Appl Microbiol Biotechnol (2000) 54: 575–580

ORIGINAL PAPER

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Commercial baker's yeast stability as affected by intracellular content of trehalose, dehydration procedure and the physical properties of external matrices

Received: 6 December 1999 / Received revision: 8 May 2000 / Accepted: 19 May 2000

Abstract The effects of vacuum-drying and freeze-drying on the cell viability of a commercial baker's yeast, *Saccharomyces cerevisiae*, strain with different endogenous contents of trehalose were analyzed. An osmotolerant *Zygosaccharomyces rouxii* strain was used for comparative purposes. Higher viability values were observed in cells after vacuum-drying than after freeze-drying. Internal concentrations of trehalose in the range 10–20% protected cells in both dehydration processes. Endogenous trehalose concentrations did not affect the water sorption isotherm nor the T_g values. The effect of external matrices of trehalose and maltodextrin was also studied. The addition of external trehalose improved the survival of *S. cerevisiae* cells containing 5% internal trehalose during dehydration. Maltodextrin (1.8 kDa) failed to protect vacuum-dried samples at 40 °C. The major reduction in the viability during the freeze-drying process of the sensitive yeast cells studied was attributed to the freezing step. The suggested protective mechanisms for each particular system are vitrification and the specific interactions of trehalose with membranes and/or proteins. The failure of maltodextrins to protect cells was attributed to the fact that none of the suggested mechanisms of protection could operate in these systems.

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Introduction

It is known that intracellular trehalose exerts a protective effect on yeasts under extreme environmental conditions such as desiccation, freezing, osmotic stress and heat shock (Hottiger et al. 1989; Van Laere 1989; Wiemkem 1990; Van Dijck et al. 1995; Hounsa et al. 1998) and also that it provides thermal stability to the cells (Attfield et al. 1992). The molecular mechanisms that relate the trehalose content of yeasts with cellular resistance against stresses are not exactly known. Nevertheless, the protective effects are linked to the stabilization of membranes and the preservation of enzyme activity (Crowe et al. 1984, 1987).

Viability and thermal stability of sensitive strains to dehydration could be improved by the addition of certain amounts of disaccharides (trehalose, maltose) to the suspending media in which the cells are to be freeze-dried (Mugnier and Jung 1985; Gadd et al. 1987; Tan et al. 1995). Trehalose, like other polyols, could act by replacing water molecules involved in the maintenance of the tertiary structure of proteins through multiple external hydrogen bonds (Arakawa and Timasheff 1982; Crowe et al. 1987, 1993, 1996, 1998; Fernández et al. 1995). The hydrogen-bonding capacity of compounds utilized as protective agents for phospholipids and membrane proteins and the accessibility of the protective substance to the interior of the cells could be critical factors for determining the survival of cells submitted to different treatments (Sun and Leopold 1997). The ability of trehalose to form glassy structures, which assure physical stability, has also been reported as an hypothesis to explain its protective effect (Slade and Levine 1991; Crowe et al. 1993; de Araujo 1996), but special interactions at a molecular level have to operate (Lodato et al. 1999). The presence of disaccharides during freeze-drying, their concentration and the moisture content of the system are also critical factors which affect the survival of the cells.

From an industrial point of view, there are increasing needs to predict and control the stability of dried yeast,

because its consumption is constantly growing due to the convenience of its storage, transport and shelf life in comparison with wet yeast. Moreover, the use of certain yeast-containing products, such as frozen dough, is constantly increasing (Attfield 1997).

In this study we have examined the survival of baker's yeast after submitting the cells to different stressful conditions: freezing, freeze-drying and vacuum-drying at 40 °C. The critical aspects analyzed were: (1) internal trehalose threshold level necessary for cell protection and its effect on the thermophysical properties of the samples and (2) the effect of external matrices of trehalose in making up for low intracellular endogenous levels of the disaccharide. A *Zygosaccharomyces rouxii* strain was also studied for comparative purposes.

Materials and methods

Microorganisms and inocula

A commercial strain of baker's yeast, *Saccharomyces cerevisiae* (Culture Collection BAFC 1066), was used throughout the main part of this study. This strain was cultured under fed-batch protocols conducive to the accumulation of different internal trehalose levels (Tivedi 1986; Evans 1990). *S. cerevisiae* (10 g of 28–30% w/w dry matter) was suspended in 50 ml of distilled water and used as inoculum. A strain of *Z. rouxii* NRRL Y 229, grown in Sabouraud broth for 48 h at 27 °C, was also employed.

