Cryoresistance of Baker's Yeast *Saccharomyces cerevisiae* in Frozen Dough: Contribution of Cellular Trehalose

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ABSTRACT

The effects of freezing on gas production of two baker's yeast strains (A and B) were studied in dough. A specific parameter, the mean volume (vol_m), obtained from the area under the gassing curve was shown to be the best expression of overall yeast dynamics during fermentation. Assessing the specific freeze-thaw and frozen storage resistances of yeasts showed that strain B was particularly cryoresistant. Immediate freeze-thaw resistance of both yeasts was unaffected by 1 or 2 hr of prefermentation before freezing, which is not the case for frozen storage resistance. The possible cryoprotective role of cellular trehalose was then examined by following the changes in yeast trehalose content in dough. Cryoresistance was not directly correlated with the initial amount of trehalose in the yeast nor with the level still present in the dough at the time of freezing. However, when the trehalose level dropped below 5%, the delayed frozen storage resistance was clearly lowered. A 4–5% trehalose content at freezing appeared to be sufficient to prevent yeast from indirect chilling injury during frozen storage. A higher quantity of cellular trehalose does not contribute to further cryoresistance.

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Over the last decades, the quality of French bread obtained from frozen dough has been markedly improved by adaptation of breadmaking technology and selection of yeast strains (Maitre 1985, Neyreneuf 1990). However, very little is known about the physiological behavior of yeast cells through the freezing stress they face, or about factors that make one yeast strain more resistant than another. The modification of water distribution in the complex dough matrix could result in strong variations of the yeast's microenvironment, leading to reversible or irreversible cellular damage (Mazur 1976) during both freeze-thaw treatment and frozen storage.

The ability of yeast to maintain maximum fermentative power through this stress (cryoresistance) is affected by both technological and cellular parameters. Technological parameters, which can now be considered as controlled parameters (Van der Plaat 1988), consist not only of the freezing treatment (freezing and thawing rates, length of frozen storage period) but also recipe and dough history up to the time of freezing. Nevertheless, French bakers are still obliged to overdose the yeast in frozen doughs. Some parameters related to the yeast, strain selection, way of growth, and physiological state of the cells when used (Kline and Sugihara 1968, Oda et al 1986, Hino et al 1987, Gelinas 1988, Dunas 1988) could probably also be improved, but the mechanisms involved still remain largely unknown.

Interest has focused on trehalose as an important physiological factor of yeast cryoresistance. This particular disaccharide, which can accumulate up to 20% of the cellular dry weight, was considered an energetic reserve as well as a stress metabolite (Van Laere 1989). Trehalose was related to resistance of yeast to several types of stress, including desiccation (Gadd et al 1987), osmotic stress (Mackenzie et al 1988), and heat stress (Hottiger et al 1987). Coutinho et al (1988) showed that trehalose could be used as a powerful cryoprotectant for yeast when frozen in water. Furthermore, several authors reported that it imparts a higher cryoresistance to baker's yeast in dough. The strains selected by Oda et al (1986) and Uno (1986) during screenings of yeasts suitable for frozen dough showed higher trehalose contents than less cryoresistant strains. Trehalose accumulated during strongly aerobic culture may have had a cryoprotective effect on *Saccharomyces cerevisiae* (Gelinas et al 1989). A high trehalose content was beneficial for a good freeze-thaw stability after extended storage (Dunas 1991). These authors also pointed out, however, that a high trehalose content was not always related to a higher cryoresistance.

The work of Hino et al (1990) on freeze-tolerant versus freeze-sensitive yeasts demonstrated that the freezing tolerance of yeast strains was, to some extent, associated with the basal amount of trehalose maintained after a prefermentation period. On studying the evolution of trehalose in yeast cells during fermentation in a liquid synthetic medium, Van der Plaat (1974) observed a rapid degradation of this disaccharide due to intracellular trehalase activation. Van der Plaat (1988) further suggested that some cryoprotective trehalose had to be present at the time of freezing. Cryoresistance might then be enhanced by reducing the trehalose consumption before freezing. Driessen et al (1991) were able to enhance sugar and drying resistances of a yeast strain by modification of the genes controlling trehalose metabolism. Neyreneuf (1993) further reported that such a modified strain showed increased cryoresistance in frozen dough.

In the present work, the influence of different freezing treatments on fermentative capacity (gas production) was studied to compare the cryoresistance of two candidate yeast strains for frozen-dough. A specific high-performance liquid chromatography method using amperometric detection was set up for quantification of trehalose. Its sensitivity was so high that, for the first time, it became possible to study the evolution of cellular trehalose in yeasted doughs. An attempt was made to verify whether or not higher trehalose contents could impart a better cryoresistance to baker's yeast in frozen dough and whether trehalose consumption before freezing could affect the posthawing gas production capacity of the cells. Assumptions were also made on the way the trehalose could help the cell to withstand the freezing stress.

