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Trehalose Levels and Survival Ratio of Freeze-Tolerant versus Freeze-Sensitive Yeasts

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Five freeze-tolerant yeast strains suitable for frozen dough were compared with ordinary commercial bakers' yeast. *Kluyveromyces thermotolerans* FRI 501 cells showed high survival ability after freezing when their resting cells were fermented for 0 to 180 min in modified liquid medium, and they grew to log and stationary phases. Among the freeze-tolerant strains of *Saccharomyces cerevisiae*, FRI 413 and FRI 869 showed higher surviving and trehalose-accumulating abilities than other *S. cerevisiae* strains, but were affected by a prolonged prefermentation period and by growth phases. The freeze tolerance of the yeasts was, to some extent, associated with the basal amount of intracellular trehalose after rapid degradation at the onset of the prefermentation period. In the freeze-sensitive yeasts, the degree of hydrolysis of trehalose may thus be affected by the kind of saccharide, unlike in freeze-tolerant yeasts.

A large number of frozen dough methods have been developed since the first reported by Godkin and Cathcart (14). Most of the reports on these methods have dealt with the gassing power and relative proof times after thawing (18, 24, 35), dough formulation, making and fermentation before freezing (21), and freeze-thaw ratio (19, 34) to stabilize the frozen dough.

However, there are few studies about yeasts that are most affected by freezing and, as a result, may damage the thawed dough. Kline and Sugihara (21) reported substantial losses in the number of viable cells in frozen dough. Wolt and D'Appolonia (38) showed that the free sulfhydryl group content in yeasted dough increased during freezing and remained in the yeast cells even after extensive freezing damage and leaching. Consequently, due to the broken gluten S-S bonds of dough by exuded sulfhydryl groups, the thawed dough cannot expand. Since fermentation of dough before freezing impairs the viability of the yeast cells, the current methods have been improved by increasing the concentrations of sugar, fat, and oxidizing agents and by reducing the time of handling and dough temperature to prevent freezing injury. However, bread produced without fermentation before freezing lacks adequate taste and flavor.

In a previous report (17), we described the characteristics of three new strains of freeze-tolerant yeasts. These strains exhibited differences in fermentation ability and gassing rate before and after freezing in several types of dough. The new freeze-tolerant yeasts retained their fermentative ability and bread-leavening activity even after prolonged frozen storage in fully fermented doughs before freezing. Frozen dough made with these yeasts gave good-quality bread similar to that made from unfrozen dough with ordinary bakers' yeast.

Tanaka et al. (33) reported that the independent existence of flour or ethanol protected the activated yeasts in dough whereas the coexistence of the two was harmful during frozen storage. Oda et al. (26) reported that *Saccharomyces* yeast strains suitable for frozen dough accumulated a higher amount of trehalose in the cells than commercial bakers' yeast. The growth conditions and trehalose content have affected the cryotolerance of bakers' yeast in frozen dough and water (13). Storage of the carbohydrate trehalose (α-D-glucopyranosyl α-D-glucopyranoside) occurs naturally and is common to various organisms such as bacteria, yeasts and other fungi, algae and lower plants, insects, and invertebrates (12, 36). Trehalose, along with glycogen, is the major storage carbohydrate and energy reserve of yeasts (15, 22, 29, 30). Recent reports indicated that the presence of trehalose in yeast cells operates as a resistance to low water activity in liquid medium (23) and that trehalose also functions as both a protectant during freezing and a membrane preservative during desiccation (4, 6, 8–10, 26).

This study was undertaken to determine which cellular compounds affect freeze injury and freeze tolerance and the survival of these yeasts in liquid media. We used a synthetic medium in order to have a simplified system for the analysis of freeze tolerance in yeasts. The results indicated that the intracellular accumulation of trehalose is associated with freeze tolerance in yeasts.

