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Effect of Growth Conditions and Trehalose Content on Cryotolerance of Bakers' Yeast in Frozen Doughs

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The cryotolerance in frozen doughs and in water suspensions of bakers' yeast (*Saccharomyces cerevisiae*) previously grown under various industrial conditions was evaluated on a laboratory scale. Fed-batch cultures were very superior to batch cultures, and strong aeration enhanced cryoresistance in both cases for freezing rates of 1 to 56°C min^{-1}. Loss of cell viability in frozen dough or water was related to the duration of the dissolved-oxygen deficit during fed-batch growth. Strongly aerobic fed-batch cultures grown at a reduced average specific rate (μ = 0.088 h^{-1} compared with 0.117 h^{-1}) also showed greater trehalose synthesis and improved frozen-dough stability. Insufficient aeration (dissolved-oxygen deficit) and lower growth temperature (20°C instead of 30°C) decreased both fed-batch-grown yeast cryoresistance and trehalose content. Although trehalose had a cryoprotective effect in *S. cerevisiae*, its effect was neutralized by even a momentary lack of excess dissolved oxygen in the fed-batch growth medium.

Improving the tolerance of microbial cells to freezing and thawing is of major concern in areas of applied microbiology such as culture collections, frozen starters, and frozen doughs. Since the 1950s, much work has been devoted to improving the quality of frozen yeast-leavened doughs. Their stability would depend to a large extent on the freeze-thaw resistance of the bakers' yeast (12, 38) which, in turn, would vary according to the commercial source (18). Current research interest has focused mainly on the isolation and development of a freeze-tolerant strain (17) that retains the baking activity of the yeast (36). However, yeast producers have not yet implemented thorough quality control standards pertaining to the cryoresistance of their product.

Most published work on cryoresistance has dealt essentially with the effects of the strain, the growth phase, and the growth medium composition in batch fermentation (26). Harvesting the cells during the stationary phase rather than the logarithmic phase, for example, improved the cryoresistance of several yeast strains (20). Aeration of batch cultures of yeasts, on the other hand, gave inconsistent results (20).

Cryoresistance in *Escherichia coli* has been correlated with its higher carbohydrate content (4). The disaccharide trehalose has protected spores of *Streptomyces griseus* against dehydration and heat (29). Similar results were obtained with dehydrated cells of *Saccharomyces cerevisiae* (42), but not with dehydrated or frozen *Phycomyces* spores (41). Generally recognized as superior to most carbohydrates of interest in cryoprotection, trehalose has been associated with membrane stabilization during freezing (35, 37) and also during lyophilization (9) and dehydration (43). According to Coutinho et al. (8), addition of a 10% trehalose solution to the freezing menstruum would have a cryoprotective effect on mutant *Saccharomyces* strains defective in trehalose-6-phosphate synthase, but would not confer extra protection on two other yeast strains, wild type or mutant. It appears that trehalose would not ensure universal cryoprotection in yeast, so one cannot rely on high trehalose content or addition to extrapolate for freeze-thaw survival. Although trehalose is found at high concentrations in cryoresistant bakers' yeast used in frozen doughs, no absolute, direct correlation between yeast cryoresistance and trehalose content has been obtained (32). In this study we examined the effect of growth conditions on the cryoresistance of bakers' yeast in dough or water and attempted to clarify the cryoprotective effect of trehalose.

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MATERIALS AND METHODS

Yeast strain. A commercial strain of bakers' yeast, *S. cerevisiae* C from the collection of Lallemand Inc., was used in all experiments and maintained on malt extract at 4°C.

Growth conditions. (i) Growth media. Batch fermentation medium consisted of 875 g of malt syrup (60 ± 2% reducing sugars; Lallemand), 125 g of beet molasses (54 ± 2% reducing sugars; Lallemand), 7 g of $(NH_4)_2SO_4$, 4 g of $(NH_4)_2HPO_4$, 1.6 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), and 6.8 liters of water (pH adjusted to 5.5 with sulfuric acid). Fed-batch fermentation medium consisted of 4.55 ml of phosphoric acid (85%, wt/wt), 1.5750 g of $MgSO_4 \cdot 7H_2O$, 0.1125 g of $ZnSO_4 \cdot 4H_2O$, 0.0100 g of calcium pantothenate, 0.0450 g of thiamine hydrochloride, D-biotin (0.5 ml of a 500-mg liter^{-1} stock solution), and 4 liters of water (pH adjusted to 3.0 with KOH). Beet molasses and cane molasses were blended in a 1:1 ratio and ajusted to 42.0 ± 0.2°Brix. Ammonia solution was prepared by diluting 20-fold ammonium hydroxide (30%, wt/vol) with water. All growth media were autoclaved at 121°C for 10 min, except molasses-containing solutions, which were kept at 121°C for 30 min.

