Manufacturers of baker's yeast are in constant pursuit of strains with improved dough-raising properties and capacity to retain fermentative ability during storage at low temperature in order to meet baking industry requirements such as the increasing use of frozen doughs. Selected yeasts have been either isolated from natural sources (5, 6, 8), found in culture collections (18, 19), or genetically manipulated (12, 16). Traditional baker's yeast is susceptible to freeze damage during storage of doughs after fermentation, and the breadmaking potential of frozen doughs decreases substantially as the storage period becomes longer (10, 11, 14). The ability of the yeast to maintain high fermentative power after periods of storage at low temperature is affected by both technological and cellular parameters. Trehalose is considered an important physiological factor of yeast cryoresistance. This disaccharide has been related to resistance of yeast to several types of stress (3, 9, 13), and a high intracellular trehalose content is reportedly advantageous for a good freeze-thaw stability after prolonged storage of dough. Although strains of *Saccharomyces cerevisiae* are generally used in bread making, *Torulaspora* species have already been reported to be more cryoresistant, and therefore studies have been developed to select freeze-tolerant strains potentially applicable in the baking industry (7, 17, 18). However, often the selected strains do not combine all the main characteristics of a baker's yeast, namely, a strong maltose fermentation, causing low leavening abilities in lean doughs. In the present work, following an isolation program of yeasts from homemade corn and rye bread doughs, several strains of *S. cerevisiae* and *Torulaspora delbrueckii* were selected on the basis of maltose fermentation and high cell yield, characteristics desirable in baker's yeast (1). The influence of dough freezing and storage both on the leavening ability of the yeast cells and on their viability was tested, and the intracellular trehalose content of the yeast cells was determined in order to detect strains combining simultaneously freeze tolerance and high leavening ability. Furthermore, comparative studies of the strains isolated with commercially available baker's yeast were performed to select alternative strains with potential application in the baking industry.

## MATERIALS AND METHODS

**Yeast strains and culture media.** A total of 12 strains were used in this study. Eight were isolated from homemade corn and rye bread doughs (1) and designated *S. cerevisiae* IGC 5317, IGC 5318, IGC 5319, IGC 5320, and IGC 5324 and *T. delbrueckii* IGC 5321, IGC 5322, and IGC 5323; strains IGC 5325 and IGC 5326 of *S. cerevisiae* were isolated from two commercial compressed baker's yeast cakes; *S. cerevisiae* IGC 4072 and *T. delbrueckii* IGC 4478 were supplied by the Portuguese Yeast Culture Collection, Gulbenkian Institute of Science, Oeiras, Portugal. Stock cultures were maintained on glucose-yeast extract-peptone-agar slants. For determination of specific growth rates, yeasts were grown on YPS medium, pH 6.0, containing 2% (wt/vol) yeast extract, 4% (wt/vol) peptone, 2% (wt/vol) sucrose, 0.2% (wt/vol) KH2PO4, and 0.1% (wt/vol) MgSO4 z 7H2O, at 308C. Maximum biomass was determined for cells grown in YPS medium up to stationary phase and estimated as dry weight. For dry weight determinations, 2-ml samples of cell suspension were collected and filtered through preweighed and washed 0.45-mm-pore-size Schleicher & Schuell filters which were dried at 808C for 24 h before the estimation of dry weight. In order to confirm if all the sucrose had been consumed, a sample was centrifuged at 17,000 3 *g* for 2 to 3 min and the sucrose concentration was determined in the supernatant following the reaction with b-fructosidase with a Boehringer Mannheim GmbH Sucrose/D-Glucose UV method test kit.

**Dough preparation and determination of leavening ability.** For the determination of leavening ability, strains were grown in an orbital shaker (160 rpm) at 308C in 500-ml Erlenmeyer flasks containing 250 ml of YPS medium, pH 6.0. Cells were harvested at stationary phase by centrifugation at 48C, washed twice with ice-cold demineralized water, and filtered to obtain the yeast cake. Commercially available wheat flour was used for dough preparation. The following ingredients were mixed manually to their optimum development (;8 min): 100 g of flour, 2 g of salt, 2.8 g of yeast (wet weight), and 52 ml of water at 208C. The dough was immediately fermented for 3 h at 308C, and the leavening ability of the yeast was determined by recording the gas volumes (CO2) evolved from the

doughs in a Reofermentometer Chopin F2 (Tripette & Renaud, Villeneuve-la- Garenne, France), which allows the automatic reading of the gas produced by the fermenting dough as a function of time.