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains used in this study were *Saccharomyces cerevisiae* FRI 413 and FRI 802 and *Kluyveromyces thermotolerans* FRI 501, described in a previous report (17), as well as *S. cerevisiae* FRI 868 and FRI 869, isolated from two commercial bakers' yeasts for frozen dough preparations (CFY), and FRI 825, isolated from a commercial bakers' yeast used for common preparations. Commercial bakers' yeasts were provided by the Japan Yeast Industrial Association. The strains were stored and maintained on YPD (0.5% yeast extract, 1% peptone, 2% dextrose, 0.5% KH_2PO_4, 0.3% $MgSO_4$) slants. Cells in the stationary (density, 2×10^8 cells per ml) and logarithmic (density, 2×10^6 cells per ml) growth phases were maintained as follows. Yeasts were grown on a slant of YPD medium for 3 days at 30°C. The cell suspension from the slant was inoculated to 100 ml of YPD broth in a 500-ml Sakaguchi flask. After 48 h of shaking at a speed of 140 rpm (10-cm amplitude) at 30°C, stationary-phase cells were collected by centrifugation, and 2 ml of the culture was transferred to the same medium. After 6 h of cultivation, loga-

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TABLE 1. Formula of LF medium

Ingredient	Concn
Glucose	1.0%
Sucrose	3.0%
Maltose	3.0%
$(NH_4)_2SO_4$	0.25%
NH_2CONH_2 (urea)	0.50%
KH_2PO_4	1.6%
$Na_2HPO_4 \cdot 12H_2O$	0.50%
$MgSO_4$	0.06%
Nicotinic acid	22.5 ppm[a]
Pantothenic acid	5.0 ppm
Thiamine	2.5 ppm
Pyridoxine	1.25 ppm
Riboflavin	1.0 ppm
Folic acid	0.5 ppm

[a] 22.5 μg/ml.

rithmic-phase cells were collected by centrifugation. Compressed yeasts were obtained by cultivation in a 3-liter jar fermentor under aerobic conditions as described in a previous report (17). However, a slight change in temperature from 30 to 35°C during the last 4 h was made to mature the yeasts under starvation conditions to obtain the same dough-making ability as that of commercial bakers' yeast.

Gas production measurements of frozen dough. A Fermograph AF-1000 (ATTO Co. Ltd., Japan), which can measure gas production in fermenting doughs automatically, was used in all gas production measurements (16). The formulation and procedure for the frozen dough used in the gas production measurements were the same as in a previous report (17). The measurement process was as follows. After freezing, the frozen dough in the reaction vessel was placed in the water bath of the Fermograph, maintained for 15 min at 30°C, and the lids were tightly capped. The total amount of CO_2 released was recorded for 120 min at 30°C.

Survival ratio of yeasts. Activated cells from compressed yeasts were obtained as follows. One gram of yeasts (70% moisture base) was fermented for 15 to 180 min at 30°C in 50 ml of a liquid fermentation (LF) medium (Table 1), which was modified from that of Atkin et al. (1) to simulate the fermenting ability in bread dough. The cells were centrifuged at 1,000 × *g* for 10 min, washed with cold distilled water, and suspended in 0.5 ml of chilled water corresponding to 2.0×10^9 to 4.0×10^9 cells per ml. The suspended cells were stored for 0 to 7 days at −20°C in a laboratory freezer (model PF-20; Tabai Mfg. Co. Ltd., Japan). The 10-ml frozen suspension was incubated in the water bath at 30°C for 3 min. Further dilution of the suspension was made before spreading on YPD plates to obtain yeast counts ranging from 500 to 2,000 cells per ml after incubation for 72 h at 30°C. Data at zero time indicated the survival ratio after freezing at −20°C for 60 min and thawing to observe the tolerance of yeasts to such treatment.

Trehalose determination. Activated cells from compressed yeasts were obtained as described above. Yeast cells were collected by centrifugation at 1,000 × *g* for 10 min and washed three times with cold distilled water. Trehalose was extracted from yeast cells with 0.5 M trichloroacetic acid and determined by the anthrone method (3) or high-pressure liquid chromatography. The trichloroacetic acid extract was deionized with an Amberlite IRA-410 and IR-120 (Organo Co., Japan) column (1.0 by 10 cm) and lyophilized. The material was dissolved in 500 μl of distilled water and applied to BIO-LC with a CarboPac PA1 column (4 by 250 mm; Dionex, Sunnyvale, Calif.) and eluted with a linear gradient of 0 to 50% 1.0 M sodium acetate in 0.1 M NaOH (flow rate, 1 ml/min). A pulsed amperometric detector (Dionex) was used.