(ii) Inoculum preparation. For batch fermentations, surface growth from malt extract agar was transferred to four test tubes containing 10 ml each of batch growth medium; the test tubes were then incubated for 24 h at 30°C. Two 500-ml Erlenmeyer flasks, each containing 300 ml of medium, were then inoculated with the contents of two test tubes each and incubated for 48 h at 30°C without agitation, giving a 640-ml liquid inoculum.

For fed-batch fermentations, 30 g (dry weight equivalent)

of bakers' yeast recovered from a nonaerated 7-liter batch culture (microaerobic) were used as the inoculum (see below).

(iii) Batch fermentations. A 10-liter fermentor (Biolafitte, Maisons-Laffitte, France) containing about 7 liters of medium was inoculated as described above. The fermentation was carried out at 30°C for 39 to 40 h with agitation at 300 rpm. This condition was designated microaerobic because steady incorporation of air was permitted only by agitation. Strongly aerobic conditions (80 to 100% dissolved-oxygen saturation) were obtained by providing the growth medium with 16 to 17 liters of air min^{-1}. Because of the evaporation of the growth medium throughout the fermentation, this aeration rate corresponded to about 2.4 and 3.1 volumes of air volume of growth $medium^{-1}$ min^{-1} (vvm), at the beginning and end of fermentation, respectively. Dissolved oxygen was measured by using a polarographic electrode (model IL 531; Instrumentation Laboratory, Lexington, Mass.), which was calibrated with nitrogen and an air-saturated water solution. Cells were harvested by centrifugation at $1,000 \times g$ for 3 min, and the pellets were washed three times before being stored at 2 to 4°C as yeast cream (ca. 10% solids).

(iv) Fed-batch fermentations. A microcomputer (TRS-80 model 100; Tandy Corp., Fort Worth, Tex.) was used to control the stepwise incremental feeding of molasses and ammonia solutions by means of peristaltic pumps (model 520; Biochem Technology, Malvern, Pa.). Ammonia addition was completed in 12 h, molasses feeding was completed 1 h later (maturation period), and the fermentation itself stopped after another 1 h (designated as the postmaturation period). Curves of substrate feeding were chosen which sustained average specific growth rates of 0.117 h^{-1}, but some tests were done at 0.088 h^{-1}. Fermentations were carried out at 30, 25, or 20°C, and all harvesting was performed as described for batch fermentations. The final cell (dry weight) concentration in the fed-batch growth medium was typically about 30 g liter of $medium^{-1}$; this means that the cell concentration was below the optimum required to keep control over the total oxygen demand of the biomass throughout the fermentation. Two aeration conditions, partial and strong aerobiosis, were used. The former was obtained by providing the fermentation medium with 0.75 to 1 liter of air min^{-1}, which was equivalent to about 0.25 and 0.16 vvm at the beginning and end of the fermentation, respectively. Under such conditions, the dissolved-oxygen concentration dropped to a constant zero after 4 to 5 h. Under strongly aerobic conditions (16 to 17 liters min^{-1} or about 3.1 vvm at the end of the fermentation), the dissolved-oxygen concentration varied between 90% (beginning of fermentation) and 35% (end of fermentation) saturation.

At the beginning of some fermentations that took place under partially aerobic conditions, ergosterol and Tween 80 were added to the growth medium. Contents of a flask (0.5 g of ergosterol and 5 g of Tween 80 in 10 ml of warm ethanol) were poured into the fermentor; the flask was then rinsed with 10 ml of ethanol, which was also added.

Freeze-thaw procedures. (i) Rapid tests. The following test was performed with yeast cells in water. A 0.3-ml portion of a suspension of 10^7 washed cells ml of $water^{-1}$ was frozen at -50 ± 1°C for 10 min in polypropylene tubes (1 by 7 cm) (Cryovials; Les Plastiques Simport, Beloeil, Quebec, Canada) in an ethanol bath (Kryostat type KT 40S; Colora Messtechnik GmbH, Lord-Württ, Federal Republic of Germany) and then immediately thawed in a water bath at 30°C for 2 min. Plate counts were done in duplicate for each freeze-thawed tube (malt extract agar [Difco]; 65 h of incubation at 30°C). Four tubes of each yeast sample were freeze-thawed. Cryoresistance (cell viability) was expressed as the ratio (percent) of the counts obtained from frozen and unfrozen suspensions.