Matrices

The following compounds were tested for their protective effect: trehalose (Sigma, St. Louis, Mo.) and maltodextrin (average molar mass 1.8 kDa; Grain Processing Corporation, Muscatine, Iowa). Solutions (40% w/v) of the mentioned compounds in distilled water were sterilized at 121 °C for 15 min.

Samples without matrices

Aliquots of inoculum of *S. cerevisiae* or *Z. rouxii* were mixed with equal volumes of distilled water and distributed in sterile vials or plates according to the dehydration procedure.

Samples with matrices

Inocula of *S. cerevisiae* containing different levels of internal trehalose or *Z. rouxii* were mixed with equal volumes of double-concentrated matrices. For systems to be freeze-dried, aliquots of 0.5 ml were distributed in sterile glass vials (2 ml capacity). For systems dehydrated under vacuum, samples were distributed in sterile flat plates (2 cm internal diameter, 1 ml capacity).

Drying

Drying of the samples was carried out by two methods: (1) freeze-drying for 24 h in a Stokes freezer-drier, model 21 (F.J. Stokes Co., Pennsylvania, Pa.), at a condenser plate temperature of −40 °C, and a chamber pressure of less than 13 Pa, with samples frozen at −26 °C prior to freeze-drying, using a cooling rate adequate for yeasts (Berny and Hennebert 1991), and (2) drying in a vacuum oven at 40 °C, in the presence of a desiccant ($MgClO_4$), for 48 h.

Adjustment and measurement of moisture

Samples were exposed at different relative humidities (RH) in atmospheres of the following saturated salts: LiCl, KCH_3COO, $MgCl_2$ or NaBr, giving RH 11%, 22%, 33% or 58%, respectively. $Mg(ClO_4)_2$ was used for RH close to 'zero'. Moisture content was determined by duplicate sampling after drying at 90 °C in a vacuum oven for 48 h.

Viability determinations

Viability values were determined as previously described (Lodato et al. 1999) and were expressed as colony-forming units/ml. The estimated error in viability counting was less than half an order of magnitude.

Heat treatment

Humidified samples in hermetically sealed vials were heated to 70 °C for 2 h or 4 h in a forced-air convection oven. After heating, at least two samples were removed from the oven for viability counts.

Glass transition temperatures

The glass transition temperature (T_g) of each system studied was measured by differential scanning calorimetry (Mettler DSC30, TA4000, graphware TA72), as the onset of the endothermal baseline shift. Hermetic aluminum pans were employed and an empty aluminum pan was used as reference; and the samples were scanned twice at a heating rate of 10 °C/min. The instrument was calibrated with indium.

Extraction and assay of trehalose

Intracellular trehalose concentration was determined according to the procedure of Attfield (1987). Trehalose in cell-free extracts was determined using the anthrone assay as described by Lillie and Pringle (1980) with trehalose (1 mg/ml) as standard. A standard curve was run in each experiment. The trehalose concentrations reported represent the average of, at least, three determinations and were expressed as % w/w of dry matter. The intracellular concentrations determined were: 4.7, 10.2 and 19.5 ± 1.0% for *S. cerevisiae* samples which, for practical purposes, are reported as 5%, 10% and 20% trehalose in this study. The trehalose content for *Z. rouxii* was 3.5 ± 1.0%.

Results

Figures 1 and 2 (dotted lines) show the survival of baker's yeast cells with three different internal trehalose content (5, 10 and 20%), either after vacuum-drying at 40 °C (Fig. 1), or after freeze-drying (Fig. 2). For a given system, lower losses of viability were observed when cells were vacuum-dried (Fig. 1) than when they were freeze-dried (Fig. 2). Figs. 1 and 2 also show that a higher viability loss was observed in both dehydration procedures in the yeasts containing 5% trehalose, as compared with the cells containing 10% or 20% trehalose. In cells containing 5% internal trehalose, viability decreased by three or four logarithmic cycles after vacuum-drying or freeze-drying, respectively. The effect of thermal treatment at 70 °C of the rehumidified samples