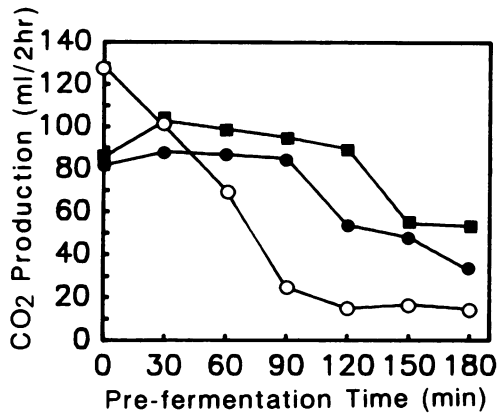

FIG. 1. Effect of fermentation before freezing on CO_2 production for frozen dough made with 20 g of flour and stored at −20°C for 7 days. Symbols: ○, FRI 825; ●, FRI 868; ■, FRI 869.

RESULTS

Gas production of yeasts for frozen dough. The tolerance to frozen storage in doughs, that is, the rates of CO_2 production by thawed doughs prefermented with three yeasts for certain periods of time before freezing, is shown in Fig. 1. The stability of FRI 868 and FRI 869, isolated from CFY, was similar to that of FRI 802 and CFY reported previously (17). In the frozen dough made with these strains, the CO_2 production by the thawed dough was relatively constant up to 90 to 120 min of the prefermentation period (that is, fermentation of doughs before freezing), though it decreased markedly after this time.

Survival ratio. Figures 2, 3, and 4 show the survival of the yeasts under varying conditions in liquid media. The survival ratio of frozen and thawed yeast cells that were fermented for some time in LF medium before freezing is shown in Fig. 2. All yeasts cultured in the jar fermentor, in which growth was arrested by the removal of the nitrogen source in the final culture phase, showed a high survival ratio of >90% in the absence of fermentation prior to freezing (Fig. 2a). The slopes, which indicate the number of surviving cells after storage at −20°C for various periods of time, differed among the strains. The survival ratio of FRI 825 rapidly decreased with duration of the fermentation period, whereas a slight decrease was observed in FRI 501. The survival patterns of the other five strains at various freezing times with periods of fermentation were intermediate between those of FRI 825 and FRI 501 (Fig. 2b and c). Figure 3 indicates the survival ratios of yeast cells cultured to the log phase and stored at −20°C for various periods of time. The ratio for FRI 501 was conspicuously different from those of the *Saccharomyces* strains in every period observed. The survival ratio of the *S. cerevisiae* strains was <50% when the cells were frozen for 1 h at −20°C to determine the tolerance to freezing and thawing treatment, while FRI 501 exhibited a growth ability 100- to 2×10^4-fold higher than that of the other strains after 7 days of frozen storage. The plots of the log survival ratio of the *S. cerevisiae* strains versus time corresponded approximately to straight lines. Although that of FRI 501 did not decrease except on the first day, the survival ability still

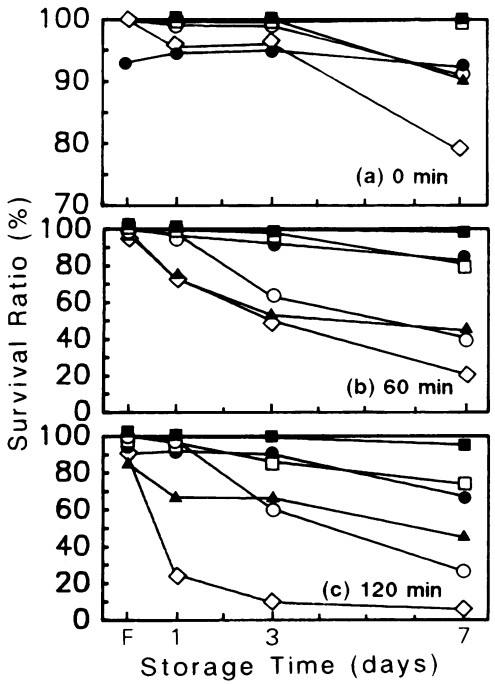

FIG. 2. Effect of fermentation before freezing on survival ratio of freeze-tolerant and freeze-sensitive yeasts stored at −20°C. Yeasts were cultivated in LF medium for 0, 60, and 120 min. F indicates 60 min (see text). Symbols: ●, FRI 413; ■, FRI 501; ▲, FRI 802; ◇, FRI 825; ○, FRI 868; □, FRI 869.

