Freeze tolerance of the yeast *Torulaspora delbrueckii*: cellular and biochemical basis

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Abstract

The freeze-stress responses to prolonged storage at - 20 °C in *Torulaspora delbrueckii* PYCC5323 were investigated. In this yeast no loss of cell viability was observed for at least 120 days during freezing at - 20ºC, whereas a loss of 80% was observed in a commercial baker's yeast after 15 days. In the former strain, freeze resistance was dependent on an adaptation process. The primary cell target of freeze stress was the plasma membrane, preservation of it's integrity being related with a lower increase of lipid peroxidation and with higher resistance to H_2O_2, but not with intracellular trehalose concentration.

1. Introduction

Frozen-dough technology is well established in the baking industry, making it easier for bakers to supply oven-fresh bakery products to consumers and improving labour conditions. However, storage of frozen bread-dough may lead to the loss of baker's yeast cell viability as well as of its baking capacity, and consequently to economic losses. Thus, bread-making industry keeps a high demand for yeast strains with improved freeze resistance [1,2]. Most research on this field has focused on strains of *Saccharomyces cerevisiae*, which is the species currently used as baker's yeast. In this species, tolerance to freezing has generally been correlated with the intracellular trehalose concentration, but no direct correlation has been found above a threshold value [3-6]. Prior to the frozen storage, once the yeast cells are mixed with flour the fermentation takes place and a rapid loss of stress resistance occurs [7]. This has also been associated with the degradation of intracellular trehalose [8]. However, it has been shown that retention of high trehalose levels in fermenting cells does not prevent the loss of fermentation capacity during freezing, and that other factors - not yet identified - are required for the maintenance of freeze stress resistance [9, 10]. Accumulation of other solutes such as amino acids and glycerol, and expression of aquaporins were also reported to increase freezing resistance [11-15]. In addition, oxidative damage has been considered to be a factor underlying freeze-thaw damage, since an oxidative burst has been predicted to occur during thawing [16]. In agreement with this, Park et al. found that respiratory ability and functional mitochondria are necessary to confer full resistance to freeze-thaw stress [17]. It has also been shown that freeze tolerance is correlated with tolerance to H_2O_2, and free radicals were detected in *S. cerevisiae* after the freeze-thaw process [5, 18].

The strain PYCC5323 of *Torulaspora delbrueckii*, isolated from traditional corn and rye bread dough from the North of Portugal, besides presenting dough-raising capacity, growth rates and biomass yields similar to commercial baker's yeast, displays high freeze and osmotic tolerance [19-21].. Therefore this yeast emerges as a powerful candidate for the bread making industry, and the elucidation of such a peculiar behaviour reveals to be of great interest.

In this work the freeze-stress responses to prolonged storage at - 20 °C in *Torulaspora delbrueckii* PYCC5323 were investigated. The results obtained were compared to the ones of a commercial baker's strain of *S. cerevisiae.* The following cellular and biochemical parameters were analysed: cell

viability, plasma membrane integrity, oxidative damages, intracellular trehalose content and trehalase(s) activity.

2. Materials and methods

2.1. Microorganisms, growth and freezing conditions

The strains used were *Torulaspora delbrueckii* PYCC5323, isolated from homemade corn and rye bread dough, and *Saccharomyces cerevisiae* PYCC5325, isolated from commercial compressed baker's yeast - both supplied by the Portuguese Yeast Culture Collection, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal. Stock cultures were maintained on glucose-yeast extract-peptone-agar (2% (wt/vol) glucose, 1% (wt/vol) peptone, 0.5% (wt/vol) yeast extract, 2% (wt/vol) agar), at 4 ºC. Yeast strains were grown on yeast extract-peptone-sucrose (YPS) medium containing 2% (wt/vol) sucrose, 4% (wt/vol) peptone, 2% (wt/vol) yeast extract, 0.2% (wt/vol) KH_2PO_4, and 0.1% (wt/vol) $MgSO_4.7H_2O$, at 30 ºC. Peptone (ref. 0118-17) and yeast extract (ref. 0127-17) were from Difco (Becton Dickinson, Sparks, MD, USA) and sucrose from Merck (E. Merck, Darmstadt, Germany).