MATERIALS AND METHODS

Yeasts

Experiments were conducted with different batches of two industrial baker's yeasts (Gist-brocades, Prouvy, France): *Saccharomyces cerevisiae* strain A (33.7% dry matter, 44%

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protein [N × 6.25]) and strain B (33.7% dry matter, 47% protein [N × 6.25]).

Frozen Dough Preparation

Doughs (1,500 g of standard French wheat flour, 900 ml of water, 37.5 g of compressed yeast, 33 g of NaCl) were mixed for 21 min in an Artofex mixer after the yeast had been first suspended for 5 min in water. Dough temperature was 22°C at the end of mixing (except where otherwise stated). In one case however, a 13°C dough was prepared with kneading at 4°C. After sampling in plastic containers (150 g for gassing power measurements and 50 g for trehalose determination), dough pieces were incubated 0–120 min at different temperatures. Dough prefreezing history represented the sequence of events taking place before freezing: yeast suspension (5 min) + dough mixing (21 min) + sampling (19 min) + preincubation times (0–120 min). Therefore, total prefreezing treatment of dough pieces could last 45–165 min. Freezing was performed in a deep-freezer, for 1 hr at –38°C and then for 5 hr at –20°C. Frozen dough samples were then stored at –20°C. Standardized defrosting was performed slowly overnight in a retarder (16 hr at 4°C).

Gas Production Analyses of Yeast in Dough

Gassing analyses were performed in a fermentometer adapted from the volumetric system of Burrows and Harrison (1959). Dough samples with core temperatures of 22°C (when unfrozen) and 4°C (when freeze-thawed) were put into glass flasks in a water bath at 28 ± 0.05°C. The flasks were connected to a graduated device filled with a liquid equilibrated with atmospheric pressure. Kinetics of yeast gas production were followed by measuring the level of this liquid every 10 min, which indicated the total volume of evolved carbon dioxide.

Trehalose Determination

Trehalose was extracted with boiling 12 m*M* H_2SO_4; 50 g of dough sample was mixed with the extraction solution and heated for 10 min at 100°C and then centrifuged-washed. The final extract was adjusted to 500 ml. The initial amount of trehalose present in the yeast was determined before the mixing step: 0.5 g of compressed yeast sample was suspended in 3 ml of the extraction solution and heated for 3 min at 100°C and then centrifuged-washed. The extract was finally adjusted to 100 ml. Extracts were filtered on 0.45-µm filters and then stored frozen at –20°C. Trehalose was separated by anion-exchange chromatography with 150 m*M* NaOH as eluant on a Carbopac PA1 column coupled with a pulsed-amperometric detector (Dionex, Jouy en Josas, France). Trehalose contents presented in Table I are the average values of two or four samples of the same dough or yeast batch.

TABLE I
Dough Treatments and Gassing Power (Vol_m)[a]

Dough Sample	Yeast Strain	Prefreezing Treatment[b]		Vol_m (ml)[c]			FS (wks)
		A	B	D	FT	FS	
D1	A	22°C	0	267	200	146	3
			60 min, 20°C		199	147	3
			120 min, 20°C		193	103	3
D2	A	13°C	0	162	178	137	3
			60 min, 10°C		184	130	3
			120 min, 10°C		181	133	3
D3	A	22°C	0	276	203	140	4
			40 min, 20°C		200	128	4
			80 min, 20°C		200	95	4
D4	A	22°C	0	250	179	123	4
			40 min, 20°C		185	113	4
			80 min, 20°C		197	85	4
D5	B	22°C	0	291	230	197	4
			40 min, 20°C		249	179	4
			80 min, 20°C		230	133	4
D6	B	22°C	0	302	240	209	3
D7	B	20°C	0	292	230	201	3

[a] Vol_m = Mean value of gas volumes measured throughout 3 hr of fermentation, obtained from the integral of the gassing curve.
[b] A = Dough temperature at the end of mixing. B = Time and temperature (bench temp.) of the preincubation period and after the sequence (5-min yeast suspension + 21-min mixing + 19-min sampling = 45 min).
[c] Gas production measurements performed in direct (unfrozen) process (D); after freeze-thaw cycle without frozen storage (FT); and after frozen storage (FS), respectively.

RESULTS AND DISCUSSION

Gassing Profiles of Yeasts in Dough

CO_2 production of the samples was represented by a gassing curve checking CO_2 production versus time. As shown in Figures 1–3, three steps could be observed during fermentation: 1) an acceleration period corresponding both to initiation of the yeast metabolism and to equilibration of the dough temperature with the 28°C of the water bath; 2) a period with constant and maximum fermentation rate; 3) a slowing down period of gas production followed by a period of weak gassing activity.