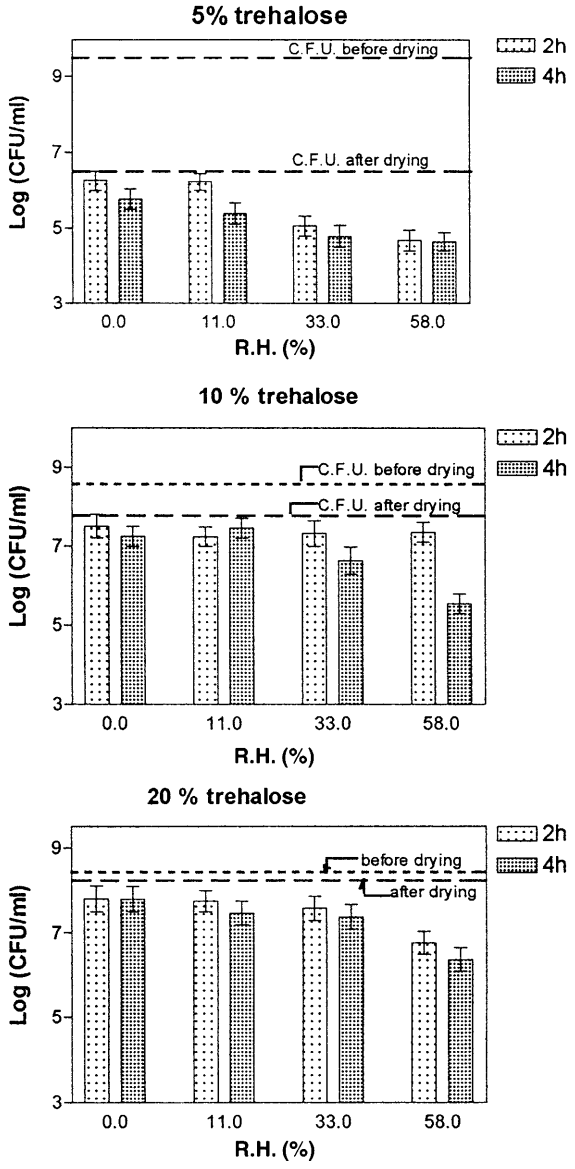

Fig. 1 Viability of baker's yeast *Saccharomyces cerevisiae* containing different intracellular levels of trehalose after vacuum-drying at 40 °C (*dotted lines*) and after heat treatment of the rehumidified samples at 70 °C for 2 h or 4 h (*bars*). *C.F.U.* and *CFU* colony-forming units, *RH* relative humidity to which samples were exposed before heat treatment

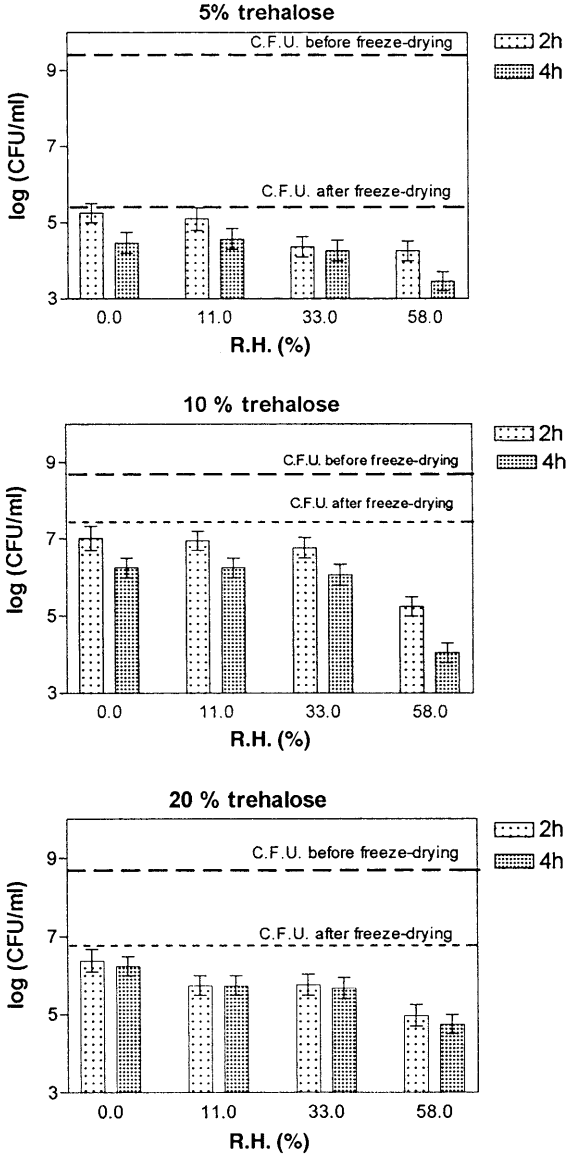

Fig. 2 Viability of baker's yeast, *S. cerevisiae*, containing different intracellular trehalose levels after freeze-drying (*dotted lines*), and after heat treatment of the rehumidified samples at 70 °C for 2 h or 4 h (*bars*). *C.F.U.* and *CFU* colony-forming units, *RH* relative humidity to which the samples were exposed before heat treatment