For freezing assays, cells were harvested at initial stationary phase (24 hours of culture, 2.4-2.7x10^8 cells ml^{-1} for *T. delbrueckii* and 1.1-1.5x10^8 cells ml^{-1} for *S. cerevisiae*), washed twice with deionised water, and suspended in a quarter of the initial volume in sterile water to an A_{640} of 12-20. Aliquots (5 ml) of cells were transferred into 15-ml polycarbonate tubes, centrifuged and the pellet (400-500 mg of wet weight) suspended in 500 μl of the storing medium: LF medium or as described in results. LF medium is a liquid medium formulated to simulate the fermenting ability of yeast in bread dough, with the following composition: 1% (wt/vol) glucose, 1% (wt/vol) sucrose, 3% (wt/vol) maltose, 0.25% (wt/vol) $(NH_4)_2SO_4$, 0.5% (wt/vol) urea, 1.6% (wt/vol) KH_2PO_4, 0.5% (wt/vol) $Na_2HPO_4.12\ H_2O$, 0.06% (wt/vol) $MgSO_4$, 22.5 ppm nicotinic acid, 5.0 ppm pantothenic acid, 2.5 ppm thiamine, 1.25 ppm pyridoxine, 1.0 ppm riboflavin and 0.5 ppm folic acid [22]. The samples were then frozen at -20 °C for different time periods (cooling rate approximately 3 °C min^{-1}) [5], and then thawed at 30 °C for 2 min. For fast freezing, 15-ml polycarbonate tubes containing the cell suspensions were directly immersed in liquid nitrogen (cooling rate approximately 200 °C min^{-1}) [5]. For pre-fermentation treatments, cells were subjected to a fermentation period before freezing as follows: the pellets in the 15-ml polycarbonate tubes were suspended in LF medium to a final $OD_{640\ nm}$ of 0.3-0.5, and incubated for

120 min, at 30 ºC. After this, the suspension was centrifuged and the pellet was suspended in LF medium and frozen as described above.

2.2. Extraction and assay of trehalose

Cells were harvested by centrifugation, washed twice with cold deionised water and sampled for dry weight contents. Trehalose was extracted from cold cell pellets with 5% (wt/vol) trichloroacetic acid (Merck, Darmstadt, Germany) for 45 min with occasional shaking. Cells were then centrifuged at 735 g, for 10 min. Extraction was repeated once more, and supernatants from the two extractions were combined and used for the determination of trehalose by high-performance liquid chromatography. The apparatus used was a Gilson chromatograph (132-RI Detector) equipped with a carbohydrate H^+ column (SS-100, H^+, Hypersil) which was maintained at 30 ºC. A solution of H_2SO_4 (0.0025 M) was used as the mobile phase at a flow rate of 0.45 ml min^{-1}. The relative values (%) of intracellular trehalose concentration after different periods of freezing were calculated by dividing the values of intracellular trehalose concentration obtained for the frozen samples, by those obtained for the unfrozen samples.

2.3. Trehalase activity and protein assay

Pellets containing 75-100 mg (wet wt) of washed cells were suspended in 1 ml ice-cold 50 μM MES (4-morpholineethanesulfonic acid) (Boehringer Mannheim, GmbH-Germany)/KOH buffer, pH 7, containing 50 μM $CaCl_2$. Cells were broken by vortexing with 500 μl of glass beads (0.5 mm diameter), for four periods of 1 min, with 1 min intervals on ice between them. The crude enzyme extract was centrifuged for 3 min at 13 200 g, at 4 ºC. The supernatant was dialysed overnight at 4 ºC against 10 mM Mes/KOH buffer, pH 7, containing 50 μM $CaCl_2$. Trehalase was assayed as described previously [23]. The glucose liberated was determined by glucose oxidase/peroxidase method (Glucose GOD – Perid, Boehringer Mannheim, GmbH-Germany). Protein determination was carried out according to Lowry et al. [24]. Specific activity of trehalase was expressed as units (U, nmol glucose released per min) per mg protein.

2.4. Measurement of cell viability and membrane integrity

The viability of yeast cells was determined by counting CFU. For this assay, yeast cell suspensions were washed twice with deionised water and, after convenient dilution, spread on YPDA

medium plates. The plates were incubated for 48 hours at 30 ºC before counting. The relative values (%) of viable cells after different periods of freezing were calculated by dividing the values of CFU counts obtained for the frozen samples, by those obtained for the unfrozen samples.