The following freezing and thawing rates and their corresponding final temperatures were used: freezing at 39.6 ± 1.1°C min^{-1} and thawing at 148.9 ± 11.3°C min^{-1} (final temperature, −50°C); freezing at 9.0 ± 0.1°C min^{-1} and thawing at 103.2 ± 7.8°C min^{-1} (final temperature, −20°C); freezing at 17.6 ± 1.1°C min^{-1} and thawing at 106.8 ± 3.4°C min^{-1} (final temperature, −30°C); freezing at 55.9 ± 1.4°C min^{-1} and thawing at 150.8 ± 12.3°C min^{-1} (final temperature, −70°C); and freezing at 1°C min^{-1} and thawing at 148.9 ± 11.3°C min^{-1} (final temperature, −50°C). The final temperature is the lowest temperature achieved during the freeze-thaw cycle.

The following test was performed with yeast cells in dough. A farinograph (model 3-S-300; C. W. Brabender Instruments, South Hackensack, N.J.) was used at high speed to combine the following ingredients: 100 g of hard red spring wheat flour, 3.5 g of sucrose, 2.0 g of salt, 1.05 g (dry weight equivalent) of bakers' yeast in the form of about 3.5 g of compressed bakers' yeast, 1,180 ppm of calcium sulfate (1,180 mg kg^{-1}) (flour basis), 33 ppm of ascorbic acid (flour basis), 25 ppm of potassium bromate (flour basis), 10 ppm of L-cysteine (flour basis), and about 65 ml of water. The resulting dough (18°C) was sheeted and divided into portions of 1.5 ± 0.1 g, which were placed at the bottom of polypropylene tubes. Twelve such tubes were immediately placed inside a proofing chamber at 30°C and 50% relative humidity for 1 h. The net risen heights of the dough samples were averaged and served as a control. Another 12 tubes were frozen for 10 min at −50 ± 1°C min^{-1} in the ethanol bath described above. The freezing rate was 20.6 ± 1.1°C min^{-1}, measured between −5 and −45°C at the center of the dough. The frozen tubes were then thawed for 5 min at 30°C (22.7 ± 3.5°C min^{-1} at the center; thawing rates were measured between −50 and −5°C) and proofed for 1 h, after which the net risen heights were mesured. Cryoresistance (shown as the gas-producing activity) was expressed as the percent ratio of the mean net risen heights of dough in frozen and unfrozen tubes.

(ii) Storage tests. Multiple series of eight tubes of dough were frozen at −30 ± 1°C for 10 min (9.9 ± 0.7°C min^{-1} at the dough center) and stored at −25°C for up to about 20 weeks; one series was later thawed (at 16.5 ± 2.3°C min^{-1}) every 2 weeks, proofed, and measured as described above.

Trehalose content. Trehalose analysis of bakers' yeast was based on the methods of Trevelyan and Harrison (39) and Lillie and Pringle (25). Freshly harvested yeast samples (20 mg [dry weight equivalent] in duplicate) were refrigerated and readily extracted three times in 3 ml of 0.5% trichloroacetic acid for 40 min at room temperature, with agitation at 150 rpm. Cell suspensions were centrifuged at $6,300 \times g$ for 5 min, and the three supernatants were combined and brought to 25 ml with water. The presence of trehalose in the extract was confirmed later by gas-liquid chromatography (19).

Yeast dry weight. In triplicate, 5 g of yeast cream in an aluminum weight boat was diluted with 5 ml of 70% ethanol solution, dried at 110°C for 4 h and cooled, and the resulting dried yeast sample was weighed.