at different relative humidities is also shown in Figs. 1 and 2 (bars). It is to be noted that for all the samples with an internal content of 5% and for most of the 10% and 20% trehalose samples, viability loss during the drying treatment (either vacuum-drying or freeze-drying) was higher than that occurring during thermal treatment under the selected conditions (4 h at 70 °C). The T_g of the systems with 5% or 20% internal trehalose, determined at the different RH values, together with the water sorption isotherms, are shown in Fig. 3a, b. The amount of internal trehalose did not affect the water sorption isotherm nor the T_g values of the samples. Since the T_g of each system was lower than 70 °C (Fig. 3a), all the samples were in a theoretically unstable supercooled-liquid state during thermal treatments (2 h and 4 h) at this temperature. However, the vacuum-dried cells with 10% or 20% trehalose retained a high viability upon heating, which only decreased considerably at 58% RH (corresponding to moisture contents higher than 8%). At this RH value, the freeze-dried samples were also more susceptible to thermal stress than the vacuum-dried ones.

The ability of either a sugar matrix (trehalose) or a polymeric matrix (maltodextrin of average molar mass 1.8 kDa) to protect baker's yeast during the dehydration procedure was investigated in relation to the physical properties of these materials. As shown in Fig. 4a, b, the

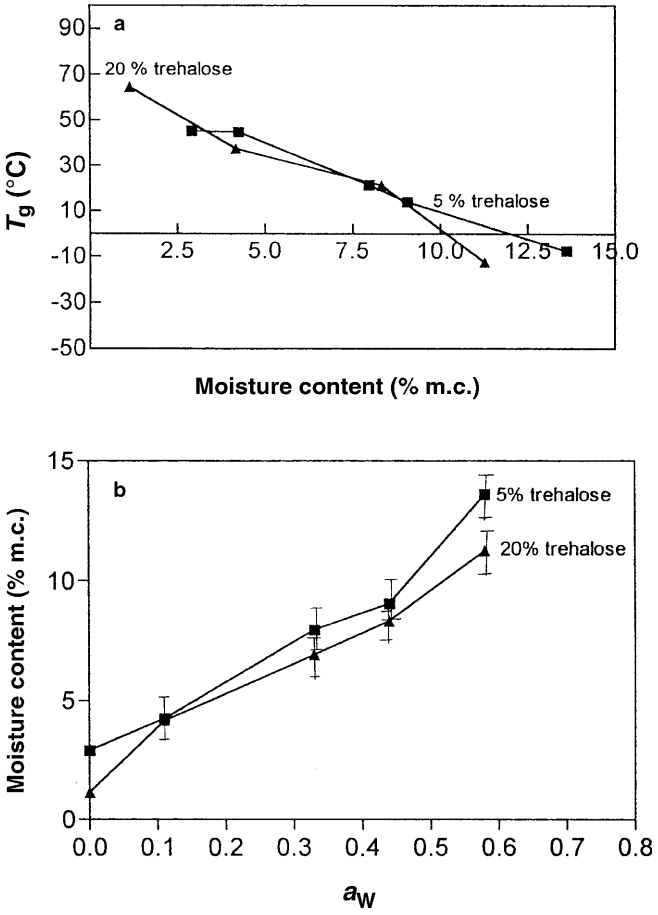

Fig. 3 Glass transition temperature (T_g; **a**) and sorption water isotherm (a_w; **b**) of freeze-dried baker's yeast with 5% and 20% internal trehalose content. *m.c.* Moisture content, dry weight basis