**Preparation of frozen dough.** For preparing the frozen dough, five times the quantity of the ingredients were mixed as described above and divided into five pieces, based on a 100-g flour weight. One of these pieces was used for the determination of leavening ability, and the others were molded by hand to 0.5-cm thickness, wrapped in aluminum foil, and stored at 22ºC for periods of 4, 8, 15, and 30 days. After the required periods, the frozen dough was left to thaw in a cabinet at 30ºC for 1 h, after which the dough was fermented and the leavening ability assessed as described previously. To test the effect of a fermentation period before freezing on the leavening capacity of the dough, bread doughs prepared by the formulation described above were prefermented at 30ºC for different periods of time (0.5, 1.5, an 2.5 h) and then stored in a freezer for 4, 8, and 15 days. After the required periods, the doughs were thawed and their leavening capacity was determined as described before.

**Viable cell counts.** Both unfrozen and frozen doughs were assayed for cell viability. The numbers of viable yeast cells were determined just before freezing and after thawing by the plate count method. For the yeast counts, 0.5 g of dough was suspended in 10 ml of sterile 0.1% (wt/vol) peptone water solution, mixed, and diluted as required. Triplicate samples of 0.1 ml were taken and spread onto plates containing 2% (wt/vol) glucose, 1% (wt/vol) Bacto Peptone, 0.5% (wt/vol) yeast extract, and 2% (wt/vol) agar, to obtain yeast counts ranging from 30 to 300 cells per plate, after incubation at 28ºC for 48 h. The colonies (CFU) were counted, and the mean was used to assess the survival ratio (percent) as follows: (CFU/100 g of dough after freezing period)/(CFU/100 g of unfrozen dough). In the case of doughs subjected to a fermentation before freezing, the survival ratio was defined as (CFU/100 g of dough after prefermentation period)/(CFU/100 g of dough frozen without prefermentation).

**Determination of intracellular trehalose content.** Cells were grown in YP medium at 308C for 24 h, harvested by centrifugation, and washed twice with cold demineralized water. The extraction of trehalose followed the procedure described by Hino et al. (7), with modifications. Trehalose was extracted from cells with 5% (wt/vol) trichloroacetic acid with occasional shaking for 45 min. Cells were then centrifuged at 1,000 3 *g* for 10 min. This procedure was repeated twice, and the supernatants were used for the determination of trehalose by high-pressure liquid chromatography. Prior to a 20-ml injection, the supernatants were filtered through 0.2-mm-pore-size sterilized Schleicher & Schuell filters. The apparatus used was a Gilson chromatograph with a Merck Polyspher OAKC (catalog no. 51270) column maintained at 858C, with ultrapure water as the mobile phase at a flow rate of 0.5 ml min21.

**Reproducibility of results.** All experiments were repeated at least twice, and some of them were repeated three or four times, the values obtained being very similar. Differences of up to 25 ml of CO2 per 3 h per 100 g of dough were not considered significant.

## RESULTS AND DISCUSSION

**General properties and leavening ability.** The wild strains used in this study, isolated from homemade corn and rye bread doughs, were the result of a first selection based on biomass production upon growth in YPS medium and maltose fermentation. Specific growth rates, biomass production, trehalose content, and leavening ability in lean dough (no addition of sugar) of the strains studied are summarized in Table 1. These characteristics are some of the properties required for a successful baker's yeast, and our term of comparison was the utilization of strains of commercial baker's yeast which, supposedly, possess values considered desirable for the baking industry. In a medium with sucrose at 30ºC, the values estimated for the specific growth rates varied between 0.51 and 0.70 h21. The highest values of biomass production and total protein content (not shown) were found in strains IGC 5321 and IGC 5323 of *T. delbrueckii*. Both presented an intracellular trehalose concentration higher than 20% (wt/wt), just like *S. cerevisiae* (IGC 5325 and IGC 5326), whereas the other strains contained from 5 to 18%. With respect to the leavening ability, half of the strains tested produced between 400 and 500 ml of CO2 per 3 h per 100 g of dough; in contrast, about one-third, including the collection strains, showed lower fermentative ability (values between 200 and 300 ml of CO2 per 3 h per 100 g of dough). Strains IGC 5318, IGC 5319, and IGC 5320 of *S. cerevisiae* and IGC 5321 and IGC 5323 of *T. delbrueckii* displayed high biomass production together with a good leavening ability in dough, with values close to the ones obtained with the industrial strains of *S. cerevisiae* (IGC 5325 and IGC 5326).