TABLE 1. Effect of growth conditions on the trehalose content and cryotolerance of bakers' yeast

Growth conditions	Mean trehalose content (% dry wt) ± SD	Cryotolerance index (%)[a] in:	
		Dough	Water
Batch			
Microaerobiosis, 30°C, logarithmic phase	–[b]	–	2 ± 1
Microaerobiosis, 30°C, stationary phase	4.8 ± 1.2	–	2 ± 1
Strong aerobiosis, 30°C, stationary phase	19.0 ± 3.7	–	17 ± 5
Fed-batch (partial aerobiosis)			
30°C	6.3 ± 1.4	44 ± 5	74 ± 10
30°C, low specific growth rate (0.088 h^{-1}), ergosterol and Tween 80 added	9.5 ± 2.1	56 ± 16	–
30°C, low specific growth rate (0.088 h^{-}), strong aerobiosis during maturation only	9.2 ± 1.7	55 ± 11	–
Fed-batch (strong aerobiosis)			
30°C	12.5 ± 1.8	100 ± 11	92 ± 8
25°C	10.9 ± 1.5	96 ± 4	–
20°C	6.4 ± 1.8	18 ± 3	–
30°C, low specific growth rate (0.088 h^{-1})	13.8 ± 0.6	110 ± 2	–
25°C, low specific growth rate (0.088 h^{-1})	13.8 ± 0.2	102 ± 3	–
20°C, low specific growth rate (0.088 h^{-1})	11.9 ± 1.1	100 ± 5	–

[a] Rapid freezing tests (−50°C for 10 min). Freezing rates in dough were 20.6°C min^{-1} (center) and 42.4°C min^{-1} (outside) and about 39.6°C min^{-1} in water. All reported results are based on a minimum of five repetitions (fermentations). Cryotolerance was calculated as the percent proofing activity (in dough) or the percent cell recovery (in water) of freeze-thawed compared with nonfrozen yeasts.

[b] –, Results not available.

RESULTS

Effect of growth conditions. Table 1 shows cryotolerance indices and trehalose content for cells grown under various conditions. The freezing menstruum (water or dough) did not greatly affect the cryoresistance indices obtained for fed-batch cultures (e.g., 100% versus 92% at 30°C), whereas yeasts from batch cultures could not raise unfrozen dough (Table 1). The growth phase (logarithmic or stationary) of batch microaerobic cultures (shaken only) did not greatly affect their cryoresistance (cryotolerance indices of about 2% for both).

Resistance of the cells to freezing and thawing appeared to be greatly conferred by aeration but moreso by controlled substrate feeding: fed-batch cultures were much more cryoresistant than batch ones, and cells provided with very intense aeration (no dissolved-oxygen deficit throughout growth) proved the most tolerant ones under batch or fed-batch conditions. Gas-producing activity in freeze-thawed doughs (no storage) was partially preserved (about 50%) in cells grown under partially aerobic fed-batch conditions and entirely preserved in cells grown under strongly aerobic fed-batch conditions, except when the growth temperature was 20°C. At this temperature, lowering the specific growth rate from 0.117 h^{-1} (cryotolerance index of 18%) to 0.088 h^{-1} (cryotolerance index of 100%) appeared to bring the characteristics of the yeast into line with the fed-batch results at 25 or 30°C in the rapid (Table 1) or the storage (Fig. 1) test. At 25°C (Fig. 2) and 30°C (Fig. 3), lowering the growth rate would also improve the long-term stability of the frozen doughs.

The freezing temperatures and rates used (1 to 56°C min^{-1}) did not greatly affect the cell viability in water, although a small dip occurred between 40 and 56°C min^{-1} (Fig. 4). The use of strongly aerobic fed-batch culture proved to be a very reliable way to ensure yeast cryoresistance in water under these freezing conditions. When the air flow to fed-batch cultures growing at 30°C was limited, the cryotolerance index fell (the mean and standard deviation for at least five fed-batch cultures at 30°C are as follows: for no oxygen deficit, the cryotolerance index is 100% ± 11%; for a deficit lasting 4.1 ± 1.8 h, the index is 53% ± 18%; and for a deficit lasting 6.7 ± 0.9 h, the index is 34% ± 4%). Clearly, the ability of the yeast to maintain gas-producing activity in freeze-thawed dough is significantly affected by the duration of the dissolved-oxygen deficit during growth. This effect appeared to be irreversible, since subsequent vigorous oxygenation during the maturation period did not significantly alter these results, nor did addition of ergosterol and Tween 80 to the medium to fulfill a theoretical lack of essential lipids in yeast cells (Table 1).