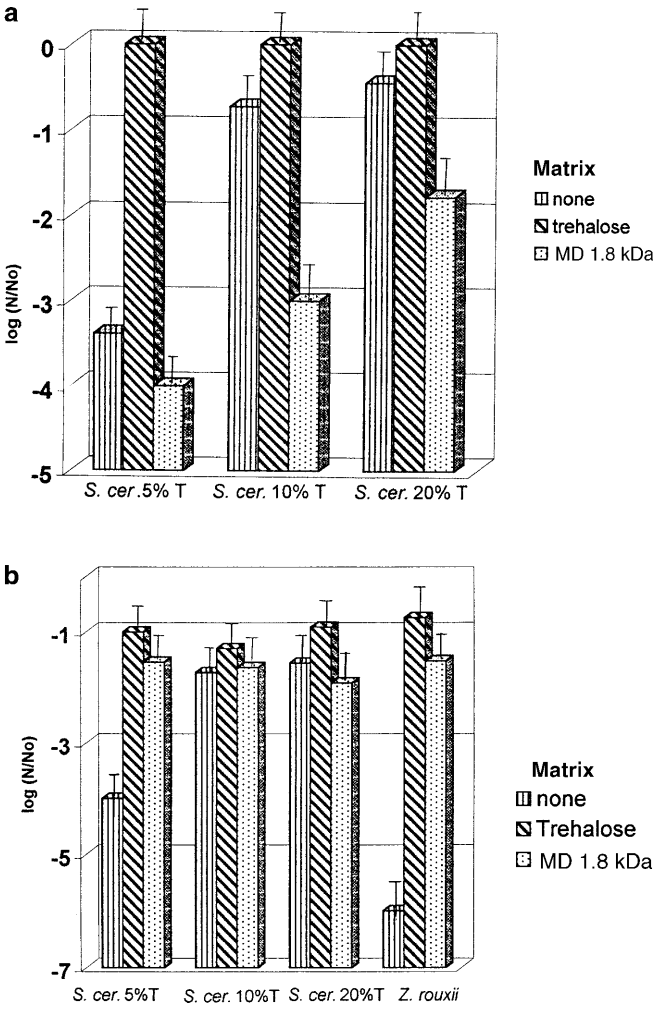

Fig. 4 Effect of intracellular trehalose content and external matrix on the survival of a strain of baker's yeast, *S. cerevisiae* after (**a**) vacuum-drying at 40 °C or (**b**) freeze-drying. *Zygosaccharomyces rouxii* (internal trehalose content = 3.5%) is included as comparison. *MD 1.8 kDa* Maltodextrin of average molecular mass 1.8 kDa. N/N_0 ratio between CFU after and before the drying processes, *S. cer. S. cerevisiae*, *T* trehalose

addition of external trehalose improved the survival of the more sensitive cells with 5% trehalose in both drying procedures (Fig 4a: vacuum-drying, b: freeze-drying). In contrast, the maltodextrin matrix failed to provide protective effect to the vacuum-dried samples at 40 °C (and even impaired the viability of the vacuum-dried cells), when compared with the control samples without matrices. Table 1 shows the moisture content of *S. cerevisiae* samples with 5% internal trehalose, after each dehydration procedure, together with the corresponding T_g values. The T_g values for trehalose and maltodextrin matrix systems were in accord with the expected values for the corresponding moisture contents (Roos and Karel 1991; Cardona et al. 1997; Miller et al. 1997). Although all samples were in the glassy state at room temperature after drying, which was coincident with their brittle physical appearance, they remained as solutions (of concentrations of 20% and above) during the first hours of the vacuum-drying process at 40 °C. Figure 4b shows that the presence of external trehalose or maltodextrin (of 1.8 kDa) greatly enhanced the survival of baker's yeast submitted to freeze-drying. In particular, for the samples containing 5% internal trehalose, the external matrices compensated for this effect. The results with *Z. rouxii* (with a trehalose content of 3.5%), which was demonstrated to be very sensitive to freeze-drying, was included in Fig. 4b to reinforce the manifest positive influence of the external matrix in the freeze-drying process.

Table 1 Glass transition temperature (T_g) and moisture content (*m.c.*) of the different systems after drying *Saccharomyces cerevisiae* cells with 5% endogenous trehalose. *T* Trehalose, *MD 1.8 kDa* maltodextrin with average molar mass of 1.8 kDa

Drying method	Matrix	m.c. ± 0.5 (%)	T_g ± 4 (°C)
Vacuum-drying	none	3	43
	T	3	36
	MD 1.8 kDa	2	87
Freeze-drying	none	2.5	45
	T	2	38
	MD 1.8 kDa	3	92

Table 2 Viability losses of strains with low trehalose content (*S. cerevisiae* and *Zygosaccharomyces rouxii*) after freezing and freeze-drying treatments. N/N_0 (CFU/ml after each treatment)/(initial CFU/ml)

Strain	Matrix	N/N_0	
		After freezing	After freeze-drying
S. cerevisiae[a]	–	10^{-3}	10^{-4}
	Trehalose	0.1	0.1
	MD 1.8 kDa	0.1	6×10^{-2}
Z. rouxii[b]	–	10^{-4}	10^{-6}
	Trehalose	0.2	0.1
	MD 1.8 kDa	8×10^{-2}	5×10^{-2}

[a] 5% of endogenous trehalose
[b] 3.5% of endogenous trehalose

Table 2 shows the remaining viability after the different steps involved in the freeze-drying process. The highest reduction of viability after freeze-drying for the most sensitive yeasts studied (*Z. rouxii* and *S. cerevisiae* with internal 5% trehalose, both grown under non-stress conditions) was due to the freezing step.