**Storage stability of frozen dough.** To test the influence of dough freezing and storage both on the fermentative capacity and on the viability of the yeast cells, the doughs were prepared and frozen at 2208C for 4, 8, 15, and 30 days, as described in Materials and Methods. After 4 days of frozen storage, gassing power decreased for most strains of *S. cerevisiae* tested (Table 1). Most strains of *T. delbrueckii* consistently showed high fermentativeability even after long periods of frozen storage: strains IGC 5321 and IGC 5323 presented a slightly weaker performance in freshly prepared dough, compared with commercial strain IGC 5325, but kept high values of CO2 production after long periods

of frozen storage. *S. cerevisiae* IGC 4072 and *T. delbrueckii* IGC 4478, although showing low values of CO2 production, seemed to be stable after the frozen storage periods, indicating tolerance to freezing (Table 1).

**Effects of prefermentation.** The effects of different prefermentation periods on the leavening ability and cell viability of selected strains were also studied. The doughs, prepared with the different yeast strains, were subjected to prefermentation periods of 30, 90, and 150 min before freezing and kept in frozen storage at 2208C for 4, 8, and 15 days. Two distinct behavior patterns were observed for strains of *T. delbrueckii* and *S. cerevisiae*. Strains IGC 5321 and IGC 5323 of the former species, although showing a slight decrease in their leavening abilities after 2.5 h of prefermentation, seemed not to be affected by fermentation before freezing as were the strains of *S. cerevisiae* (Fig. 1). The same strains of *T. delbrueckii* were subjected to further periods of freezing to test whether the storage period affected their CO2 production. Both strains kept a high leavening ability in doughs stored for 15 days and prefermented for 150 min (results not shown).

**Effects of freezing and prefermentation on cell viability.**

Freshly mixed doughs gave viable cell counts of similar magnitude for all the yeasts studied. Values ranged from 4 3 105 to 6 3 105 CFU/g of dough for *S. cerevisiae* and 6 3 105 to 8 3 105 CFU/g of dough for *T. delbrueckii*. After 4 days of frozen storage a remarkable decrease in survival ratio may be observed in all strains except strain IGC 5321 of *T. delbrueckii* (Fig. 2). For the subsequent periods of frozen storage (8, 15, and 30 days) the observed values stayed approximately the same in all the strains studied, indicating that cell viability was not so significantly affected by the length of the freezing period. Prefermentation also reduced the viability of yeasts, depending on the strains. We could observe a pronounced decrease in viable cells after 2.5 h of prefermentation mainly in *S. cerevisiae* IGC 5320 and *T. delbrueckii* IGC 5321 and IGC 5323 (Fig. 3). The same pattern was not followed by strains IGC 5318, IGC 5319, and IGC 5325 of *S. cerevisiae*, which kept a high survival ratio after that prefermentation period. The results indicate that survival ratio and leavening ability are not directly correlated, since strains IGC 5321 and IGC 5323 of *T. delbrueckii* displayed high values of CO2 production after 4 days of

frozen storage and 2.5 h of prefermentation (Fig. 1). In summary, with respect to freezing tolerance the leavening ability of all the strains of *S. cerevisiae* except IGC 4072 decreased considerably as the period of frozen storage increased. Similarly, in these strains, when the corresponding dough was subjected to a bulk fermentation before freezing, a negative effect on the yeast leavening ability was observed. Moreover, as shown in Fig. 1, the yeast fermentative ability decreased with the increase of prefermentation time, a reduction to about one quarter of the original value in fresh doughs being obtained in three of the strains after 2.5 h of prefermentation. Apparently, some strains were more sensitive to the freezing process itself than to storage, while others (IGC 5318 and IGC 5319) seemed to be more sensitive to long periods of storage than to freezing since they kept a good leavening ability for up to 8 days of storage, decreasing considerably after 15 days. In contrast, and as one would expect taking into account reports from other. authors (7, 17, 18), strains of *T. delbrueckii* (IGC 5321, IGC 5323, and IGC 4478) seemed to be the most tolerant to freezing since they displayed approximately the same leavening ability after 30 days of frozen storage. Although presenting a significant decrease in the survival ratio after prefermentation, two of those strains kept a good leavening ability in dough after 2.5 h of prefermentation and 15 days of storage.