Membrane integrity was analysed by flow cytometry using the membrane exclusion dye, propidium iodide (PI). In these assays, cells with preserved membrane integrity are not permeated by propidium iodide (PI^- cells) while those that lost their membrane integrity do incorporate the fluorochrome (PI^+ cells) [25]. Cell suspensions (about 10^7 cells ml^{-1}) were incubated for 10 min in the dark with a 20 μg/ml PI solution (ratio of 2:1, respectively) and injected on a Partec PAS III flow cytometer equipped with an argon-ion laser emitting a 15 mW beam at 488 nm . From each sample $2x10^4$ cells were analysed. Control suspensions of membrane-disrupted cells were prepared by boiling cell suspensions. The relative values (%) of PI^- cells after different periods of freezing were calculated by dividing the values of PI^- cells obtained for the frozen samples, by those obtained for the unfrozen samples.

2.5. Oxidative stress evaluation

For evaluating the effect of pre-treatment with the radical scavenger N-tert-butyl-α-phenylnitrone (PBN) (Aldrich Chem. Co. Milwaukee, WI 53201), cells were grown for 24 hours to initial stationary phase in YPS medium at 30 °C. PBN was added to the culture at the final concentrations of 0.5, 5.0 and 15 mM, and cells were cultured for 30 min before being harvested.. PBN was removed by washing cells twice with deionised water and cells were frozen in LF medium as described above. In assays where a pre-fermentation was performed, PBN was added to the fermenting cell suspensions 30 min before the end of the fermentation period.

For treatment with oxidising agents, cells were grown to mid-exponential ($OD_{640\ nm}$ 0.5-1.0) and initial stationary phase (24 hours of culture), harvested and washed twice with deionised water. Subsequently, these cells were suspended in water in order to achieve a concentration of OD_{640nm} 0.6-0.7, and 0.1 ml of this suspension was spread on solid YPDA plates (50 mm diameter). A paper disc (6 mm diameter - BBL, 231039, Becton Dickinson, Sparks, MD, USA) containing 10 μl of one oxidising agent, was laid on each inoculated plate. The oxidants used were: menadione at concentrations of 0.05, 0.10, 1.0 mM; diamide at concentrations of 0.3, 3.0 M; hydrogen peroxide at 1.1 and 11 M, all

purchased from Sigma Chemical Co (St. Louis, MO). The plates were incubated at 26 ºC during two days, after which the diameters of the inhibition halos around the paper disks were measured. The value obtained was the average of two perpendicular diameters, excluding the disc diameter [26].

2.6. Thiobarbituric acid (TBA) reaction for lipid peroxide analysis

For TBA-reactive substances (TBARS) quantification, pellets containing 350-400 mg (wet weight) of cells were washed with ice-cold deionised water and suspended in 0.75 ml ice-cold sodium phosphate (E. Merck, Darmstadt, Germany) buffer, pH 7.2. Cells were broken by vortexing with 500 μl of glass beads (0.5 mm diameter) for six periods of 1 min with 1 min intervals on ice between them. TBARS were then determined according to Buege and Aust [27].The TBARS concentration was expressed as μmol malondialdehyde per mg protein.

2.7. Reproducibility of the results

All the experiments were repeated at least three times, and the data reported are mean values and SD. When statistical analyses were performed, the significance was tested by analysis of variance (Anova, Microsoft Excel 2000).

3. Results

3.1. Cell viability and membrane integrity along freeze storage

To characterise the freeze resistance of *T. delbrueckii* PYCC5323, we first studied cell viability, assessed as colony-forming units (CFU), during freezing at -20 ºC for up to 120 days as described in materials and methods (Fig. 1A). For a comparative analysis, a commercial baker's yeast, *S. cerevisiae* PYCC5325, was used as a reference strain (Fig. 1B). In *T. delbrueckii* no loss of cell viability was observed for the entire storage period, whereas a loss of 80% was obtained in *S. cerevisiae* after 15 days. In both species, viability loss was faster when cells were stored in freezing medium without sugars, even when glycerol was added to keep the osmotic pressure. These results are consistent with previous reports showing that the presence of the disaccharide trehalose in the extracellular medium has a protective effect in cell viability during freezing [28].

