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Lipid Content and Cryotolerance of Bakers' Yeast in Frozen Doughs†

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The relationship between lipid content and tolerance to freezing at −50°C was studied in *Saccharomyces cerevisiae* grown under batch or fed-batch mode and various aeration and temperature conditions. A higher free-sterol-to-phospholipid ratio as well as higher free sterol and phospholipid contents correlated with the superior cryoresistance in dough or in water of the fed-batch-grown compared with the batch-grown cells. For both growth modes, the presence of excess dissolved oxygen in the culture medium greatly improved yeast cryoresistance and trehalose content (P. Gélinas, G. Fiset, A. LeDuy, and J. Goulet, Appl. Environ. Microbiol. 26:2453–2459, 1989) without significantly changing the lipid profile. Under the batch or fed-batch modes, no correlation was found between the cryotolerance of bakers' yeast and the total cellular lipid content, the total sterol content, the phospholipid unsaturation index, the phosphate or crude protein content, or the yeast cell morphology (volume and roundness).

Fed-batch is the common feeding regimen for the industrial production of bakers' yeast, but batch fermentation is also used in the first stages of its propagation. In the batch process, all nutrients are added at the beginning of the fermentation. Under fed-batch, nitrogen and carbon sources are added at a lower rate than the normal yeast assimilation rate; the whole yeast biomass is harvested after about 14 h. Cell physiological needs are probably better met under fed-batch than batch growth, resulting in better dough proofing activity and higher biomass yield, both of major economic importance in industrial baker's yeast production (8).

In a companion study (12), special growth conditions for the relationship between yeast trehalose content and its adaptation to freeze-thaw stress have been reported. However, information on the effect of growth conditions on other yeast characteristics and cryotolerance is scarce in the literature. For example, in the yeast industry, protein and phosphate levels are usually correlated with baking activity and storage stability of commercial bakers' yeast (8). The influence of growth conditions on size and morphology of yeast cells has been shown to be species and strain dependent (20), but higher agitation in continuous culture (31) and a lower magnesium level (30) enhance cell volume. Cell elongation following anaerobiosis has also been observed (11).

There have been numerous reviews on yeast lipid composition (14), but few workers have examined the relationship between lipid content and resistance to freezing in *Saccharomyces cerevisiae*. Modification of growth conditions allows the lipid profile to be altered, but such changes are difficult to associate with cryoresistance. One of the major benefits of a better understanding of the mechanisms of cryoresistance in *S. cerevisiae* is the improvement of preservation techniques for culture collections. In the present study, however, the primary concern was the survival of bakers' yeast incorporated in frozen doughs. Improving the cryotolerance of bakers' yeast would significantly contribute to the stability of leavened bakery products which can be baked outside the principal production facilities in specially designed installations. The survival of yeast after freezing is considered among the most important factors in frozen dough stability (15). This study focuses not only on the lipid but also the nitrogen and phosphorus composition of *S. cerevisiae* grown under different conditions (batch and fed-batch) and sheds light on the relative importance of growth conditions on biomass yield, cell morphology (volume and roundness), and the dough proofing activity of bakers' yeast.

MATERIALS AND METHODS

Yeast strain and growth conditions. All fermentations were done in duplicate by using a commercial bakers' yeast strain, *S. cerevisiae* C, supplied by Lallemand Inc. (Montréal, Quebec). Batch fermentations were conducted under microaerobic (agitated only) or strongly aerobic conditions (dissolved oxygen maintained at 80 to 100% saturation) at 30°C. Fed-batch fermentations (with partial limitation of nitrogen and glucose) were carried out at 30°C under either strong (35 to 90% oxygen saturation) or partial (0% oxygen saturation during the latter two-thirds of the growth cycle, approximately 9 to 10 h) aerobiosis. Strongly aerobic fed-batch fermentations were also carried out at 20°C. The complete fermentation protocols were described previously (12).