Effect of trehalose content. Relatively high levels of trehalose were found in all cryoresistant yeasts (Table 1). However, it was equally apparent that cryoresistance was greatly reduced in yeasts grown under partially aerobic

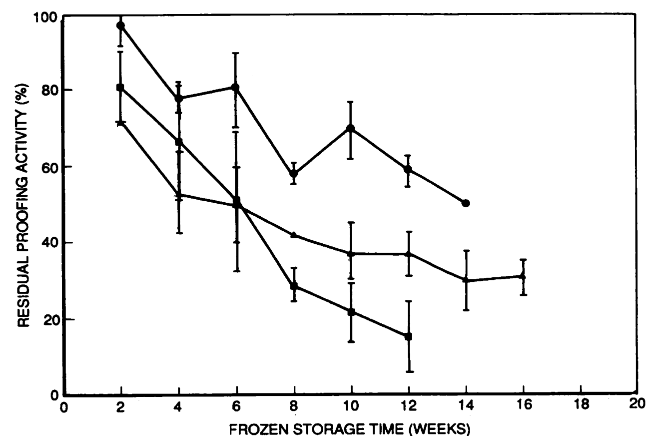

FIG. 1. Long-term frozen-dough stability Yeast grown at 20°C. *S. cerevisiae* was grown under fed-batch conditions at 20°C with strong aerobiosis at growth rates of 0.088 h^{-1} (●) and 0.117 h^{-1} (▲) or partial aerobiosis at 30°C and 0.117 h^{-1} (■). The doughs were frozen at −30°C (9.9 ± 0.7°C min^{-1} at center) and stored at −25°C. Residual proofing activity of frozen doughs was obtained by comparison with nonfrozen standards.

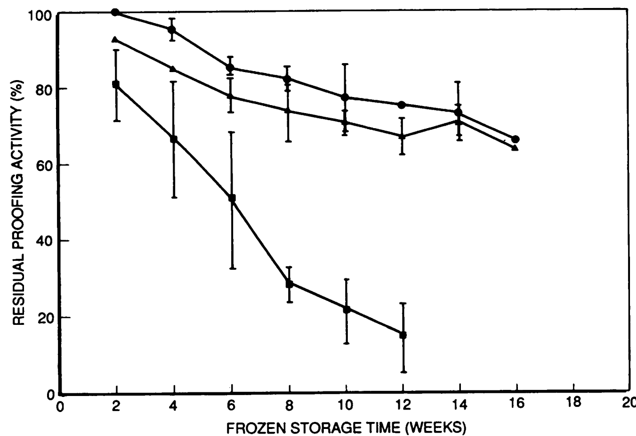

FIG. 2. Long-term frozen-dough stability. Yeast grown at 25°C. *S. cerevisiae* was grown under fed-batch conditions at 25°C with strong aerobiosis at growth rates of 0.088 h^{-1} (●) and 0.117 h^{-1} (▲) or partial aerobiosis at 30°C and 0.117 h^{-1} (■). See the legend to Fig. 1 for the other test conditions.

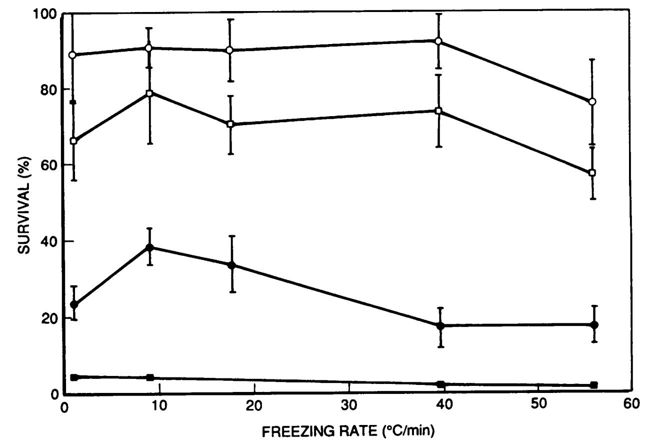

FIG. 4. Effect of freezing rate on the cryoresistance of bakers' yeast in water. *S. cerevisiae* was grown under fed-batch conditions at 30°C with strong (○) or partial (□) aerobiosis and under batch conditions with strong aerobiosis (●) or microaerobiosis (■). Temperatures (corresponding to freezing rates) were −50°C (freezing rate, 1°C min^{-1}), −20°C (freezing rate, 9.0 ± 0.1°C min^{-1}), −30°C (freezing rate, 17.6 ± 1.1°C min^{-1}), −50°C (freezing rate, 39.6 ± 1.1°C min^{-1}), and −70°C (freezing rate, 55.9 ± 1.4°C min^{-1}). Results obtained at 39.6°C min^{-1} are from Table 1.