To elucidate whether the loss of membrane integrity along freezing was directly conditioning cell viability, membrane damage was monitored by flow cytometry using propidium iodide (PI) cell staining.

The decrease of cells with preserved plasma membrane (PI^- cells) was much less pronounced in *T. delbrueckii* (Fig. 2 A) than in *S. cerevisiae*. As shown in Fig. 2B, there was a direct correlation between the percentage of PI^- cells and the percentage of CFU counts for both *T. delbruekii* and *S. cerevisiae* yeast strains. This was evident either in cells subjected to a short fermentation period (120 min) or not subjected to fermentation before freezing. The results show that, independently of the physiological state of the cells, membrane integrity is directly conditioning cell viability, expressed as CFU counts, and therefore the plasma membrane seems to be one of the first freezing targets.

3.2. Cell oxidative stress responses during the freeze-thaw process

The results described above indicate that *T. delbrueckii* displays a higher freeze resistance when compared with *S. cerevisiae*, which is mainly due to plasma membrane integrity. To examine whether this capacity to preserve plasma membrane integrity was correlated with oxidative stress resistance, we directly assessed oxidative damage in the membranes of both *T. delbrueckii* and *S. cerevisiae* during frozen storage by measuring TBA-reactive substances (TBARS). The TBARS test quantifies lipid peroxides in the thiobarbituric acid derivatized form. As shown in Table 1, for both yeast strains, frozen cells presented higher amounts of products of lipid peroxidation than control cells (cells before freezing). However, the percentage increase in TBARS levels was significantly enhanced ($P < 0.05$) much earlier in *S. cerevisiae* (approximately 61% for the fifth day), which was associated to its rapid decrease in cell membrane integrity. As shown in the previous section, the presence of glycerol did not protect cells of *T. delbrueckii* during freezing. Therefore we tested *T. delbrueckii* under these conditions to avaliate the increase in the percentage of TBARS. The results showed that the values of percentage increase of TBARS in *T. delbrueckii* frozen in a medium with glycerol, for 60 and 84 days, were similar to those observed for *S. cerevisiae* (respectively 64% and 83%). Moreover, samples of *T. delbrueckii* frozen under conditions where no loss of cell viability was observed, presented much lower percentage increase in TBARS production (Fig. 1, Table 1).

The role of oxidative stress during freezing, was examined by using the oxygen radical scavenger PBN. In *S. cerevisiae* PYCC5325, pre-incubation of cells with PBN (1 or 5 mM) resulted in higher levels of membrane integrity preservation for the first days of frozen storage, but no protective effect by PBN was observed in cells frozen after a period of pre-fermentation (results not shown). This result might reflect the different physiological status of the cells and it is conceivable that oxidative stress is

being carried over by other factors [17]. Next, the oxidative stress responses of both strains to hydrogen peroxide (H_2O_2, toxicity mainly due to hydroxyl radicals), menadione (a superoxide-generating agent) and diamide (thiol-oxidizing drug) were evaluated. The results obtained in a disk diffusion assay are shown in Fig. 3. *T. delbrueckii* was more sensitive to menadione ($P < 0.001$) and diamide ($P < 0.05$) while more tolerant to H_2O_2 ($P < 0.01$) when compared with *S. cerevisiae*. Therefore, like it was previously reported for *S. cerevisiae*, in *T. delbrueckii* freeze resistance is also correlated with H_2O_2 resistance [5].