**Trehalose content.** Intracellular trehalose values ranging from 5 to 29% (wt/wt) were found, the highest values corresponding to one industrial strain of *S. cerevisiae* (IGC 5325) and to strains IGC 5321 and IGC 5323 of *T. delbrueckii*, corresponding the highest values of protein found, also in these strains (Table 1). The trehalose content of commercial baker's yeast is generally believed to be a critical parameter for its resistance to stress (2, 20), and culture conditions have been optimized over the years in order to obtain a higher trehalose content. Trehalose levels of 15 to 20% of the dry weight are common, 10% being considered a critical threshold (4). Furthermore, protein content appears to be one of the factors that affects the leavening ability of the yeast, and a good yeast performance after freezing has been associated with a high protein content (10). Our results seem to indicate that other factors could play an important role in freeze tolerance, since the highest trehalose values found did not always correspond to the

highest tolerance (Table 1). Also, when freshly prepared doughs were subjected to a fermentation prior to freezing, a negative effect on the leavening ability was observed even in *S cerevisiae* IGC 5325, a strain with a high intracellular trehalose content. These results are apparently in accordance with those reported by other authors (4, 15), indicating that cryoresistance was directly correlated neither with the initial amount of trehalose in the yeast cell nor with the level still present in the dough at the time of freezing. Summarizing, we observed that of the strains isolated from traditional bread doughs, most showed high leavening ability in lean doughs, while collection strains IGC 4072 and IGC 4478 had low leavening ability compared with that of commercial baker's yeast. Furthermore, two strains of *T. delbrueckii*, IGC 5321 and IGC 5323, kept a strong capacity to produce CO2 under all conditions tested, showed high intracellular concentrations of trehalose and protein, and presented the highest tolerance to freezing. In conclusion, these properties make them candidates of potential value for the baking industry.

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FIGURES

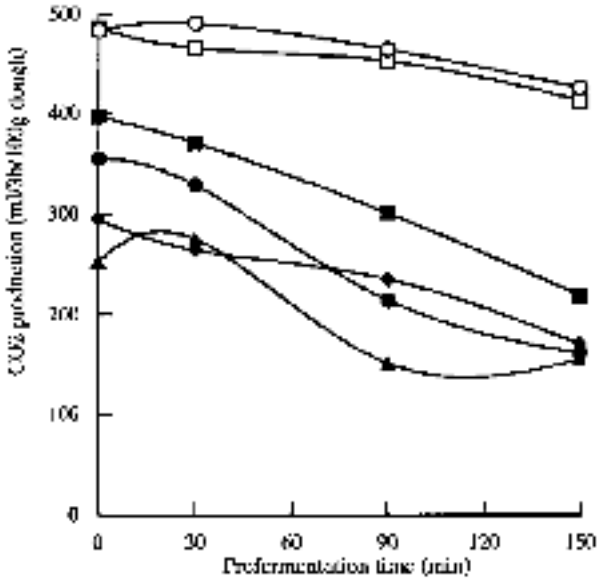


FIG. 1. Effect of prefermentation on $CO_2$ production by yeast strains in frozen dough stored at −20°C for 4 days. Strains: ■, *S. cerevisiae* IGC 5315; ●, *S. cerevisiae* IGC 5316; ▲, *S. pastorianus* IGC 5330; ◆, *S. cerevisiae* IGC 5329-1; *T. delbrueckii* IGC 3221; ○, *T. delbrueckii* IGC 3225.

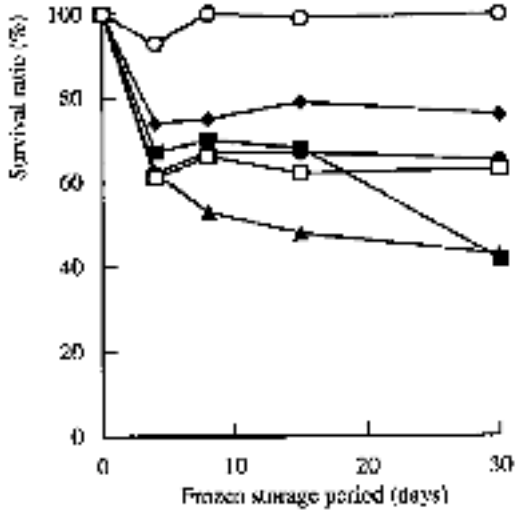


FIG. 2. Effect of the storage period on the yeast survival ratio in frozen dough stored at −20°C for up to 30 days without prefermentation. Strains: ■, *S. cerevisiae* IGC 5316; ●, *S. cerevisiae* IGC 5319; ▲, *S. cerevisiae* IGC 5322; ◆, *S. cerevisiae* IGC 5328; □, *T. delbrueckii* IGC 3221; ○, *T. delbrueckii* IGC 3225.