Freeze-thaw procedures. Cryotolerance of the cells was measured in duplicate in water or dough. Small pieces (1.5 ± 0.1 g) of white bread dough were transferred to polypropylene tubes (1 by 7 cm) and frozen at −50°C for 10 min (freezing rates of 20.6 ± 1.1°C min^{-1} at the center of the dough and about 42.4°C min^{-1} at the internal wall of tube). Immediately after freezing, thawing was done for 5 min at 30°C. After incubation at 30°C for 1 h, proofing volumes of frozen-thawed doughs were compared with nonfrozen con-

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TABLE 1. Yield, dough proofing activity, protein and phosphate content, and cryotolerance of bakers' yeast grown under different feeding, aeration, and temperature conditions[a]

Growth conditions	% Cryotolerance[b]		% Yield (wt/wt)[c]		Dough proofing activity (cm)	Crude protein (% dry wt)	Phosphate (% dry wt)[d]
	Dough	Water	Molasses	Fermentables			
Fed-batch							
Strong aerobiosis (30°C)	100a	92a	89.6a	55.4a	2.3a	53.6a	3.1a
Partial aerobiosis (30°C)	44b	74b	81.3b	50.2b	2.5b	59.2b	3.4b
Strong aerobiosis (20°C)	18c		70.0c	44.2c	2.4c	56.9c	3.6c
Batch							
Microaerobiosis (30°C)		2c		5.7d	0	57.2c	3.0a
Strong aerobiosis (30°C)		17d		10.9e	0	36.7d	3.6c

[a] All values are means. Means within each column followed by the same letter (a to e) are not significantly different ($P < 0.05$), according to the Student t test for paired samples.
[b] Results were obtained from reference 12.
[c] Yeast solids (27%) were used. Data were obtained from mean fermentable sugar concentrations in molasses (54%) and malt syrup (60%), as used in growth media.
[d] Phosphate = P_2O_5.

trol samples (12 replicates). Cryotolerance (stated as the gas-producing activity) was expressed as the ratio (%) of the mean net risen dough heights of frozen and unfrozen doughs.

Mainly because batch cultures could not raise doughs, the freeze-thaw evaluation was also performed in water with four replicates of 0.3-ml cell suspensions (10^7/ml) kept at −50°C for 10 min (freezing rate of 39.6 ± 1.1°C min^{-1}). Thawing was set at 30°C for 2 min, and cell viability was estimated by plate count (malt extract agar; Difco Laboratories) done in duplicate for each tube; the plates were incubated for 65 h at 30°C before counting. The complete freeze-thaw procedures were described by Gélinas et al. (12).

Dough proofing activity. Dough proofing activity of freshly produced yeast was recorded as the mean proofing height (centimeters) of 12 replicates.

Nitrogen, phosphorus, and total fermentables analysis. Duplicate samples of freshly harvested yeast were frozen at −25°C, lyophilized, and kept at −25°C in a desiccator prior to analysis. The nitrogen content (reported as crude protein; N × 6.25) was measured by the Kjeldahl method (Kjel-Foss 16210 A/SN; Foss Electric, Hilleroed, Denmark). Phosphorus analysis in duplicate was based on the method of Allen (2) and reported as P_2O_5 (P × 2.29). Total fermentable sugars in molasses and malt syrup were determined in triplicate by the method of Lane-Eynon (3) and used to calculate the biomass yields as the amount of dried yeast (grams) produced either by 100 g of fermentable sugar or by 100 g of molasses (on the basis of a 27% yeast dry weight). All data were analyzed by using the paired Student t test.

Lipid analysis. Fresh yeast biomass was washed, frozen at −25°C, lyophilized, and kept at −25°C in a desiccator. The cells were disintegrated by the method of Kaneko et al. (17), and lipids were extracted and purified by the method of Folch et al. (10); the first extraction and the phase separation were done by the method of Bligh and Dyer (4). All extractions were done in duplicate from two different yeast samples. The purified extracts were dried after the addition of a few drops of methanol to eliminate traces of water, and the dried lipids were weighed. The extracts were stored in the dark at −25°C in 2 ml of $CHCl_3$ under nitrogen. Total phospholipids were estimated (in duplicate) by the method of Allen (2). Lipid extracts were separated (in duplicate) by thin-layer chromatography on silica gel type G using hexane-diethyl ether-glacial acetic acid (80:20:1) (1).