Due to the various conditions applied during the present study, it was difficult to directly compare the numerous gassing curves obtained. A general parameter expressing the overall gassing activity of yeast was necessary. Although the baker's preoccupation is most often focused on the level of loaf volume obtained at a given time, a single value such as total gas production in a given period does not describe the kinetics of the entire proofing process and does not give access to the dynamics of fermentation. Consequently, it would not provide any information on physiological reactions of yeast to freezing stress. Several characteristics of the gassing curves were assessed for the ability to better describe this phenomenon (maximum speed, time to reach the maximum gas production rate, etc.). Considering the general aspect of gassing curves obtained in the present work, the most relevant parameter appeared to be the mean volume (vol_m) of CO_2 produced, given by the area under the gassing curve divided by the time, which takes into account the shape of the curves during fermentation.

The vol_m of CO_2 depends on the intensity of activity at each moment and especially during the first critical period of gas production. It is related to intrinsic factors (yeast properties) as well as to extrinsic factors (temperature, yeast dosage). For comparisons between different conditions and different strains, vol_m was determined after a constant period of 180 min of fermentation, an usual time for French bread dough leavening. For example, the

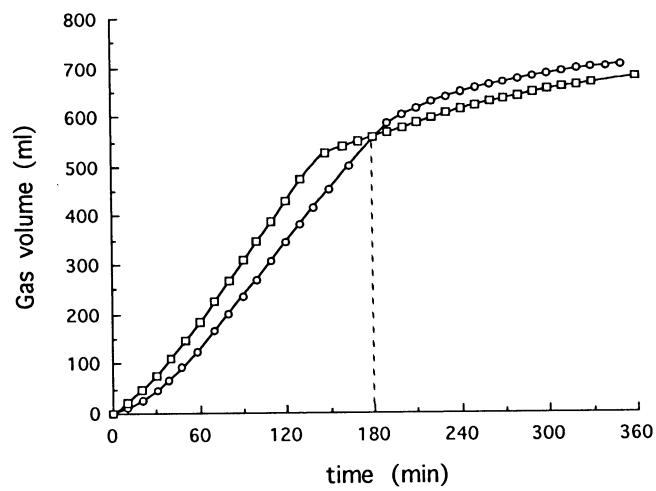

Fig. 1. Gas production in unfrozen doughs at 28°C (150-g dough samples). Yeast strain A (D4, ○). Yeast strain B (D5, □). Initial dough temperature was 22°C.

vol_m given by the gassing curve of strain B without freezing was 16% higher than that obtained with strain A (291 vs. 250 ml). These values express the higher initial gassing rate of this yeast, whereas the final volumes after 3 hr of fermentation were similar for both strains (Fig. 1). A good correlation was observed with dough fermentation in practice, because strain B allowed a reduction of 14% of proofing time during breadmaking.

Effect of Freezing Treatments on Gassing Behavior

Gas production profiles of dough prepared with yeast strains A and B subjected to different freezing treatments are presented in Figures 2 (strain A) and 3 (strain B). Table I presents the vol_m values expressing the gassing behavior of several dough samples, together with their sample history. About 80 samples were analyzed, and Table I shows only the most representative ones. Values obtained were compared for direct testing (without freezing), after a simple freeze-thaw cycle (FT), and after a freeze-thaw cycle associated with a frozen storage time (FS). This allowed us to distinguish between direct chilling injury (caused by freezing and thawing) and indirect chilling injury (caused by frozen storage).

The vol_m between a nonfrozen dough at 22°C and a freeze-thawed dough was reduced from 21% (for strain B and one sample of strain A) to 28% (for several samples of strain A). This decrease in fermentative activity was not caused only by yeast damage because the core temperature of the thawed dough was 4°C at the beginning of the gas production analyses. The real effect of the freeze-thaw cycle on yeast intrinsic capacities was distinguished by applying cold treatment to unfrozen dough (Fig. 4). The reduction of vol_m between an unfrozen cooled dough allowed to stand for 6 hr at 4°C before transfer into the fermentometer and a freeze-thawed dough was only 5–10%, which showed a weak loss in yeast integrity. The 6-hr period corresponded to the end of the defrosting process, during which yeast cells had time to adapt their metabolism to the medium. In fact, when a dough was rapidly (30 min) cooled down to 4°C and immediately transferred into the fermentometer without this 6-hr period, the activity was much more reduced: compared to the 22°C dough, the residual vol_m was 70%, versus 85% for dough after 6 hr at 4°C. As shown in Figures 2 and 3, a frozen storage period of three or four weeks led to a slowing down of the fermentation. This was validated by a further and more significant decrease in the vol_m. With no preincubation period, indirect chilling injury resulted in a reduction of vol_m from 25 to 30% with strain A and from 10 to 15% with strain B.