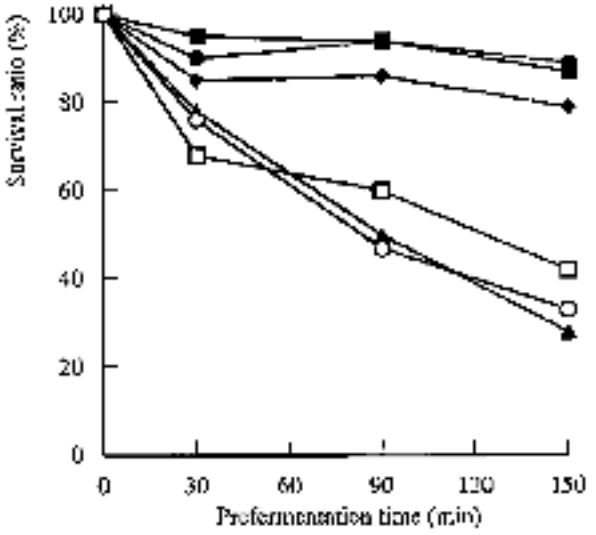


FIG. 3. Effects of different prefermentation periods on the yeast survival ratio in frozen dough stored at −20°C for 4 days. Strains: ■, *S. cerevisiae* IGC 5218; ●, *S. cerevisiae* IGC 5219; ▲, *S. cerevisiae* IGC 5325; ◆, *S. cerevisiae* IGC 5326; □, *S. delbrueckii* IGC 5321; ○, *S. delbrueckii* IGC 5323.

TABLES

TABLE 1. Growth characteristics, trehalose content, and leavening ability of selected yeasts grown in rich medium with 2% (w/vol) sucrose (YT5 medium)

| Yeast species (source[a]) | Specific growth rate ($h^{-1}$) | Final biomass (g/liter) | Trehalose content (mg/g [dry wt]) | Leavening ability | | | |
|---|---|---|---|---|---|---|---|
| | | | | Before freezing (ml of $CO_2$/3 h/100 g of dough) | After freezing (80% [v/v]) for: | | |
| | | | | | 4 days | 15 days | 30 days |
| *S. cerevisiae* IGC 5317 (BD) | 0.58 | 9.4 | ND[b] | 228 | ND | ND | ND |
| *S. cerevisiae* IGC 5318 (BD) | 0.57 | 10.6 | 102.0 | 414 | 96.9 | 41.6 | 20.5 |
| *S. cerevisiae* IGC 5319 (BD) | 0.61 | 11.9 | 173.7 | 402 | 88.5 | 47.3 | 38.6 |
| *S. cerevisiae* IGC 5320 (BD) | 0.64 | 10.0 | 51.9 | 429 | 59.0 | 46.0 | 44.1 |
| *S. cerevisiae* IGC 5324 (BD) | 0.62 | 10.0 | ND | 432 | ND | ND | ND |
| *T. delbrueckii* IGC 5321 (BD) | 0.51 | 12.6 | 259.4 | 427 | 113.8 | 75.4 | 76.0 |
| *T. delbrueckii* IGC 5326 (DD) | 0.62 | 10.2 | ND | 233 | ND | ND | ND |
| *T. delbrueckii* IGC 5323 (DD) | 0.53 | 13.2 | 227.5 | 369 | 124.1 | 139.3 | 108.5 |
| *S. cerevisiae* IGC 5325 (F) | 0.60 | 10.5 | 250.0 | 470 | 74.2 | 62.0 | 50.7 |
| *S. cerevisiae* IGC 5326 (F) | 0.70 | 11.6 | 206.1 | 447 | 66.0 | 46.5 | 47.7 |
| *S. cerevisiae* IGC 4072 (IGC) | 0.65 | 10.4 | 131.5 | 222 | 104.0 | 94.2 | 98.2 |
| *T. delbrueckii* IGC 4474 (IGC) | 0.53 | 16.3 | 166.7 | 270 | 104.9 | 96.6 | 106.3 |

[a] BD, bread dough; F, commercial compressed yeast; IGC, Portuguese Yeast Culture Collection, Gulbenkian Institute of Science, Oeiras, Portugal.
[b] Calculated on the basis of the leavening ability before freezing at −80°C.
[c] ND, not determined.