To determine the fatty acid profile of the phospholipids, 1.0 mg of heptadecanoic acid was added as an internal standard to the silica gels containing the phospholipids. The lipids were transmethylated with 11% boron trifluoride in methanol (22). The esters were transferred in amber vials with 0.5 ml of carbon disulfide and separated by gas-liquid chromatography. The unsaturation indices (Δ/mole) were calculated by the method of Kates and Baxter (18) as follows: Δ/mole = % monoenes + 2(% dienes) + 3(% trienes). All analyses were done in triplicate.

Total sterols were saponified in duplicate by the method of Parks et al. (23). Each sample was transferred with methanol and dried, and the sterols were determined by the Lieberman-Burchard method as described by Taylor and Parks (25). Free sterols were extracted three times from the silica scraped from the plates by using 3 ml of $CHCl_3$-CH_3OH (4:1, vol/vol), dried, and determined colorimetrically by the Lieberman-Burchard method as described previously (25). The free-sterol-to-phospholipid (S/P) ratios were calculated by using molecular weights of 700 for phospholipid and 397 for ergosterol.

For the analysis of the free sterols profile, 0.25 mg of cholesterol was added as an internal standard to the free-sterol-containing silica gel. The gel was extracted three times with 3 ml of $CHCl_3$-CH_3OH (4:1, vol/vol) by the method of McKersie et al. (21) and separated by gas-liquid chromatography.

Cell dimensions. The cell length and width were measured under light microscopy as follows: 20 each of large and small cells were selected at random with an hemacytometer for volume measurements according to the equation of an ellipse ($V = 4/3 \cdot \pi \cdot a \cdot b^2$, where a = length/2 and b = width/2).

RESULTS

Yield, crude protein, phosphate, and dough proofing activity. Maximum biomass yields were obtained with fed-batch cells grown at 30°C under strongly aerobic conditions (no dissolved oxygen deficit during fermentation), and growth at 20°C gave lower yields compared with those at 30°C (Table 1). In comparison with regular industrial practice (partial aerobiosis, fed-batch, 30°C), strong aerobiosis significantly improved biomass yields. Poor biomass production was obtained in batch fermentations (agitated only), and intense aeration increased yields twofold under these conditions.

TABLE 2. Effect of growth conditions on lipid composition and cryotolerance of bakers' yeast[a]

Growth conditions	Total lipids (% dry wt)	Total sterols		Free sterols		Total phospholipids		S/P ratio (mol/mol)
		% Total lipids	% Dry wt	% Total lipids	% Dry wt	% Total lipids	% Dry wt	
Fed-batch								
Strong aerobiosis (30°C)	4.87a	10.37b	0.51b	2.40b	0.12b	48.46b	2.36b	1/20a
Partial aerobiosis (30°C)	5.06a	11.69b	0.59b	2.46b	0.13b	46.50b	2.35b	1/18a
Strong aerobiosis (20°C)	5.22a	9.38b	0.49b	2.11b	0.11b	44.97b	2.35b	1/21a
Batch								
Microaerobiosis (30°C)	5.28a	4.62a	0.25a	1.39a	0.07a	35.57a	1.89a	1/27b
Strong aerobiosis (30°C)	6.30b	12.92c	0.81a	1.25a	0.08a	36.08a	2.27a	1/28b

[a] All values are means. Means within each column followed by the same letter (a to c) are not significantly different ($P < 0.05$), according to the Student t test for paired samples.

Crude protein (total Kjeldahl nitrogen) and phosphate (total phosphorus) contents of baker's yeast were not directly or inversely correlated to cryotolerance. Strong aerobiosis (no oxygen deficit) in fed-batch yeast cells gave a slightly lower proofing activity, while batch-grown cells could not raise doughs at all. Under fed-batch conditions, fermentation at 20°C gave an intermediate dough proofing activity.