Increasing the delay before freezing had no influence on gas production after a freeze-thaw cycle because no change could be observed in the vol_m values. However, this delay caused a general loss in gassing ability (vol_m) after prolonged exposure at –20°C. After three to four weeks, doughs that had been allowed to ferment for 40–60 min between sampling and freezing showed a loss in gassing power of 9% (excepted sample D1). When an additional prefermentation step was introduced (from 40 or 60 min to 80 or 120 min), the decrease in vol_m reached 25% (D4), 26% (D3 and D5), and 30% (D1). For D2, no influence of prolonged prefreezing period on gassing power could be seen because the low dough and bench temperatures (13 and 10°C, respectively) prevented any significant yeast activity. Throughout the literature, and since the first report of Godkin and Cathcart (1949), frozen-dough workers most often stated that avoiding any yeast activity before freezing is a *sine qua non* condition when preservation of the gassing power is to be achieved through the freezing stress. However, as shown in Table II, the preincubation period had to last more than 1 hr in the present work for the subsequent cryoalterations to be of some importance. The study of trehalose might suggest some explanation to this apparent discrepancy.

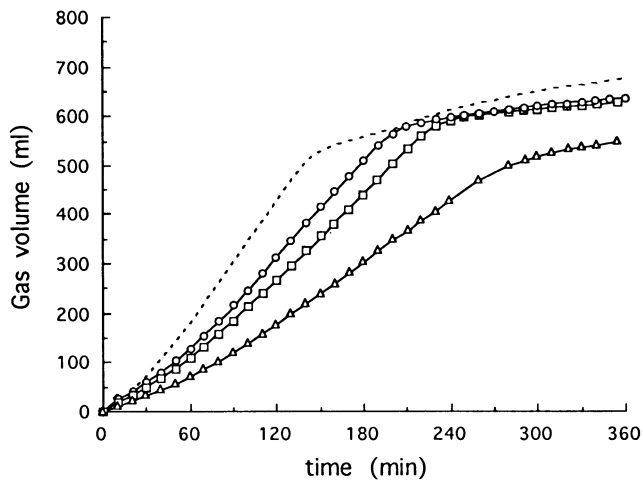

Fig. 3. Gas production in dough at 28°C (150-g samples) for D5 with yeast strain B: ○ = after freeze-thaw cycle; □ and Δ = after four weeks of storage at –20°C; ○, □ = frozen after sampling; Δ = frozen after 80 min more at 20°C; - - - - - = control.

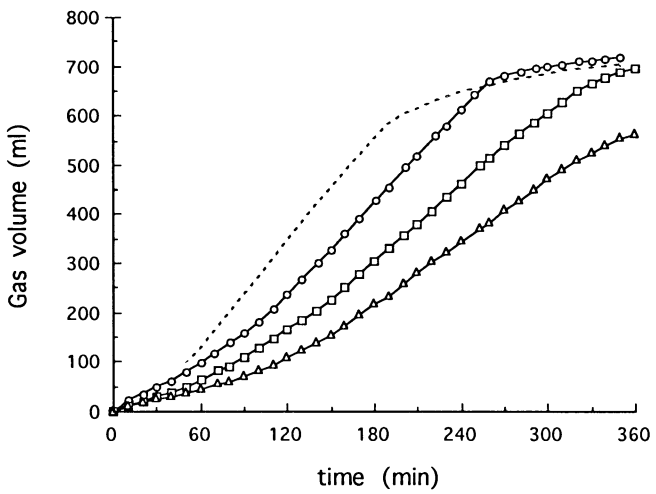

Fig. 2. Gas production in dough at 28°C (150-g samples) for D4 with yeast strain A: ○ = after freeze-thaw cycle; □ and Δ = after four weeks of storage at –20°C; ○, □ = frozen after sampling; Δ = frozen after 80 min more at 20°C; - - - - - = control.

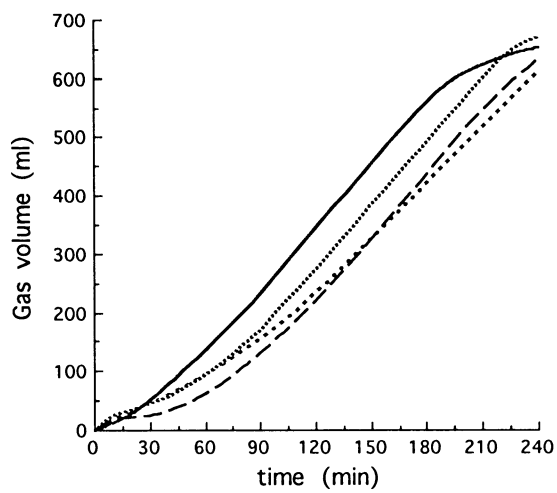

Fig. 4. Influence of initial temperature on gas production in dough at 28°C (150-g samples) with yeast strain A: —— = without any cold treatment (core temperature 22°C); – – – = after rapid cooling down to 4°C; ----- = after 6 hr at 4°C; - - - = after a freeze-thaw cycle.

