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Exploring differential scanning calorimetry as a tool for evaluating freezing stress sensitivity in Baker's yeasts

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Abstract

Thermal transitions were analyzed after freezing and thawing of two commercial baker's yeast cells of *Saccharomyces cerevisiae* having high and low tolerance to sugar. Yeast in stationary phase of growth were exposed to two cycles of freezing (20 h and 4 h) at −20 °C after being exposed to various physical and chemical mild stresses and differential scanning calorimetry (DSC) runs were performed at 10 °C/min from 20 °C to 100 °C. Thermograms showed two peaks that were attributed to low and high protein onset denaturation temperatures: 47–58 °C and 67–77 °C, respectively. Apparent enthalpy changes showed that the sugar tolerant strain had a relatively higher resistance to freezing and thawing than the non-tolerant one. On the other hand, some pre-stress treatments (*e.g.*, temperature, ethanol and NaCl) increased both enthalpy values and survival after freezing and thawing. The analysis of data clearly showed that DSC can be a very useful tool to analyze the behavior of yeast cells after freezing and thawing stress.

Keywords: Frozen yeast cells; Denaturation enthalpy; Freezing stress sensitivity; Differential scanning calorimetry; Trehalose

1. Introduction

Frozen-dough technology is well established in the baking industry saving time, space and equipment costs to bakers [1,2]. The stability of yeast in frozen dough (its cryoresistance) is the key to obtain good results in the product. On the other hand, baker's yeast, mainly *Saccharomyces cerevisiae* strains, is an appropriate microorganism for studying cellular parameters that affect freeze–thaw damage produced during frozen-dough process.

Ice crystallization during freezing could be considered as a stress factor for cells, and during this period protein denaturation or cell injury could occur. Freezing of proteins solutions may result in irreversible denaturation and protein aggregation and severe loss of catalytic activity of enzymes [3]. A study by tryptophan phosphorescence did provide direct evidence that solidification of water causes important alterations of the native globular fold in all examined protein [4], as was observed for a copper-free azurin from *Pseudomonas aeruginosa* by Gabellieri and Strambini [5].

In *S. cerevisiae*, freezing-thawing resistance has been generally associated with the concentration of intracellular trehalose, although no direct correlation has been found above a certain threshold value [6–8]. Accumulation of other solutes such as aminoacids and glycerol and expression of aquaporins [9–11] or synthesis of stress proteins [12] were also informed to increase the yeast freeze-thaw resistance.

With respect to trehalose, many authors showed that intracellular concentrations of the disaccharide exerts a protective effect on yeasts under extreme environmental conditions, such as cold or heat shock, anoxia desiccation and others [13–17]. Singer and Lindquist [18] found a dual effect of trehalose, which acts directly during heat shock to stabilize proteins in their native

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state, but also reduces aggregation of proteins that have already been denatured (which is a function previously assigned only to heat shock proteins). Nevertheless, the mechanisms by which trehalose protects yeast cells are not completely clear yet. The ability of trehalose to form glassy structures, which assure physical and chemical stability, is one of the hypotheses to explain the protective effect of trehalose. The maintenance of a glassy state (below the glass transition temperature, T_g) avoids sugar crystallization and delays chemical reactions. On the other hand, trehalose may play specific key roles during environmental stress conditions, due to its capability to interact with proteins and other biomolecules through multiple hydrogen bonds [19,20]. Another role attributed to trehalose is that this sugar can act as a water-replacing agent during yeast cells dehydration, conferring high resistance to water stress in these cells [6]. Previous work conducted in our laboratory [21] showed that trehalose was an effective protectant in freeze-dried commercial baker's yeast. Nevertheless, inconsistencies between trehalose levels and stress tolerance in yeasts have been reported [22–24].

It is well known that during its production, application and storage in the baking industry, baker's yeast encounters different stressful conditions (i.e., hyperosmolarity, supraoptimal temperatures, freezing, oxidation, etc.) [1,25,26]. There is some degree of commonality in the nature of damage resulting from the various stresses encountered. For this reason it was proposed that pretreatment of yeast cells with mild stresses can predispose them to protection against subsequent lethal exposure to the same or other types of stresses. This phenomenon is called "cross protection" [12,27–30], and it is very important to induce cell robustness during industrial processes where yeasts are employed. The availability of adequate analytical tools for a rapid evaluation of freezing and thawing stresses would improve the selection of plausible biomaterials and pre-treatments in the preparation of frozen dough for the baking industry [31–33].

