界から冷凍耐性酵母を検索し，そのパン生地発酵特性を検討した．麹汁を分離用培地として種々の試料から合計 144 株の発酵性酵母を分離し，そのうち $D_{2-4}$, $E_2$, $F_6$, および $A_2$ の4株が比較的高い冷凍耐性と生地膨脹力をもつことを明らかにした．それらの冷凍耐性率は70〜92%で，いずれも −30℃ で数日間貯蔵した前発酵冷凍生地で強い発酵能を示した．とくに，$D_{2-4}$ 株は高糖生地中で最高の発酵力を示したが，中種生地 (無糖生地) ではマルトース発酵能がないため発酵後期でのガス発生量が減少する傾向がみられた．$D_{2-4}$ 株を用いてつくった冷凍生地パンの品質は *S. cerevisiae* 2001 のそれよりもすぐれていた．

キーワード：冷凍耐性酵母，冷凍耐性酵母の検索，冷凍生地製パン法，冷凍耐性率.