3.3. Intracellular trehalose content during the storage period

The results described above pointed to the possibility that oxidative stress was not the only condition influencing the cell freeze resistance, mainly in cells frozen after a short period of pre-fermentation. Intracellular trehalose accumulation has been described to protect cells from oxygen radicals and also to be involved in the stabilisation of the plasma membrane structure during freezing [29-31]. Hence, it was also investigated whether trehalose was involved on the higher capacity of *T. delbrueckii* to maintain plasma membrane integrity compared to *S. cerevisiae*. Immediately before freezing, the values of the intracellular trehalose content were high and similar in both species: 109.34 ± 8.72 and 112.79 ± 20.97 mg g^{-1} dry wt for *S. cerevisiae* and *T. delbrueckii*, respectively. During the entire freezing storage period, these values were kept high and constant in *T. delbrueckii* but decreased quickly in *S. cerevisiae*. For both species the relative values of the intracellular trehalose content during freezing followed closely the percentage of PI^- cells (Fig. 4). In *S. cerevisiae*, the decrease in the intracellular trehalose content was accompanied by an increase of the extracellular trehalose content and of the medium OD_{260nm} (used as a measure of leakage of cell contents) (Fig.4B). At the end of the assay, the amount of trehalose found in the media was about the same as the one lost from the cells, indicating that total trehalose amount (intracellular + extracellular) remained unchanged. Thus, cell leakage appears to be responsible for the decrease in trehalose content of the cells, which is consistent with the observed loss of plasma membrane integrity. In addition, a decrease in the intracellular trehalose content was observed in cells that were subjected to a short fermentation period before freezing (cells more sensitive to freeze stress), which was similar for both strains (from 109.34 ± 8.72 to 9.79 ± 0.93 mg g^{-1} dry wt for *S. cerevisiae* and from 112.79 ± 20.97 to 17.16 ± 6.24 mg g^{-1} dry wt for *T. delbrueckii,* during a 120 min fermentation period).

These results seemed to indicate that a similar activation pattern of trehalase(s) by glucose was present in both yeast strains. To address this point, trehalase(s) activities were monitored in cell free extracts prepared from cells in the absence of glucose (stationary phase cells) as well as after a glucose pulse. A similar behaviour was presented by both strains with a two to three-fold increase in activity almost immediately after glucose addition (from 15.0 ± 0.4 to 32.1 ± 1.3 mU/mg protein for *S. cerevisiae* and from 11.1 ± 0.2 to 31.0 ± 0.7 mU/mg protein for *T. delbrueckii,* respectively before and after glucose addition). The results are in agreement with the mobilisation of trehalose observed during the pre-fermentation period.

The results described above are consistent with the conclusion that the higher freeze resistance displayed by *T. delbrueckii* can not be attributed to higher intracellular trehalose contents.

3.4. Cell adaptation during the freeze period

We evaluated the ability of *T. delbrueckii* and *S. cerevisiae* to adapt to freezing by inhibiting protein synthesis with cycloheximide. The results obtained when cells were frozen at – 20 °C, showed that for *T. delbrueckii*, contrasting with *S. cerevisiae*, the presence of cycloheximide in the freezing medium increased the loss of cell viability throughout frozen storage (Fig. 1). To assess if cycloheximide could have a toxic effect in *T. delbrueckii* cells, being responsible for the observed decrease in cell viability, a cell suspension of this yeast was incubated in LF medium with cycloheximide, and the number of CFU counts was determined without previous freezing the suspension. No differences were found between the number of CFU counts estimated for this cell suspension and for the control without cycloheximide. These results indicated that at the concentration tested, cycloheximide was not having a toxic effect. In addition, when cells of *T. delbrueckii* were frozen at a much faster rate, in liquid nitrogen (-196 °C), and subsequent storage at -80°C, loss of cell viability rapidly increased, CFU counts dropping aproximatly 50% after one day of frozen storage. Together these results suggest that cell viability of *T. delbrueckii* is dependent on *de novo* protein synthesis, and therefore that yeast cells can adapt in a slow freezing process, most probably during the initial cooling period. In accordance with these findings, the capacity of *T. delbrueckii* cells to adapt might be essential for the yeast high freeze tolerance phenotype.