showed the same ratio afterwards. Of the *S. cerevisiae* strains, the survival ratios of FRI 413 and FRI 869 correspondingly decreased and gave higher values than those of other strains (Fig. 3). Similar results were obtained for the survival ratio of stationary-phase cells (Fig. 4). The survival ratio of FRI 501 was 10- to 500-fold higher than that of other strains when stationary-phase cells were frozen for 7 days, as in the case of the log-phase cells. Although the survival ratio of strain FRI 825 did not decrease quickly after the first day compared with that of the log-phase cells, the value always remained the lowest. The characteristics of FRI 413 and FRI 869 differed from those of other strains in the following respects. The survival ratio of FRI 413 after freezing for 1 day was the same as that without frozen storage, but decreased at the same speed as that of the other strains. On the contrary, the survival ratio of FRI 869, which decreased rapidly on the first day as in the case of the other strains, remained relatively unchanged afterwards. Therefore, after being frozen for 7 days, the FRI 413 and FRI 869 cells retained a higher growth ability than cells of the other *S. cerevisiae* strains.

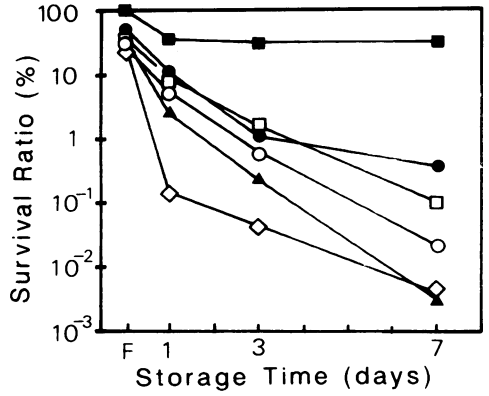

FIG. 3. Survival ratio of logarithmic-phase cells of freeze-tolerant yeasts and freeze-sensitive yeasts stored at −20°C. F indicates 60 min (see text). Symbols: ●, FRI 413; ■, FRI 501; ▲, FRI 802; ◇, FRI 825; ○, FRI 868; □, FRI 869.

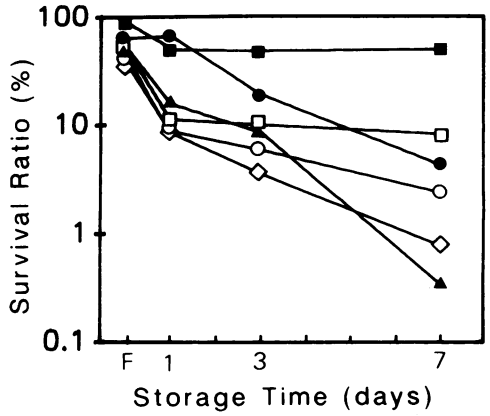

FIG. 4. Survival ratio of stationary-phase cells of freeze-tolerant and freeze-sensitive yeasts stored at −20°C. F indicates 60 min (see text). Symbols: ●, FRI 413; ■, FRI 501; ▲, FRI 802; ◇, FRI 825; ○, FRI 868; □, FRI 869.

Trehalose degradation. When compressed yeasts fermented in LF medium for 15 to 180 min at 30°C, changes in trehalose accumulation in cells occurred (Fig. 5). By high-pressure liquid chromatography (see Materials and Methods), we determined that 80 to 95% of the sugars detected by the anthrone method were trehalose (data not shown). Therefore, we used the anthrone method for the detection of trehalose. Although all *S. cerevisiae* strains accumulated a large amount of trehalose at zero time, the amount decreased very quickly during the fermentation period. The change in the amount of accumulated trehalose can thus be classified into two types based on this experiment. In the first type, i.e., in strains FRI 825 and FRI 868, trehalose was degraded

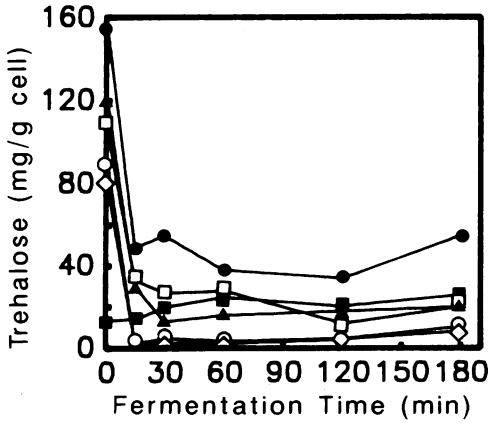

FIG. 5. Changes in accumulation of trehalose (on a dry-weight basis) during fermentation of freeze-tolerant and freeze-sensitive yeasts. Symbols: ●, FRI 413; ■, FRI 501; ▲, FRI 802; ◇, FRI 825; ○, FRI 868: □, FRI 869.