fed-batch conditions, with a only slightly reduced trehalose content, and nearly eliminated in yeasts containing the largest amounts of trehalose measured (19% trehalose; batch-grown cells under strongly aerobic conditions and in the stationary phase). Higher levels of trehalose in fed-batch cultures appeared to be related to the slower specific growth rate (0.088 h^{-1} compared with 0.117 h^{-1}) and the higher growth temperature (25 or 30°C compared with 20°C) (Table 1). At 20°C, a lower trehalose content was associated with low cryoresistance, whereas a reduced growth rate enhanced trehalose accumulation (Table 1) and restored the cryoresistance of the cells (Table 1; Fig. 1). However, this positive effect of trehalose was limited to fed-batch-grown cells with strong aerobiosis; a high trehalose content could ensure superior cryoresistance only in yeast cells grown under ideal conditions (control of sugar and ammonia addition; strong aerobiosis).

Studies on trehalose depletion in yeast cream under storage at 4°C for several weeks confirmed that freeze-thaw tolerance was lowered as the trehalose content disappeared

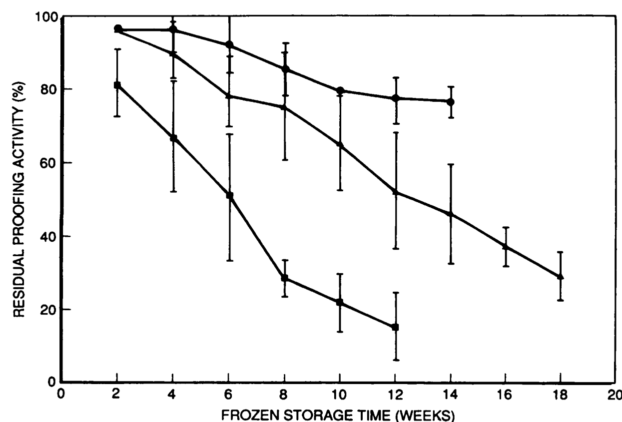

FIG. 3. Long-term frozen-dough stability. Yeast grown at 30°C. *S. cerevisiae* was grown under fed-batch conditions at 30°C with strong aerobiosis at growth rates of 0.088 h^{-1} (●) and 0.117 h^{-1} (▲) or partial aerobiosis at 30°C and 0.117 h^{-1} (■). See the legend to Fig. 1 for the other test conditions.

(Table 2). However, although the correlation between these two factors was good, it was not as precise as might have been expected: although cryotolerance declined from 100 to about 74% after 11 weeks of storage, the trehalose content was lowered from 12.7 to 7.0%, which represents a drop to 55% of the original level. Furthermore, the rate of endogenous trehalose metabolism during storage of yeasts produced under different growth conditions was quite variable (Fig. 5). Trehalose reserves appeared to be depleted more slowly in cryotolerant yeasts. Measured levels after 4 weeks corresponded to losses of 25% for tolerant yeasts, compared with 60 to 70% for intolerant yeasts.

DISCUSSION

Growth conditions ensuring a higher freeze-thaw tolerance in microorganisms have been poorly defined in the literature. For example, batch fermentations are often used to grow cells intended for culture collections, but specific recommendations are usually scarce. In the baking industry, there is a need to improve the freeze-thaw tolerance of bakers' yeast. According to our results, the use of the fed-batch process, which is the regular final process for bakers' yeast production, rather than the batch process was the best factor for improving the cryotolerance of *S. cerevisiae*. The fed-batch process allows a thorough control over yeast cell growth, close to that obtained in a chemostat, because of incremental feeding which is devised to end up

TABLE 2. Effect of trehalose content on the cryotolerance of *S. cerevisiae* during storage of the yeast cream at 4°C[a]

Storage period (weeks)	Mean trehalose content (% dry wt) ± SD	Cryotolerance index in dough (%)
0	12.7 ± 2.5	100 ± 8
7	7.7 ± 1.7	91 ± 8
11	7.0 ± 1.0	74 ± 12

[a] Fed-batch fermentations at 30°C with strong aerobiosis.