Differential scanning calorimetry (DSC) has been successfully applied to analyze the influence of different stresses like high hydrostatic pressure and thermal effects on the denaturation of structural components in relation to microbial cell injury and death [34–39]. DSC can be considered also a powerful technique for studying the thermodynamics of transition in biological molecules [40,41].

In the present study, DSC was explored as a tool for evaluating freezing and thawing sensitivity of two industrial strains of *S. cerevisiae* previously submitted to mild stresses. Trehalose accumulation and protein levels in cells were also evaluated in order to establish the possible relationship of these variables with cell freezing tolerance and/or enthalpy changes.

2. Materials and methods

2.1. Yeasts

The *S. cerevisiae* strains used were BAFC 3084 and BAFC 3083 (Facultad de Ciencias Exactas y Naturales, Buenos Aires), originally provided by CALSA (Argentina); they were recommended for high sugar content doughs (O) and for plain doughs (NO), respectively. Strains were preserved at −70 °C in 30% (v/v) glycerol. Volumes of 50 mL YEP medium (0.5%, w/v yeast extract, 0.5% peptone, 1% glucose, 0.3% $(NH_4)_2SO_4$ and 0.3% KH_2PO_4) contained in 250 mL Erlenmeyer flasks were inoculated from a fresh yeast slope of the same medium and incubated overnight at 28 °C in an orbital shaker (160 rpm) for 24 h. These cultures were used to inoculate 500 mL Erlenmeyer flasks containing 100 mL of YEP broth, in order to obtain an initial optical density of 0.025 at 640 nm. After 48 h incubation, cells were harvested by centrifugation (6800 × *g* for 10 min), washed twice with 0.1% peptone–water, and finally suspended in YEP without glucose (YEPNG). Then, samples of cell suspensions (∼10^7 CFU/mL) were exposed to different physical and chemical mild stress treatments (pre-stresses) according to [7,42] modified: (a) heat: 40 °C for 2 h and then cooled in water at 25 °C; (b) ethanol: 2% (v/v) ethanol at 28 °C for 3 h; (c) oxidative: 5 mM H_2O_2 at 28 °C for 2 h; (d) salt: 0.5 M NaCl at 28 °C for 3 h. These pre-stresses were chosen in order to obtain sub-lethal conditions, i.e., more than 80% survival after pre-treatments.

After being submitted to the pre-stress treatments, samples were immediately filtered through cellulose acetate membranes (pore size = 0.45 μm), and submitted to freezing and thawing (F/T). A non-pre-stressed sample was also carried out.

2.2. Freezing and thawing (F/T)

F/T stress was performed by exposing 5 mL of the cellular suspension (∼10^7 CFU/mL) in YEPNG to successive cycles of freezing for 20 h and 4 h at −20 °C and thawing at 25 °C.

2.3. Biochemical determinations

All determinations were carried out at least by duplicate and repeated three times.

2.3.1. Cell viability

Viable counts (CFU/mL) were determined by spotting 20 μL of serial dilutions of the cell suspensions onto YEP-agar plates in quadruplicate. Plates were incubated at 28 °C for 48–72 h. For each culture, survival was recorded as the percentage of cell viability ratio after F/T treatment respect to control. Control corresponds to non-pre-stressed and non-frozen cells.

2.3.2. Extraction and assay of trehalose

Intracellular trehalose determination was carried out in non- and pre-stressed cells before F/T, according to [15]. Aliquots of 5 mL of cell suspensions were centrifuged (6800 × *g* for 10 min); cell pellets were washed with phosphate buffer 0.1 M (pH 6), frozen immediately in liquid N_2 and maintained at −80 °C until analysis. Trehalose was extracted from the samples by boiling in 1 mL 0.25 M Na_2CO_3 for 20 min. Aliquots of 0.2 mL were neutralized by adding 0.1 mL of 1 M acetic acid and 0.1 mL of buffer T (0.3 M acetate and 0.03 M $CaCl_2$, pH 5.5). Two hundred microlitres of this mixture were incubated in the presence of the same volume of trehalase (Sigma no. T-8778, diluted 1/3) for 1 h at 40 °C. Released glucose was assayed by the glucose

oxidase–peroxidase (Wiener lab., Argentina) as recommended by the supplier. All reagents used were analytical grade.