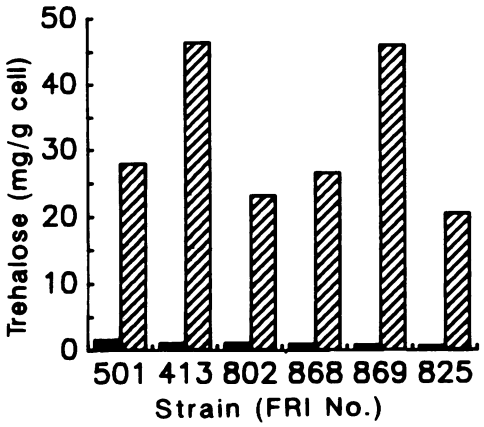

FIG. 6. Comparison of trehalose accumulation (on a dry-weight basis) at different growth phases in freeze-tolerant and freeze-sensitive yeasts. Symbols: ■, logarithmic phase; ▨, stationary phase.

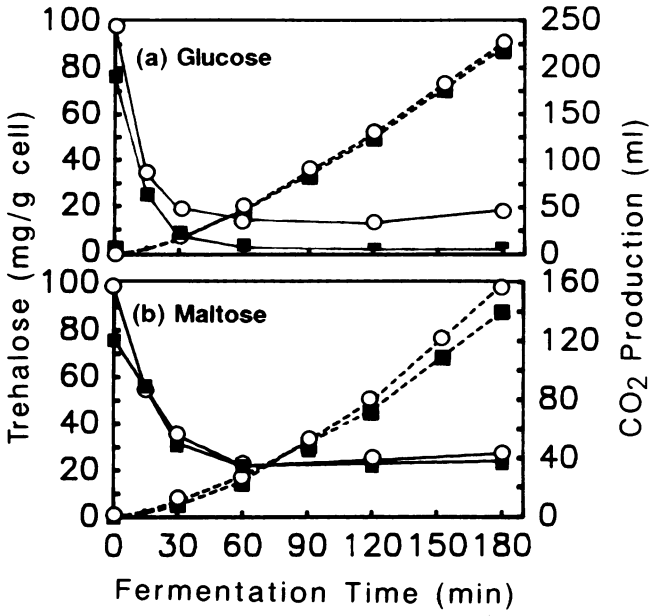

FIG. 7. Changes in accumulation of trehalose during fermentation of freeze-tolerant and freeze-sensitive yeasts on two carbon sources. Symbols: broken lines, CO_2 production at 30°C for 2 h; solid lines, changes in trehalose content; ■, FRI 825; ○, FRI 869.

almost completely below 0.5% after the first 15 min, and thereafter it accumulated slightly with time. The second type was observed in strains FRI 413, FRI 802, and FRI 869; after the onset of fermentation, the retention of trehalose exceeded 1 to 5% and remained relatively constant after the initial degradation. On the other hand, *K. thermotolerans* FRI 501 accumulated a small amount of trehalose but kept it at the same basal level as that of the *S. cerevisiae* freeze-tolerant cells during fermentation.

Trehalose accumulation in various yeasts, when grown on YPD, was determined in various strains (Fig. 6). None of the strains accumulated trehalose at the logarithmic phase of growth. At the stationary phase, however, the amount of trehalose accumulated by strains FRI 413 and FRI 869 was higher than that accumulated by other strains and the amount in FRI 825 was the lowest.

Effect of carbon source on trehalose preservation. The effects of glucose and maltose on trehalose degradation of the freeze-sensitive strain, FRI 825, and the freeze-tolerant strain, FRI 869, were compared. Figure 7a and b shows the trehalose degradation and CO_2 production of the above two strains when they were fermented with glucose or maltose as carbon source, respectively. In maltose fermentation, the medium (LF-maltose) also contained 0.3% glucose because FRI 869 required a long induction time for maltose fermentation. Although both strains immediately degraded the trehalose when fermentation started, regardless of the carbon source, the rates of trehalose hydrolysis and CO_2, production during fermentation with maltose were lower. The pattern of the basal level contrasted between FRI 825 and FRI 869 after the first decrease. The freeze-sensitive FRI 825 strain retained about 10-fold more trehalose in maltose fermentation than in glucose fermentation. In contrast, the basal level of trehalose in maltose fermentation of the freeze-tolerant strain, FRI 869, was not much different from that in glucose, being only 1.5- to 1.7-fold higher.