Discussion

Drying and freeze-drying, two useful industrial treatments for yeast cell preservation, may result in a decrease in viability. In this work we observed a lower loss of viability in vacuum-dried samples of *S. cerevisiae* cells (Fig. 1), as compared with freeze-dried cells (Fig. 2). These results could be explained by the fact that, during freezing/freeze-drying, the cells did not have the opportunity to compensate for the osmotic stress produced by this procedure, this reaccommodation only being possible during drying at 40 °C. Cell viability after drying was dramatically dependent on the internal content of trehalose, as was demonstrated for samples with endogenous trehalose levels of 5%. The results obtained in this study showed that trehalose, endogenous or externally added, provided protective effects on dehydrated baker's yeast cells. de Araujo et al. (1996) and Diniz-Mendes et al. (1999) pointed out that to provide a protective effect to cells during freeze-drying, trehalose has to be present on both sides of the cell membrane, and evidence has been presented that yeast cells have a constitutive trehalose carrier through the plasma membrane (de Araujo et al. 1996). *Z. rouxii* showed to be extremely sensitive to freeze-drying, despite its reported osmotolerance; and the presence of external trehalose during freeze-drying increased its survival.

The present results demonstrate for the first time that the internal amount of trehalose, which dramatically affected cell viability, had only a minor effect on the T_g of the samples. Consequently, the physical properties of the systems (i.e. vitrification) are not sufficient to explain per se the higher viability values obtained in samples with 10% and 20% trehalose content. Maltodextrin matrix provided protection during freeze-drying but not during vacuum-drying at 40 °C. The lack of protection to the vacuum-dried samples observed with maltodextrin could be due to the fact that none of the mechanisms of protection postulated to provide stability to membranes or proteins (i.e. vitrification and specific molecular interactions; Crowe et al. 1998) could operate in the maltodextrin systems under the drying conditions employed. Even if the glass structure could have stabilizing properties, its late formation in the vacuum-dried systems could not prevent the loss of viability during the initial stages. The presence of maltodextrin also delayed the drying of the cells, thus allowing them to be subjected to a wet environment at 40 °C for a longer period of time than control samples in which no solutes were incorporated and whose drying occurred at a faster rate.

Both trehalose and maltodextrin matrices protected the cells during freeze-drying. In frozen systems, the T_g value of the maximally concentrated matrix (T_g') has important implications for freeze-drying processes; and it has been reported as a variable related to the stability of frozen biomaterials (Levine and Slade 1986; Lim and Reid 1991). In the conditions of the present experiments, the formation of a glassy matrix (vitrification) could account for the observed protection provided by maltodextrin during the freezing of cells, since the freezing process was carried out at a temperature (−26 °C) below the T_g' of the maltodextrin employed (−23 °C; Lim and Reid 1991; Roos and Karel 1991). Its stabilizing effect could be attributed, in this case, to the formation of a glassy matrix upon freezing. In the case of the trehalose matrix, the reported T_g' values are between −22 °C (Miller et al. 1997) and −35 °C (Roos 1993), so the complete vitrification at the freezing temperature employed in this work (−26 °C) is difficult to assess.

The stability of yeasts (i.e. cell viability) during drying or thermal treatment did not seem to be determined by the overall physical properties of the cells (i.e. sorption behavior, T_g values), but were affected by the concentration of internal trehalose. On the other hand, the physical properties of the external matrices seemed to affect yeast stability during the drying processes employed. From the results of this work, the protective mechanisms that can be proposed for each particular system are: (1) vitrification, in frozen maltodextrin and possibly in trehalose systems, and (2) specific interactions of trehalose with membranes and/or proteins, for both vacuum-drying and freezing during the freeze-drying process.

Acknowledgements The authors acknowledge financial support from the University of Buenos Aires (Project IX05) and the International Foundation for Science (E2393-2, Sweden). This work is part of the CYTED Project XI.13.

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