Total lipids, sterols, and phospholipids. Under our experimental conditions, no direct correlation was observed between total lipid weight and resistance of *S. cerevisiae* to freezing (Table 2). Cells grown in batch mode under strongly aerobic conditions had little cryoresistance, although they contained more lipid on a cell dry weight basis than cells from fed-batch cultures. Intensely aerated batch cells had a total lipid content nearly 20% higher than that found for the microaerobic yeasts (agitated only). Under fed-batch conditions, the cells contained nearly the same amounts of total lipids, even though the more resistant cells seemed to accumulate slightly less lipid than the others. Because important differences in intracellular trehalose reserves might influence the results on a dry weight basis (12), all results are presented on a dry weight and on a total lipids basis. However, only in the case of strongly aerobic batch cells might the weight of the lipid reserves have been underestimated because of a high cellular trehalose content.

Under strongly aerobic conditions, batch-grown yeasts had a higher total sterol content than fed-batch-grown cells (Table 2). Aeration increased the sterol content of batch cells, causing a threefold difference. Under fed-batch fermentation, no significant differences in total sterol content was observed between intense aeration and partial aerobiosis experiments. Growth at 20 compared with 30°C seemed to reduce sterol accumulation. However, no relationship was observed between cryotolerance and total cellular sterol content.

Free sterol formation and cryotolerance were lower in batch than in fed-batch cells (Table 2). No noteworthy differences were observed between cultures of fed-batch cells. As a rule, the main free sterol was ergosterol (35 to 45%) and, even though the four other sterols detected were not formally identified, no major differences in their proportions were noticed under the growth conditions tested.

The total phospholipid content was lower in batch-grown compared with fed-batch-grown cells (Table 2). For batch-grown cells, the intense aeration considerably increased the phospholipid levels on a total lipid basis. Among the fed-batch-grown cells, phospholipids were at quite similar levels on a cell dry weight basis, although strongly aerobic growth at 30°C appeared to increase phospholipids as a percentage of the total lipids. The S/P ratio was lower in batch-grown (range, 1/27 to 1/28) than in fed-batch-grown (1/18 to 1/21) cells and correlated with cryotolerance.

Fatty acid profile. The predominant fatty acid chains of the phospholipids were $C_{16:1}$ and $C_{18:1}$ (Table 3). Under fed-batch conditions, these made up over 75% of the total fatty acids, but they made up only 68 or 60% for batch growth with and without direct intense aeration. Batch-grown cells were characterized by slightly higher levels of saturated and polyunsaturated fatty acids, while fed-batch-grown cells showed a larger proportion of unsaturated chains, although the unsaturation index remained fairly constant for both fermentation types. No correlation was obtained between the resistance of the yeasts to freezing and their unsaturation indices (Δ/mole), percent unsaturation, or percent polyunsaturation.

Aeration of batch-grown cells caused a decrease in polyunsaturated fatty acids and a large increase in C_{16} unsaturated chains. Under fed-batch conditions, decreasing the temperature to 20°C resulted in somewhat increased proportions of $C_{14:0}$, $C_{14:1}$, and $C_{15:0}$. At 30°C, intense aeration brought a general decrease in unsaturation (Δ/mole, percent unsaturation, and percent polyunsaturation) and an increase in C_{16} chains compared with the results with the partially aerated cells. The fatty acid profile of the phospholipids was not associated with the observed cryotolerance of baker's yeast (Tables 1 and 3).

Cell morphology. Yeast cell roundness and volume were greatly affected by growth conditions (Table 4) but were not related to cryotolerance. Intense aeration enhanced volumes by 30 to 50% in batch-grown cells. Batch fermentations produced larger cells compared with fed-batch ones (all cells were under stationary phase). Even if the growth rates were similar among fed-batch cells, lower growth temperature (20°C) also enhanced the cell volume. Fed-batch fermentation under strong aeration provided the roundest cells.