Defining Cryoresistance of Baker's Yeast in Frozen Dough

In literature, terms like *freeze-thaw tolerance, cryotolerance,* or even *cryoresistance* are widely used, but attempts to precisely define these terms and distinguish between them are very scarce. *Yeast cryoresistance* can be defined as the yeast's ability to withstand and resist the overall freezing treatment (freezing, thawing, cold-storage stresses). Cryoresistance has also been expressed as the percentage of survival after freezing in a cell suspension. In a medium such as dough, cellular death is not the sole cause of the loss of gassing power, and nonlethal cryodamage should also be taken into account. Takasaki and Karasawa (1992), for example, observed a decrease in the fermentation ability of doughs during frozen storage, whereas no change in the survival rate of yeast could be detected. These workers postulated that some decrease of the activity per yeast cell had occurred, but further investigations in this field are needed. Different levels of cryoresistance may be characterized according to how the yeast preserves its initial gassing power throughout the overall freezing treatment. Two expressions for a freeze-tolerance ratio of baker's yeast have been proposed (Gelinas 1988, Murakami et al 1994): the maintenance of gassing power in a thawed yeasted sample versus an unfrozen yeasted sample measured in a given time. The control parameter was the increase in dough volume for Gelinas (1988) and the total gas evolved during fermentation in a liquid medium for Murakami et al (1994). The increase in dough volume during fermentation can be considered a key parameter for the baker, but it does not provide much information on the yeast behavior from a physiological point of view.

In the present work, the maintenance of vol_m throughout the freezing stress was regarded as an index of yeast cryoresistance. The kinetics of the gassing power recovery just after defrosting represent the most critical point for expressing yeast cryoresistance. The vol_m properly takes this characteristic into account. Consequently, the percentage of remaining vol_m for 3-hr fermentation after thawing was finally selected as a suitable cryoresistance index for comparing the resistance of different yeasts in dough or analyzing the resistance of a given yeast subjected to different freezing treatments. A distinction should be made between the resistance to simple freeze-thawing (FT vol_m/Direct vol_m) and the resistance to frozen storage (FS vol_m/FT vol_m), because the phenomena involved in these two steps are unlikely to be the same. The consequences of direct chilling injury (caused by freezing + thawing) and indirect chilling injury (caused by prolonged exposition to subzero temperature) on the yeast performance can then be analyzed separately.

Considering the FT resistance and FS resistance index presented in Table II, the effect of freezing stress on the remaining gassing power was more pronounced on strain A than on strain B, and the latter appeared to be clearly more cryoresistant. Whatever the conditions of dough preparation, strain B preserved much more of its initial gassing power than did strain A. For example, while strain B maintained 86% of the vol_m through four weeks of frozen storage, strain A maintained only 69%. However, comparing the vol_m values after storage of samples D3, D4, and D5 showed that both strains were equally sensitive to the prefermentation time before freezing, even though strain B appeared to be a more rapid yeast. After three weeks of frozen storage, this strain could recover almost 90% of its initial fermentative capacity, provided the prefermentation was not prolonged. The major difference between strains A and B seemed to be in the capacity of strain B to rapidly express optimal activity. This led us to suggest that the two strains might have different energetic metabolisms. In a previous work, Neyreneuf and Van der Plaat (1991) showed that yeasts with rapid initial fermentative capacity were more sensitive and less stable to freezing than yeasts with slower initial gassing power. The new data presented in this article demonstrate that at least some strains have the ability to be both cryoresistant and quite active before freezing.

Evolution of Trehalose Content of Yeast in Dough

The determination of trehalose content was performed on 18 different batches of yeast strain A and four different batches of yeast strain B. Different growth conditions caused the initial trehalose level in the block of compressed yeast of strain A ranged from 9 to 17% (dry matter basis) with an average value of 14%. Cellular trehalose for strain B was much lower (6–8%).

Yeasts incorporated into doughs were then subjected to differ-

TABLE II
Cellular Trehalose Content, Dough Treatments, and Cryoresistance Index

Dough Sample	Yeast Strain	Initial Trehalose Content (%)[a]	Prefreezing Treatment[b]		Trehalose Content at Freezing (%)[c]	Cryoresistance Index (%)[d]	
			A	B		FT–RI[e]	FS–RI[f]
D1	A	9.6 ± 0.0	22°C	0	7.9 ± 0.3	75	73
				60 min, 20°C	5.0 ± 0.3	75	74
				120 min, 20°C	3.9 ± 0.1	72	53
D2	A	11.3 ± 0.0	13°C	0	10.0 ± 0.2	109	77
				60 min, 10°C	7.6 ± 0.1	113	71
				120 min, 10°C	6.0 ± 0.1	111	73
D3	A	9.6 ± 0.1	22°C	0	7.2 ± 0.1	74	69
				40 min, 20°C	4.7 ± 0.1	73	64
				80 min, 20°C	3.6 ± 0.1	72	47
D4	A	13.7 ± 0.1	22°C	0	8.7 ± 0.2	72	69
				40 min, 20°C	4.6 ± 0.2	74	61
				80 min, 20°C	3.6 ± 0.1	79	43
D5	B	7.0 ± 0.1	22°C	0	5.7 ± 0.1	79	86
				40 min, 20°C	3.4 ± 0.2	85	72
				80 min, 20°C	2.5 ± 0.1	79	58
D6	B	8.2 ± 0.1	22°C	0	4.9 ± 0.1	79	87
D7	B	7.4 ± 0.2	20°C	0	5.2 ± 0.2	79	88