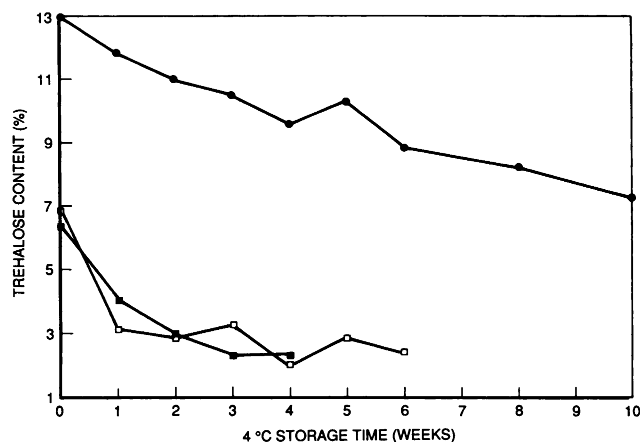

FIG. 5. Trehalose content during yeast storage at 4°C. *S. cerevisiae* was grown under fed-batch conditions at 30°C with strong (●) and partial (■) aerobiosis or at 20°C with strong aerobiosis (□).

after 12 to 14 h of fermentation, for harvesting the biomass. It is probable that the more balanced growth of fed-batch compared with batch yeast cells helped them survive the freeze-thaw process.

Most published work about the effect of aeration of microorganisms on their cryoresistance has been conducted with batch fermentations. Under these conditions, there was no consensus on improving cell cryotolerance by shaking flasks or by direct aeration of the growth medium. Variations due to yeast strains and species have been reported (20): shaken (quoted aerobic) compared with static (anaerobic) cultures improved the cryoresistance of 8 of 15 species or strains, but the reverse has been found for 4 other strains.

Unfortunately, no reported study of the cryotolerance of *S. cerevisiae* has clearly established the effect of aeration on qualitative or quantitative grounds; aeration conditions have rarely been defined (1, 21) or have been limited to unclear shaking conditions (20). Direct air bubbling of the growth medium of *Serratia marcescens* or *Escherichia coli* has been described, but no direct measurement of dissolved oxygen has been done in the medium, even if O_2 was probably in excess (14, 15, 31, 33). As discussed above, batch growth conditions in themselves (e.g., nutrient and biomass concentration, temperature, pH, and O_2 concentration) would not allow a thorough control over the fermentation process (11).

Growth medium composition, physiological and genetic differences between strains (27), and the quality of aeration would probably explain most poor results obtained with *S. cerevisiae* (5, 20) or *E. coli* (33) aerated batch cultures. The positive effect of aeration usually has been associated with high freezing rates (around 100°C min^{-1}) (31), but results presented in this report have shown the benefits of intense aeration for freezing rates between 1 and 56°C min^{-1}. Under intense aeration, lower survival levels of cells in batch compared with fed-batch cultures are difficult to explain from these results, because higher trehalose levels (19% compared with 12.5%) in batch cultures would not, in themselves, be determinant. Under fed-batch conditions, the aeration rate of the yeast growth medium would certainly have an effect on the cryoresistance of the cells. The continuous presence of excess dissolved oxygen improved cell cryotolerance for the tested freezing rates (1 to 56°C min^{-1}). Published data (24, 28) indicate that the optimal freezing rates are about 7 to 10°C min^{-1} for yeast cells and that survival of *S. cerevisiae* is quite low around 20 to 40°C min^{-1} at temperatures of −95 to −196°C. In this study, however, fed-batch cultures under strong aerobiosis appeared to be the most tolerant to the freezing stresses found in the frozen-dough industry (temperatures varying from −20 to −70°C). A close relationship was also found between freeze-thaw resistance of yeast cells in water and in dough. The conclusions of this study for the baking industry are likely to apply to frozen culture collections.

From the results obtained for fed-batch cultures, the duration of the dissolved-oxygen deficit in the growth medium had a direct influence on cell survival after the freeze-thaw process. In continuous culture, the general physiology of *S. cerevisiae* did not seem to be much disturbed by such oxygen deficits; only very poor oxygenation conditions, close to anaerobiosis, had noticeable effects (3). Most microorganisms are significantly affected by oxygen tensions below 1% of an air-saturated medium (critical oxygen concentration), at which the respiration rate (Q_{O_2}) and general cell metabolism are truly disturbed (16, 30). It is estimated that during the oxygen-deficit period in fed-batch culture, the oxygen tension (8 to 10 mm Hg [1,066 to 1,333 Pa]) was well above the critical level (1 to 3 mm Hg [133 to 400 Pa]). Thus, it suggests that the critical oxygen level for cryoresistance of yeasts is different from that for respiration and general metabolism.