DISCUSSION

Gélinas et al. (12) reported that yeast cryotolerance was greatly improved when cells were grown under fed-batch compared with batch conditions, but also when there was no dissolved oxygen deficit during cultivation. Trehalose was certainly of importance in cryoprotection, but its effect was neutralized by even a momentary lack of excess dissolved oxygen in the fed-batch growth medium. The present study checked whether other biochemical analysis (nitrogen, phosphate, or lipid composition) could be involved in cryoprotection besides trehalose. Moreover, we looked at the influ-

TABLE 3. Effect of growth conditions on the fatty acid profile of phospholipids of bakers' yeast[a]

Growth conditions	% Wt of fatty acid chain types																Δ/mole	% Unsaturated	% Polyunsaturated	% Saturated	% Total C_{16}	% $<C_{16}$
	12:0	13:0	14:0	14:1	15:0	15:1	16:0	NI	16:1	NI	NI	17:1	18:0	18:1	18:2	18:3	20:0					
Fed-batch																						
Strong aerobiosis (30°C)	tr	ND	0.57	0.17	0.28	0.30	14.29	0.54	44.74	ND	ND	1.97	4.47	30.63	1.73	0.65	ND	0.83	80.19	2.38	19.61	59.03	1.32
Partial aerobiosis (30°C)	tr	tr	0.39	0.25	0.20	0.27	10.93	0.58	42.08	ND	ND	2.14	4.83	35.52	2.42	0.63	ND	0.87	83.30	3.04	16.35	53.01	1.11
Strong aerobiosis (20°C)	0.23	tr	0.93	0.32	0.54	0.30	13.55	0.40	45.18	ND	ND	1.90	3.77	30.52	1.49	0.72	1.08	0.83	80.43	2.21	20.09	58.73	2.30
Batch																						
Microaerobiosis (30°C)	0.24	ND	0.62	0.59	0.46	tr	18.96	0.54	10.21	0.40	0.30	1.74	4.82	49.65	11.04	1.08	ND	0.86	74.31	12.31	25.11	29.17	1.91
Strong aerobiosis (30°C)	0.25	ND	0.69	tr	0.23	0.40	15.15	0.67	26.25	0.27	0.24	1.79	7.12	41.72	4.02	0.99	3.38	0.81	75.18	5.01	26.83	41.41	1.58

[a] NI, Peak not identified. ND, Peak not detected; tr, <0.1%.

ence of yeast growth conditions not only on its yield and dough proofing activity but also on cell volume and roundness.

High and low protein levels have already been associated with freeze-thaw resistance in *Escherichia coli* and baker's yeast, respectively (6, 15). Under the conditions of the present study, crude protein (total Kjeldahl nitrogen) and phosphate (total phosphorus) contents of bakers' yeast were not correlated with cryotolerance. Overall protein content varied more than phosphate content under the growth conditions tested. Industrial yeast manufacturers associate low protein and high trehalose contents with low dough proofing activity (8). It was therefore expected that strong aerobiosis would give a slightly lower proofing activity but a higher biomass yield. It is also probable that lack of oxygen during yeast growth is beneficial for baking activity because its enzymatic pool is then better prepared for such anaerobic conditions encountered in doughs.

All batch-grown yeasts were relatively cryosensitive and contained less free sterols and phospholipids than fed-batch-grown cells. The same tendencies in the lipid contents of batch versus fed-batch cells were noted by Hunter and Rose (16), who suggested that less extensive intracellular or mitochondrial membrane development in batch-grown cells might be involved. The S/P ratios of batch-grown and fed-batch cells were also different. A high ratio would exert a considerable condensing effect at the membrane level, decreasing fluidity. A positive correlation between a low S/P ratio and a high resistance to freezing has been obtained with rye seedlings (28). The concept of a connection between the S/P ratio and cryoresistance has been questioned by de la Roche (9), and Thompson (26) cast some doubt about the role of membrane fluidity in the resistance of plants to freezing. On the other hand, increasing the cytoplasmic membrane stability of *E. coli* via a greater protein-to-phospholipid ratio has however been shown to have a cryoprotective effect during freezing at −10 and −30°C (24).