DISCUSSION

It appears that the survival ratios of the freeze-tolerant yeasts, strains FRI 501, FRI 413, and CFY, were higher than that of FRI 825 when cultivated in LF medium to investigate the physiological state of activated yeasts during fermentation (Fig. 1 to 3). The stability of the yeast cells after freezing can be classified into two types based on these experiments. In the first type, FRI 501 showed an exceptionally high tolerance to freezing compared with *S. cerevisiae* strains under every condition tested.

On the contrary, in the *S. cerevisiae* strains, the survival ratios of all resting yeast cells decreased with the increase in duration of the period of fermentation prior to freezing. It seems that the following two types also were identified: (i) FRI 802 and FRI 868 were affected by the period of freezing, although the values were higher than that of FRI 825; (ii) FRI 413 and FRI 869 were not significantly affected. It appears that the survival ratio for the logarithmic- and stationary-phase cells after freezing decreased logarithmically with duration of the freezing period. It is known that cells at the stationary phase of growth are more resistant to high temperatures than actively growing cells (20, 25, 32). We also showed that stationary-phase cells are more freeze tolerant than logarithmic-phase cells.

The intracellular component significantly affected by the change in environmental conditions was mainly trehalose. We also carried out an analysis of other components (glycogen, ethanol, organic acid, amino acids, and sugar alcohol). While the accumulation of glycogen under the same conditions also changed, the ranges were comparatively smaller than those of trehalose (data not shown). Moreover, the content of other components neither changed noticeably nor differed greatly among the strains under the same conditions (data not shown).

Trehalose has been successfully used as a cryoprotectant for plant cells (2), bacteria (11), and yeasts (5). The mechanisms of membrane protection for dehydration by trehalose were reported by Crowe et al. (7). *S. cerevisiae* can accumulate the disaccharide trehalose depending on the environmental conditions and the life cycle stage (22, 31). Large amounts of accumulated trehalose were broken down as soon as the resting cells were reinoculated into fresh LF medium for fermentation irrespective of the strain used. In

recent studies, the activities of trehalose-6-phosphate synthase (EC 3.1.3.12) and trehalase (EC 3.2.1.28), involved in the biosynthesis and degradation of trehalose, have been shown to be regulated by interconversion of forms mediated by a monocyclic cascade system (26–28, 30, 37).

Nevertheless, FRI 413 and FRI 869 were able to retain about one-fourth of the stored trehalose, while other *S. cerevisiae* strains retained barely 10% of it. The cellular level of trehalose in strains FRI 413 and FRI 869 at the stationary phase was twice as high as in other strains. Strain FRI 501, which has an exceptionally high tolerance to freezing, kept a high basal level of trehalose during fermentation, although it did not accumulate a high level of it at zero time. This result is in agreement with the fact that these strains showed a higher growth activity than others after severe freezing conditions. Although the correlation between trehalose concentration and survival ratio is not direct, the freeze tolerance of yeasts should be closely associated with the basal level of intracellular trehalose. We think that the mechanisms of freeze tolerance in yeasts should not depend solely on trehalose, because strain FRI 501, which was most tolerant to freezing in dough and liquid media, should have another mechanism.

Although we have shown that the accumulated trehalose was quickly catabolized during fermentation, irrespective of the carbon source, freeze-tolerant strains were able to retain a higher basal level than the sensitive strains after fermentation (Fig. 5). The results suggested that these freeze-tolerant yeasts may be defective strains in the trehalose metabolic system regulated by the cyclic AMP-dependent protein kinase or may have individual metabolic systems of trehalose, as in bacteria (12).

These suggestions are supported by the experiment on the effect of carbon source on trehalose preservation. Panek et al. (31) demonstrated, that when maltose was used as carbon source, trehalose accumulated parallel to growth. This difference may be due to the cell conditions; that is, they used yeast cells that do not accumulate a high amount of trehalose. Although a detailed comparison may be required, it is suggested that in freeze-sensitive strains the trehalose metabolism can be changed by the carbon source, as shown by the results of Panek et al., but this cannot occur in freeze-tolerant strains.

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