[a] Initial trehalose content in block of compressed yeast (% of yeast dry matter).
[b] A = Dough temperature at the end of mixing. B = Time and temperature (bench temp.) of the preincubation period and after the sequence (5-min yeast suspension + 21-min mixing + 19-min sampling = 45 min).
[c] Trehalose content of yeast in dough at the time of freezing (% of yeast dry matter). Average values and standard deviation of four samples.
[d] Gas production measurements performed in direct (unfrozen) process (D); after freeze-thaw cycle without frozen storage (FT); and after frozen storage (FS), respectively. Vol_m = Mean value of gas volumes measured throughout 3 hr of fermentation, obtained from the integral of the gassing curve.
[e] FT–RI = Freeze-thaw resistance index (FT vol_m / D vol_m).
[f] FS–RI = Frozen storage resistance index (FS vol_m / FT vol_m).

ent prefreezing treatments (13–22°C core temperature, 0–120 min of bench time after mixing and sampling; i.e., 45 min further to yeast rehydration). Table II gives details about dough treatments and lists trehalose determinations that were performed at the exact times of freezing with four batches of strain A and three batches of strain B. The gradual decrease of trehalose concentration throughout prefreezing is presented in Figure 5. Compared with the initial content in the yeast samples, trehalose started to be used as soon as yeast was incorporated into dough once its metabolism started. In strain A, after 80 min of bench time (preincubation) at 20°C (125 min after yeast rehydration) trehalose (initially 13.7–9.6%) was degraded to a level of 3.6%, or 30–40% of the initial stock. With strain B (7% initial trehalose) treated in same conditions, 35% of the disaccharide remained (with 2.5% as absolute value). Briefly, trehalose catabolism seemed to be equally active in the two strains. In contrast, the strains selected by Hino et al (1990) showed very different rates of trehalose degradation. In a liquid medium exposed at 28°C, Van der Plaat (1974) observed a more severe decrease in trehalose because <20% of the initial level could be detected after only 40 min of fermentation. In opposition to what Dunas (1991) suggested, trehalose was never completely degraded in the strains used in the present work, whatever the prefermentation time.

Trehalose and Cryoresistance

As prefreezing treatments yielded dough samples with intracellular trehalose contents ranging from 2.5 to 10%, it was possible to study whether or not trehalose could help preserve yeast's gassing power and therefore be related to cryoresistance. Immediate and delayed cryoresistances, expressed respectively by remaining vol_m values through a freeze-thaw cycle (%FT/Direct) and frozen storage (%FS/FT), were analyzed in terms of trehalose contents determined at time of freezing (Table II).

Direct freeze-thaw resistance was not affected by trehalose level as shown by vol_m obtained with five dough samples (four with strain A and one with strain B) after different prefreezing treatments and therefore different trehalose levels. For strain A, minimum and maximum values of FT resistance index (72 and 79%) were both obtained with 3.6% trehalose content. With strain B, highest and lowest trehalose contents (5.7 and 2.5%) both yielded an FT resistance index of 79%.

Frozen storage resistance, as well as cellular trehalose content at the time of freezing, decreased as prefermentation periods increased. However, the first preincubation step (40 or 60 min) that allowed a consumption of 2–4% trehalose (35–45% of the content) led to a loss in gassing power (vol_m) of <10%. During the second preincubation step (from 40 or 60 min to 80 or 120 min), only 1% of extra trehalose (21–26% of the remaining disaccharide) was degraded, and the FS resistance index was 27–30% lower for strain A and 20% lower for strain B. In fact, vol_m values were not much changed by prefermentation and trehalose breakdown as long as the disaccharide in dough remained >4% (Fig. 6). FS resistance index of yeasted doughs with lower levels of this disaccharide (3.9, 3.6, 3.4, 2.5%) were significantly decreased (25–32% less CO_2). In the present work, yeast activity before freezing (prefermentation) was detrimental to the cryoresistance of both strains only insofar as it induced excessive trehalose degradation. Results obtained with a dough sample at 13°C and processed at 10°C (D2) strengthened this view: vol_m values were not affected by the sequence of 120 min preincubation + frozen storage (Table I, 133 ml compared with previous values of 130 and 137 ml); freezing trehalose content was 6%.