2.3.3. Protein extraction and isolation

Cells were pelleted by centrifugation at 6800 × *g* (supernatant was discarded), and 1/10 of the original volume of chilled lysis buffer were added. The composition of the lysis buffer was as follow: Tris–HCl, 50 mM (pH 7.5); NaCl, 50 mM; $MgCl_2$, 1 mM, DTT (dithiothreitol) 5 mM, 1 mM PMSF (phenylmethanesulfonyl fluoride, 1 mg/mL, DNAse in H_2O).

Cell suspensions, ca. 1–5 × 10^8 UFC/mL, were transferred to a French press (French Pressure Cell Press, SIM-AMINCO, Spectronic Instruments) and a pressure of 1.3 × 10^8 Pa were applied. These steps were carried out at room temperature. Cell lysates were centrifuged at 20,000 × *g* for 15 min at 4 °C in the JA14 rotor of a J2-21 Beckman centrifuge, to remove cell debris and the supernatant was precipitated with $(NH_4)_2SO_4$. The solid salt was added to 80% saturation and the precipitated proteins collected by centrifugation at 20,000 × *g*, for 45 min in a Sorvall RC-5B refrigerated Superspeed Centrifuge. The precipitate was suspended in a minimal volume of lysis buffer (without DNAsa), and dialyzed overnight at 4 °C against the same buffer. All reagents used were analytical grade.

2.3.4. Protein determination

Protein concentration was determined before and after F/T, according to [43] using bovine serum albumin as standard.

2.3.5. Cell dry matter

Aliquots of 30 mL were filtered trough cellulose acetate membranes (0.45 μm pore size) previously weighed. Filters were washed with distilled water and dried at 105 °C to constant weight.

2.4. Differential scanning calorimetry (DSC)

Thermal transitions were determined from the peak areas of the corresponding endothermic peaks by differential scanning calorimetry (DSC) using a Mettler Toledo 822 equipment (Mettler Toledo AG, Switzerland) with the STARe Thermal Analysis System version 6.1 software (Mettler Toledo AG). The temperatures of denaturation were recorded as the onset and peak temperatures of the endothermic peaks. The instrument was calibrated using standard (indium, zinc and lead) compounds of defined melting point and heat of melting. The melting point of pure water (0 °C) was determined in order to validate the extrapolation of the calibration curve. All measurements were made with 10–25 mg sample mass, at a scanning rate of 10 °C/min using hermetically sealed aluminum pans of 40 μL inner volumes (Mettler Toledo AG) and an empty pan was used as a reference. Samples consisted of whole cell pellets, obtained after filtration trough cellulose acetate membranes (0.45 μm pore size); all samples had enough water to allow protein denaturation (water contents were higher than 150 g H_2O/100 g solids). Enthalpy values (J/mg of fresh sample) was normalized by protein content (mg of protein/mg of fresh sample), thus, the results were expressed as J/mg of protein. Also, cell protein extracts obtained by fractionation procedures were analyzed for comparative purposes. The scans were performed from 20 °C to 100 °C and reported data are the average of at least four determinations.

3. Results

Differential scanning calorimetry (DSC) thermograms of two different strains of *S. cerevisiae* (O, osmotolerant and NO, no osmotolerant) before and after freeze/thawing (F/T) are shown in Fig. 1. Two endothermal transitions attributed to protein denaturation were observed for each strain, one of them with onset temperatures in the range of 47–58 °C (fraction 1) and the other one between 67 °C and 77 °C (fraction 2). Denaturation process may occur during both F/T stress and DSC scan. A higher denaturation enthalpy value obtained in the DSC scans implies that a lower amount of proteins were denatured during the F/T step, and thus most of protein denaturation has the opportunity to occur during scanning. On the contrary, a lower enthalpy value corresponds to a greater degree of denaturation, which could occur for example, when the cells were submitted to stress, leaving a less amount of native protein to be denatured during the DSC scan. This figure also shows that the enthalpy associated to fraction 1 in the controls (non-frozen cells) was higher for the osmotolerant strain O than for strain NO. DSC runs were also performed on the protein fraction extracted from yeast cells. The thermogram corresponding to cell proteins extracted from NO (Fig. 1, inset) meaning that the two endothermal peaks appearing in the thermograms of the cells could be associated to protein populations, with low and high denaturation temperatures. Similar results were found for O strain (data not shown). The analysis of endothermal transitions corresponding to the cells was also performed after F/T treatment. F/T promoted a change in the shape of the endothermal peaks of both fractions: while control peaks were sharper, after F/T they became wider, which may indicate greater dispersion in size or types of proteins. This change was