Some findings have linked cryotolerance to the fatty acid composition of yeast (7) or lactic acid bacteria (13) from batch experiments without aeration. However, Kirsop (19) could not differentiate cryotolerant yeasts (grown under agitation and described as aerobic) from cryosensitive ones (grown without agitation) according to their total fatty acid profile. Our results, which confirm these findings, show that the fatty acid profile of batch-grown *S. cerevisiae* would have no role in determining its cryoresistance. The results presented here also show that aeration intensity had little influence on the lipid profile of fed-batch-grown cells. In carbon-limited continuous cultures of *S. cerevisiae*, oxygen tensions greater than 3-mm Hg did not significantly alter cell composition or metabolism (5).

In batch-grown cells, cell volume increments confirm published data indicating that cell volumes are considerably less when cells are grown near anaerobiosis (11). Batch fermentations produced larger cells compared with fed-batch ones (all under stationary phase), and this effect was probably due to their higher growth rate (27, 29). Cell volume had little effect on yeast cryotolerance. Theoretically, one may assume that cell survival after freeze-thaw conditions is inversely related to water content, since smaller cells are likely to dehydrate sooner than large ones. This was not demonstrated by our findings, since cryosensitive cells were generally smaller than resistant cells.

Cell elongation following fed-batch growth under poor aeration or lower temperature conditions has not been reported elsewhere. Fed-batch growth of *S. cerevisiae* under

TABLE 4. Effect of growth conditions on bakers' yeast cell dimensions[a]

Growth conditions	Width (μm)		Length (μm)		Width/length ratio		Vol (μm³)	
	Small cells	Large cells	Small cells	Large cells	Small cells	Large cells	Small cells	Large cells
Fed-batch								
Strong aerobiosis (30°C)	5.09a	6.84a,b	5.51a	7.76a	0.93a	0.88a	75.64a	191.45a
Partial aerobiosis (30°C)	5.23a,b	6.46a	6.21b,c	8.67b	0.85b	0.75b	91.28a,b	191.52a
Strong aerobiosis (20°C)	5.19a,b	7.02b	6.67c	8.99b	0.78b	0.78b	95.68b	235.65a,b
Batch								
Microaerobiosis (30°C)	4.91a	6.42a	5.86a,b	8.74b	0.84b	0.74b	74.82a	192.24a
Strong aerobiosis (30°C)	5.58b	7.27b	7.09d	9.13b	0.79b	0.80b	117.08c	255.47b

[a] All values are means. Means within each column followed by the same letter (a to d) are not significantly different ($P < 0.05$), according to the Student t test for paired samples.

optimal fermentation conditions (intense aeration at 30°C) produced almost spherical cells (width/length ratio: range, 0.88 to 0.93). Under suboptimal conditions, cells developed higher specific volumes (surface/volume ratio), and in the case of inefficient oxygenation, this could be explained by an increase of the cell surface in response to a lack of dissolved oxygen in the growth medium (11). Yeast cell dimension characteristics are likely to be a very dynamic phenomenon, quite dependent on growth conditions but not related to cryotolerance.

In conclusion, the S/P and free sterol and phospholipid contents were correlated with yeast cryotolerance. The accumulation of reserve lipids (total cellular lipids or total sterols) was not involved in the resistance of yeast to freezing. For a given growth mode (batch or fed-batch), the intensity of aeration and lowering of growth temperature under fed-batch conditions did not significantly alter the lipid composition, in spite of their significant effect on cryotolerance. Under our experimental conditions, phosphate and crude protein levels could not explain yeast cryotolerance variations in response to growth temperature, aeration, and feeding regimen. Cell roundness and volume are dynamic characteristics of growing *S. cerevisiae* but constitute morphological responses to the environment without significant impact on yeast cryotolerance. Lipid analysis alone appears to be of limited value in screening cryotolerant industrial fed-batch-grown bakers' yeast, even though these analyses are important in yeast cryobiology studies.

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