Results obtained in doughs confirmed what was observed in a liquid medium by Hino et al (1990), who subjected some Japanese yeasts to 30 min of prefermentation before freezing. These workers showed that the retention of trehalose exceeded 1–5% (≈25% of initial level) in freeze-resistant strains, whereas the disaccharide was degraded almost completely (<0.5%) in the freeze-sensitive strains. A high level of trehalose initially accumulated by the yeast was not a prerequisite for ensuring cryoresistance. This was also shown by the results of the present work because no direct relationship could be established between the initial trehalose content (which was always ≥7%) and cryoresistance. However, lowering trehalose accumulation <4–5% would probably lead to lower yeast-delayed cryoresistance as observed by Dunas (1991) with some Danish yeasts that had only 1.3–8.9% initial trehalose before incorporation into dough. A high initial trehalose content in these yeasts seemed to moderate the general decrease in gas production caused by prefermentation before freezing. Results presented here clearly indicate that until the trehalose level fell below 5% at the time of freezing, the yeast

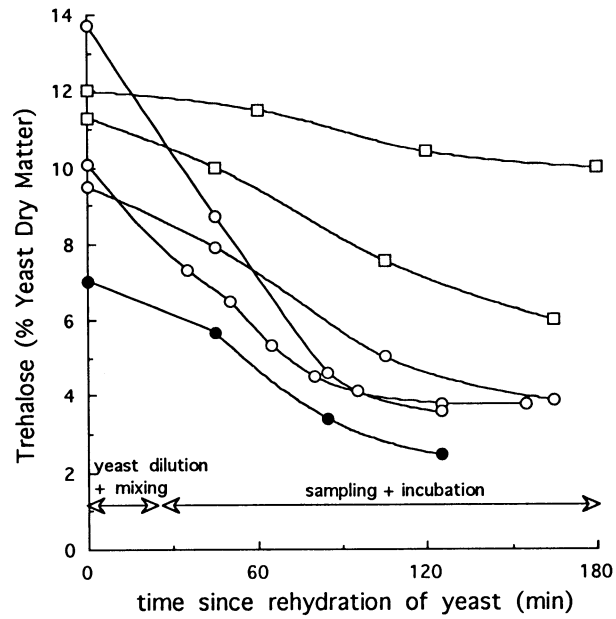

Fig. 5. Evolution of trehalose content of yeast in dough with yeast strain A (○, □) or yeast strain B (●). Dough at 22°C incubated at 20°C (○ ●); dough at 13°C incubated at 10°C (□). Each curve represented yeast from a different batch. Initial values are trehalose contents measured in compressed yeast.

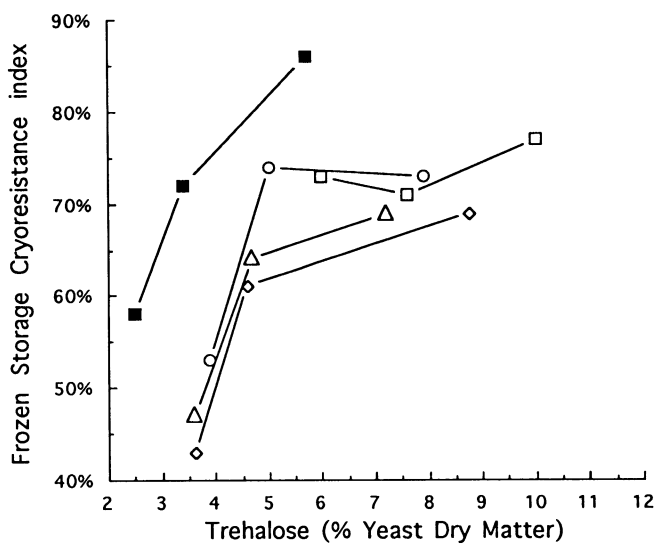

Fig. 6. Frozen Storage Cryoresistance Index after three or four weeks related to the cellular trehalose content in dough at the time of freezing. Strain A: D1 (○), D2 (□), D3 (Δ), D4 (◇). Strain B: D5 (■).

was protected from excessive indirect chilling injury. Quantities >5% did not contribute to higher cryoresistance. On studying the hydric stress in yeast cells, Mackenzie et al (1988) came to similar conclusions: "An entire population was protected at a minimum mean value of around 50 mg trehalose / g dry yeast; . . . at levels higher than this, the population was demonstrably homogeneous with respect to stress resistance since trehalose was supposed to be uniformly distributed." The idea of a threshold for trehalose to impart cryoresistance was proposed by Attfield et al (1992), who stated that strains producing >5% trehalose should be more tolerant to cold stress (8–28% survival through freezing stress in liquid medium) than strains producing <4% (<2% survival). The high level of cryoresistance of strains A and B, both presenting >5% initial trehalose, confirmed Attfield's view. If there is no doubt concerning the high cryoresistance of strain B, the same is true for strain A, because the properties of this yeast were demonstrated to meet the high cryotolerance requirements of frozen-dough producers.