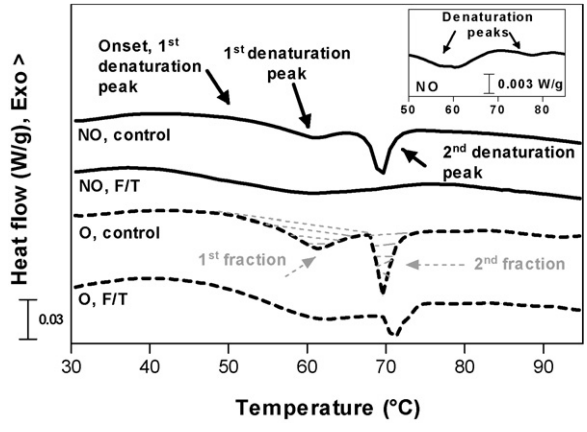

Fig. 1. DSC thermograms obtained from cells of strains O and NO. Denaturation peaks corresponding to fractions 1 and 2, respectively, are marked by arrows. Grey dotted areas in the graph show the criteria followed to define baselines for the calculation of denaturation enthalpies from the endothermal areas. The inset figure shows the thermogram of cell protein extract obtained by fractionation procedures. F/T: freezing at −20 °C for 20 h and 4 h, and thawing at 25 °C; controls are defined as systems without freezing and thawing treatment.

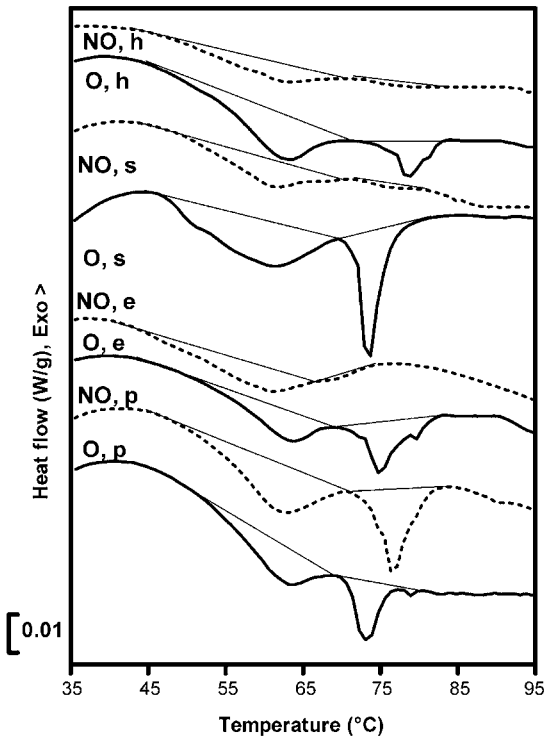

Fig. 2. DSC thermograms obtained from cells of strains O and NO submitted to different pre-stresses, frozen at −20 °C for 20 h and 4 h, and thawed at 25 °C. Grey lines areas in the graph were plotted to show the criteria followed to define baselines for the calculation of denaturation enthalpies. Pre-stresses: h: heat; s: salt; e: ethanol; p: peroxide.