Attempts to replace the positive effect of strong oxygenation on the cryoresistance of bakers' yeast have been fruitless. Trehalose supplementary accumulation from lowering the specific growth rate, as well as addition of ergosterol and oleic acid (as Tween 80) to the growth medium, did not improve the cryoresistance of partially aerated cultures. Although this last procedure is used along with weak aeration to ensure yeast viability in batch cultures in the beer and wine industries (6, 23), it did not restore the freeze-thaw resistance of the yeasts used in the baking industry. Even if the maturation period is very important for the accumulation of trehalose (13), oxygen excess only during this period did not induce any supplementary trehalose formation or better cryoresistance.

For batch cultures provided with intense aeration, the higher trehalose levels were correlated with better cryotolerance compared with the situation for poorly aerated cells (Table 1). Under fed-batch conditions with intense aeration, the use of a growth temperature of 20°C instead of 30°C caused a loss of trehalose content as well as a drop in the cryotolerance of yeast cells. At 20°C, lower growth rates (0.088 compared with 0.117 h^{-1}) were expected to enhance trehalose formation (22), and this brought back high cryotolerance. The optimal temperature for trehalose formation would be around 40 to 45°C, which would explain its poor accumulation at 20°C (7, 13). Utilization of the reserve carbohydrates has been associated with the long-term stability of bakers' yeast stored in the fresh state (10). A drop in the trehalose content of yeast cream stored at 4°C for up to 11 weeks was matched by a drop in the cryotolerance in dough, but not as closely as could be expected even if the precision of the methodology used was involved (Table 2). Furthermore, freeze-thaw-tolerant yeast cells (strongly aerobic) would use the trehalose reserves more slowly than incompletely oxygenated cells would (Fig. 5). Trehalose appears to be a very labile cell constituent. Perhaps the complete analysis of the storage carbohydrates of bakers' yeast might help to better interpret these results. Glycogen, for example, could be transformed under some conditions into trehalose and vice versa, even if it is quite improbable that glycogen acts as a cryoprotectant itself (9, 34). It is not

clear why high cryoresistance is associated with low assimilation of trehalose reserves. Trehalose analysis alone would not be recommended for quality control or screening of commercial samples of cryotolerant bakers' yeast.

Trehalose would protect yeast cells against freezing only when they were grown under ideal oxygenation conditions. This would indicate the existence of another cryoprotective factor, likely to be more important than trehalose itself but linked to trehalose formation mechanisms and oxygenation characteristics of the medium. Ethanol accumulated in fed-batch yeast cells grown under slight dissolved-oxygen limitation might be involved in their lower cryotolerance. Although precise intracellular ethanol measurements would help, it has been estimated that oxygen-limited cells yielded only slightly more ethanol in the growth medium (less than 5% more) than the total amount of ethanol obtained from cells grown under strongly aerobic conditions (P. Gélinas, Ph.D. thesis, Université Laval, Sainte-Foy, Quebec, Canada, 1988). Furthermore, the yeast biomass was thoroughly soaked and washed with water, a process which is known to rapidly extract ethanol from *S. cerevisiae* cells (2).

According to Uno (40), the cryoresistance of certain yeast strains would be related to the system that regulates trehalose mobilization or cell cycle because freeze-tolerant batch cultures would accumulate more trehalose and proceed more rapidly to the stationary phase than nonresistant yeast cells would. These results (40) have been obtained from incomplete or limited oxygenation experiments, so cryoresistance of these yeast strains might be related to their resistance to partial or momentary lack of oxygen in the growth medium. Identification of this resistance factor associated with intense oxygenation would probably explain the nature of the cryoresistance of some yeast strains whose superiority appears to be related to trehalose metabolism. For example, yeast mitochondrion characteristics might be worth considering; a more detailed description of bakers' yeast cryotolerance has been made (Gélinas, Ph.D. thesis) and will be published in forthcoming papers.

In conclusion, this study has permitted us to explain the nature of cryoresistance in *S. cerevisiae*. Fed-batch growth conditions provided with strong oxygenation gave the best results. In these fermentations, lower specific growth rate further improved the cryoresistance of bakers' yeast in frozen water or dough. Trehalose would certainly play an important cryoprotective role in freeze-thawed yeasts, but its real impact would be essentially limited to yeasts grown with strong oxygenation. The trehalose cryoprotection was neutralized by a lack, even momentary, of excess dissolved oxygen present in the growth medium.

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