Because results reported here indicate that <5% trehalose could help the yeast to withstand the freezing stress, some of the theories on cryoprotection mechanisms were reviewed. The most often accepted theory of stress protection by trehalose is the water-replacement hypothesis (Clegg et al 1982): molecules of the disaccharide protect the cellular membrane by creating interactions with hydroxyl head-groups of phospholipids and consequently maintaining sufficient integrity of the membrane exposed to damage due to the water removal. This was demonstrated in drying experiments during which stabilization of bilayers required direct interaction between the sugar and polar head-groups of the phospholipids (Crowe et al 1990). In frozen dough, only about half of the water is frozen. Nonfreezable water remains at the membrane surface, and water replacement by a structuring solute such as trehalose is probably not necessary for membrane stabilization. Membranes, however, could need some protection against the possible toxicity of ethanol produced in prefermented doughs and cryoconcentrated. Mansure et al (1994) showed that trehalose could inhibit ethanol effects on membranes. They observed a positive correlation between cell survival in the presence of ethanol and trehalose concentration (with values <6%). Low concentrations of added trehalose have been able to protect thylakoid membranes by reducing solute permeability and so lowering the solute loadings of the vesicles that would occur during freezing (Bakaltcheva et al 1994). Some cryoprotection might also be achieved by an increase of the intracellular concentration of the disaccharide. Accumulation of trehalose, a cytosolic disaccharide (Keller et al 1982), should lead to a large increase of the cellular osmotic pressure, limiting the loss of water caused by the freezing of extracellular water and cell shrinkage. However, our opinion is that the relatively high osmotic pressure in the dough medium does not require large osmotic protection. Piper (1993) suggested that trehalose might contribute to increasing the structurization of intracellular water, thus enhancing stress tolerance. In frozen doughs, apart from these hypotheses on cryoprotection through molecular interactions, trehalose reserves would probably act as an energetic starter at the end of thawing and could facilitate the mechanism of freezing-damage repair, allowing the yeast to recover optimal activity in a minimum of time.

These assumptions do not conflict with the idea of a threshold level for trehalose as a protectant, because all these possible favorable functions of trehalose could take place at concentrations <5%. At >5% of the yeast dry matter trehalose should not have any additional role.

CONCLUSION

Cryoresistance of baker's yeast can be defined as its ability to withstand the overall freezing stress that occurs in breadmaking using frozen-dough technology. A reliable indicator of cryoresistance is represented by the proportion of vol_m of CO_2 produced, determined over a 3-hr fermentation period following the freezing treatment compared with the gas production of a control sample. Using this parameter, it was possible to demonstrate that a rapid strain could show better resistance during frozen storage than a yeast with slower activity. To be of technological interest, a high resistance to prolonged frozen storage should be associated with a high fermentative ability and a poor sensitivity to the freeze-thaw cycle. Strain B was particularly suitable for frozen-dough processing because it presented each of these three main properties at a better level than strain A, which was already regarded as satisfactory for frozen-dough technology. Gas production of strain B was 22% higher than that of strain A after a freeze-thaw cycle; the difference reached 48% after four weeks of frozen storage.

The possible role of trehalose as a physiological factor of cryoresistance received particular attention. For the first time we could report on the gradual changes of this disaccharide in dough from mixing to freezing. For both strains, after dough preparation up to 2 hr at 20°C before freezing, the maximum degradation of cellular trehalose achieved on freezing was not more than 60–70% of the initial stock. The resistance of baker's yeast through frozen storage was not related to its cellular trehalose content in a simple way. A cellular trehalose degradation down to 2.5% (yeast dry matter basis) during mixing and prefermentation did not significantly affect the behavior of yeast cells when a simple freeze-thaw cycle (no storage period) was applied. On the other hand, a 4–5% (yeast dry matter basis) stock at freezing was required for protection against the sequence of prefermentation + freezing + frozen storage + thawing. Above this level, more trehalose did not clearly confer higher cryoresistance. At a concentration of <4–5%, trehalose degradation was linked with a greater decrease of gassing power. The main conclusion is, therefore, that 4–5% is a threshold level sufficient for this disaccharide to ensure protection for membrane or cytoplasm; and this level is also sufficient to play a role in the recovery of metabolic activity and possible repair of damage after the freezing stress.

Current frozen-dough technology recommends low dough-processing temperature and fast handling to minimize yeast activity before freezing. Results presented here show that making yeasted dough pieces entering the freezer with at least 4–5% remaining trehalose optimized the yeast performance after thawing. Efforts of yeast manufacturers to increase the initial trehalose content above this level would probably be ineffective.

Not only the accumulation of trehalose but also the control of its catabolism up to freezing, should be taken into account in obtaining more cryoresistant yeasts.

The cellular trehalose reserves are not the sole factor of yeast protection against freezing injury in dough. Strain B was able to preserve 62% of its initial gassing ability after four weeks of freezing stress; whereas capacity of strain A under the same conditions, and with similar trehalose level (3.6% compared to 3.4%), dropped to 34%. One of the other mechanisms that should have contributed to high cryoresistance in strain B would probably be the functionality of cellular membranes. The consequences of freezing stress on membranes in relation to their phospholipidic and sterolic composition, and the possible role of trehalose as a structurating agent, requires further research.

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