4. Discussion

Our findings clearly indicate that the strain *T. delbrueckii* PYCC5323 exhibits a high freeze tolerance, when compared with the baker's yeast *S. cerevisiae*, which reinforces previous reports claiming for its useful exploitation in baking industry. Evidence was presented that the primary cell target of freezing stress is the plasma membrane and that the capacity to preserve membrane integrity displayed by *T. delbrueckii* PYCC5323 is correlated with higher resistance to lipid oxidative damage. Hydroxyl radicals appear to be the agents responsible for cell membrane damage in freeze stress in this yeast. This is the opposite of a previous report on *S. cerevisiae* where superoxide radicals were considered as the agents responsible for cell damage in these stress conditions [18]. To ascertain the key role of these radicals during freezing we are developing a catalase null mutant in *T. delbrueckii* PYCC5323. Loss of cell viability seems to be correlated with the percentage increase in TBARS levels and not with their absolute values, suggesting that a higher content in unsaturated fatty acids could allow the cell to cope with higher absolute levels of lipid oxidation without compromising membrane integrity. This hypothesis would also agree with previous work, showing that freeze-tolerant yeast strains have larger amounts of unsaturated fatty acids when compared with sensitive strains [32]. Previous studies have pointed to a lack of capacity in *S. cerevisiae* to adapt to cold stress [17], although evidence for cold-induced expression changes associated with improved cryoresistance has also been provided more recently [33, 34]. According to our results, in *T. delbrueckii*, contrasting with *S. cerevisiae*, the surviving capacity (evaluated by cell viability) is dependent on *de novo* protein synthesis. An adaptation process during slow freezing appears to be determinant for the yeast high freeze tolerance phenotype. In the light of these observations, the yeast response to freeze stress seems to be strongly dependent on the yeast strain, culture and freeze conditions. However, further studies will be necessary to clarify the molecular basis of cell adaptation to cold/freeze. In the case of the strain *T. delbrueckii* PYCC5323, the observed behaviour is particularly relevant in view to its utilisation for frozen dough production, and implies that the dough should be frozen at a slow, rather than at a fast rate.

In addition and noteworthy from a methodological point of view, the results regarding the correlation observed along freezing between the loss of membrane integrity and cell proliferative capacity, validate the application of flow cytometry and the use of the fluorochrome PI as a measure of viability of cells subjected to freeze stress. Therefore, and contrary to other stress conditions [25], the

assessment of PI^- cells by flow cytometry, as a method to determine cell viability either in *T. delbrueckii* or *S. cerevisiae*, can replace the more laborious and time consuming determination of CFU counts.

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Fig. 1. Relative values (%) of colony forming units (CFU) in stationary growth phase cells of *T. delbrueckii* (A), and *S. cerevisiae* (B) during frozen storage at –20 ºC, for 120 and 30 days, respectively. Cells were grown in YPS medium during 24 hours at 30 ºC, harvested, washed with water and suspended in different storage media: (□) LF medium; (●) LF medium + 0.01% cycloheximide; (❍) LF medium without sugars + 0.16 M glycerol; (∆) Sugars solution (1% glucose + 1% sucrose + 3% maltose).

Fig. 2. Relative values (%) of negative propidium iodide cells (PI^- cells) of *T. delbrueckii* (close symbols) and *S. cerevisiae* (open symbols) during storage at -20 ºC (A) and correlation between relative values (%) of PI^- cells and colony forming units (CFU) (B). Cells grown in YPS medium during 24 hours at 30 ºC (initial stationary growth phase), harvested, washed with water and suspended in LF medium were frozen directly (▲, ∆) or after a pre-fermentation period of 120 min (●, ❍), as described in material methods. In Fig. 2A, for some results error bars are within the data point labels.

Fig. 3. Effect of oxidising agents on the radial growth of *T. delbrueckii* (black bars) and *S. cerevisiae* (open bars). Cells were grown in YPS medium at 30 ºC, to mid-exponential growth phase (B) or to initial stationary growth phase (A), and 0.1 ml of a suspension ($OD_{640\ nm}$ 0.6-0.7) of these cells was spread on YPDA plates. A paper disc, 6 mm diameter, containing 10 μl of one oxidising agent was laid on each inoculated plate. The plates were incubated during two days, after which the diameters of the inhibition halos were measured. The values shown are those obtained for the highest concentration of oxidant tested.

Fig. 4. Cells of *T. delbrueckii* (A) and *S. cerevisiae* (B) were grown in YPS medium during 24 hours at 30 ºC, harvested, washed with water, suspended LF media and frozen at -20 °C. During freezing samples were taken and assessed for: (❍) relative values (%) of negative propidium iodide cells (PI^- cells); (▲) relative values (%) of intracellular trehalose concentration (T_{in}); (□) extracellular trehalose concentration (T_{out}, g/l); (●) medium optical density at 260 nm, used as a measure of leakage of cell contents ($OD_{260\ nm}$) The relative values (%) were estimated as described in material and methods.