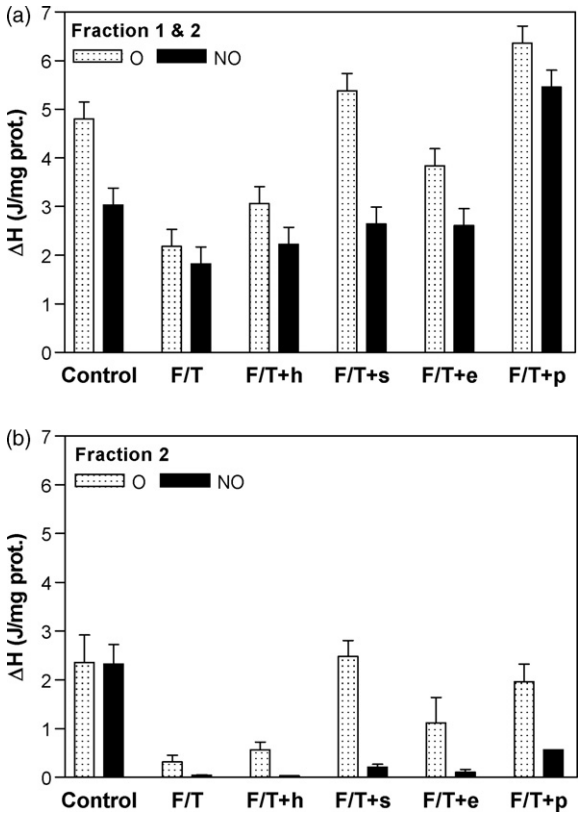

Fig. 3. Total denaturation enthalpy per mg of protein including fractions 1 and 2 (a) and endothermic area in the temperature range corresponding to fraction 2 (b) for O and NO strains cells before and after F/T. Control: cells without freezing and thawing treatment; F/T: freezing at −20 °C for 20 h and 4 h, and thawing at 25 °C; h: heat; s: salt; e: ethanol; p: peroxide. The segments over bars indicate standard deviation values.

more pronounced in the NO strain. In the thermograms corresponding to these cells, fraction 2 almost disappeared and/or overlapped with fraction 1. Due to this possible overlapping, quantification of enthalpies was difficult to assess in NO cells in fraction 2. The osmotolerant strain O had clearly two peaks after F/T. It is also to be noted that the strain O showed higher denaturation temperatures (onset and peak) and higher enthalpy values than the NO strain (Fig. 1).

As shown in Fig. 2, DSC thermograms of cells submitted to the different pre-stresses also showed that the enthalpy values of fraction 1 were higher than the corresponding to fraction 2 for both strains, and the overall effect of the applied pre-stresses was to increase the enthalpy values, when compared to those of not pre-stressed frozen samples shown in Fig. 1. Fig. 3 shows the total denaturation enthalpies, including fractions 1 and 2 (Fig. 3a) and the endothermic area in the temperature range corresponding to fraction 2 (Fig. 3b) for both strains before and after F/T, both normalized by the protein content. After F/T, enthalpies decreased and this fact was more pronounced in fraction 2 for both strains, remarking its sensitiveness to F/T treatment. This figure also shows the enthalpy values associated to fractions 1 and 2 corresponding to O and NO cells submitted to various pre-stressing conditions before F/T treatments.

Cell survival and trehalose content of O and NO yeast cells were determined with or without previous pre-stress application. Table 1 shows the results obtained when none or heat, ethanol, salt or oxidative pre-stresses were applied. The O strain constitutively had a higher trehalose content compared with the NO one ($4.5 \pm 0.2\%$ and $2.9 \pm 0.1\%$ g/g dry matter, respectively) that correlated with a higher intrinsic survival to F/T. Heat, saline and ethanol application enhanced cell survival of the yeasts submitted to F/T, and except for the case of the ethanol treatment, an increase in trehalose concentration was also observed (Table 1). Total protein concentrations were 0.12 ± 0.02 mg protein/mg sample for O, and 0.10 ± 0.01 mg protein/mg sample for NO for control cells and 0.15 ± 0.02 mg protein/mg sample for O, and 0.15 ± 0.03 mg protein/mg sample for NO for F/T treated

Table 1
Cell survival after and trehalose content before F/T of O and NO yeast cells previously exposed to different pre-stresses

Pre-stress	O		NO	
	Survival (% respect to control)	Trehalose concentration (%, p/p)	Survival (% respect to control)	Trehalose concentration (%, p/p)
None	27 ± 1	4.5 ± 0.2	7 ± 1	2.9 ± 0.1
Heat	74 ± 4	5.6 ± 0.1	30 ± 5	3.1 ± 0.1
Ethanol	81 ± 6	4.3 ± 0.1	50 ± 1	2.6 ± 0.2
NaCl	62 ± 2	6.0 ± 0.1	42 ± 2	3.40 ± 0.01
H_2O_2	14 ± 1	4.5 ± 0.2	5 ± 1	2.75 ± 0.04

Control corresponds to non-pre-stressed and non-frozen cells. Standard deviation values are indicated.

cells. Protein content of both strains was also increased about 12% and 23%, in O and NO strain, respectively, after the application of heat, saline or ethanol pre-stresses. DSC profiles seemed to reflect cell protein stability that in turn was mainly translated in an increase in cell viability. It is to be noted that although heat application did not produce a noticeable increase in enthalpy of fraction 2 (Figs. 2 and 3b), an increase in onset and peak temperatures in both strains of about 6 °C was observed (Fig. 2). On the contrary, the other pre-stresses did not produce any effect on temperatures in comparison to F/T treatment (Fig. 2).

It seemed worthwhile to investigate oxidative pre-stress conditions that are known to produce stable cross-linked modifications of proteins [44–46]. At the assayed concentration of H_2O_2, survival after F/T, trehalose levels and protein concentration did not significantly differ from non-pre-stressed cells. Nevertheless, higher enthalpy values were observed (see Figs. 2 and 3).

4. Discussion

The thermograms obtained by DSC of two representatives commercial baker's strains of *S. cerevisiae* (osmotolerant and no osmotolerant), showed two endothermal peaks that can be associated to protein denaturation. A correlation between denaturation temperatures and the involved enthalpies could not be found. It is to be noted that both parameters, enthalpy and denaturation temperature, have different meanings. While the denaturation enthalpy is related to the equilibrium between native and denatured protein states (at higher enthalpy value, the folded protein form is favoured), the denaturation temperatures are related to the dynamics of the unfolding process (a higher denaturation temperature indicates a lower rate of irreversible unfolding) [47]. The global analysis of the results indicates that DSC thermograms can be used to differentiate cells even of the same species with different biochemical or phenotypical characteristics like protein and/or trehalose content. DSC profiles were significantly changed, concomitantly with physico-chemical modifications produced in the cells (stresses like F/T, for example). The effect of protein denaturation due to F/T process on yeast cells was clearly reflected on the DSC scans, as it is shown in Fig. 1. F/T induces conformational changes in proteins that could produce a greater molecular heterogeneity in protein populations that is reflected in changes in the enthalpy values and in the onset and peak temperatures.

Changes in enthalpy values (Figs. 2 and 3) after F/T with previous pre-stresses application were also observed. The application of pre-stresses like heat, saline and ethanol produced a significant increase in cell survival that correlated with an increase in total enthalpy values and with a temperature increase in heat pre-stressed cells of both strains, but only in the case of heat or saline pre-stresses a significant trehalose accumulation was observed. The protective effect of ethanol to F/T, evidenced in cell survival and in a concomitant increase of the enthalpy values, seemed to be independent of trehalose intracellular content, supporting the idea that trehalose levels and stress tolerance are not strictly correlated. Of all considered pre-stress factors, the oxidative one (H_2O_2) promoted increased enthalpy values in both fractions in both strains, without promoting cell survival. Moreover, protein and trehalose concentrations after F/T were lower in H_2O_2 pre-treated cells. It is possible to suggest that if a pre-stress factor provokes oxidations or conformational changes of proteins (for example, by generating more structurally stable cross-linked products), higher denaturation enthalpy can be observed although no benefit to cell survival were achieved. In the particular case of H_2O_2 pre-stress, the increase in enthalpy could be attributed to protein cross-linking due to key protein carbonylation caused by oxidative damage as it was reported for yeasts as well as for higher eukaryotes [44–46], that although producing stable protein based aggregates [48] made microbial cells non-culturable [49].

As in this work, several authors reported that there is not a good correlation between trehalose content and yeast cells survival to stresses [22–24,50]. Instead, DSC determinations clearly correlated with protein stability and a decrease in the enthalpy values obtained from the thermograms can provide valuable information about the corresponding effect on yeast viability. In this way, DSC can be considered a useful complementary tool to analyze or predict the impact of different stress factors on yeast cells.

5. Conclusions

- Several authors reported that there is not a good correlation between trehalose content and yeast cells survival of yeast cells to stresses. Instead, DSC determinations clearly correlated with protein stability and a decrease in the enthalpy values obtained from the thermograms can provide valuable information about the corresponding effect on yeast viability.
- Apparent enthalpy changes showed that the sugar tolerant strain had a relatively higher resistance to freezing and thawing than the non-tolerant one.
- Some mild pre-stress treatments (*e.g.*, temperature, ethanol or saline) increased both enthalpy values and survival after freezing and thawing.
- The osmotolerant strain O had clearly two peaks after F/T. It is also to be noted that the strain O showed higher denaturation temperatures (onset and peak) and higher enthalpy values than the NO strain.
- The analysis of data clearly showed that DSC can be a very useful tool to analyze the behavior of yeast cells after freezing and thawing stress.

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