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EXPERIMENTAL ARTICLES

Anhydrobiosis in Yeast: Stabilization by Exogenous Lactose[1]

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Abstract—We have found that incubation in lactose solutions (0.75 M) of yeast culture *Saccharomyces cerevisiae* sensitive to dehydration damage increased the stability of the cells during dehydration. Simultaneously with this increase in viability, a decrease in plasma membrane permeability during rehydration was seen. Using Fourier transform infrared spectroscopy to measure lipid phase transitions, we observed that the lactose treatment depressed the membrane phospholipid phase transition temperature in a sensitive culture of dry yeast. As a result, it leads to the decrease in the damages of molecular organization of membranes during rehydration of dry yeast cells, thus reducing leakage from the cells.

Key words: yeast, lactose treatment, stabilization of plasma membrane, dehydration-rehydration, lipid phase transition temperature.

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In recent years, a great deal has been learned about the mechanism by which yeasts survive in the state of anhydrobiosis. It has been established that synthesis of the disaccharide trehalose in stationary phase of growth is essential for survival [1]. This sugar is responsible for stabilization of membranes [2–3] and proteins [4] in the dry cells maintaining their potential functional activity in dry cell. Further, a trehalose transporter in the plasma membrane also seems to be required [5–6]. In strains of the yeast that lack this transporter, addition of exogenous trehalose improved survival during drying, leading to the suggestion that the transporter may play a function in allowing trehalose, synthesized inside the cell in stationary phase, to leak to the outside [6].

Stabilization of the plasma membrane has seen the most extensive study, with the following adaptations reported in the literature: (1) an ordered folding of the plasma membrane during drying [7–10], (2) depression of the lipid phase transition temperature in the dry cells [11], probably due to synthesis of trehalose [12].

In the present paper we have investigated effects of adding exogenous lactose on the stability of yeast cells during drying. The fundamental question being addressed is whether trehalose is essential for improving the cells' survival or will other disaccharides suffice.

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MATERIALS AND METHODS

Yeast strain *Saccharomyces cerevisiae* 14 was received from culture collection of the Laboratory of Cell Biology, Institute of Microbiology and Biotechnology, University of Latvia, Riga. Cultures were grown on standard nutrient media containing (g l^{-1}): molasses, 20; $(NH_4)_2SO_4$, 3.7; NaCl, 0.5; KH_2PO_4, 1.0; K_2HPO_4, 0.13; pH 5.0 in flasks with total volume 250 ml in an orbital shaker (140 rpm) at 30°C. In our preliminary experiments it was shown that the strain *S. cerevisiae* 14 grown under these conditions can be sensitive to dehydration damage. We have taken advantage of this phenomenon to investigate effects of exogenous lactose addition for the stabilization of sensitive cultures.

One part of the yeast biomass after centrifugation (3000 *g*, 10 min) and separation from cultural liquid was compressed, suspended in lactose solution (0.75 M) and then incubated in this solution at 30°C in flasks in an orbital shaker for 2.5 h. A duplicate sample was incubated in sterile water in the same conditions as a control.

Yeast biomass was compressed, extruded through the sieve, and subjected then to convective dehydration in an oven at 30°C for 24 h. Residual moisture was 8–10%, determined by drying to constant weight at 105°C. At such residual moisture (if adequately dehydrated) yeast can maintain its viability being in the state of anhydrobiosis.

Survival rates of the dehydrated organisms were determined using the method of fluorescence microscopy with fluorochrome primulin [13].

The dry cells were rehydrated in sterile, distilled water for 10 min at room temperature; the supernatant was then separated from the cells by centrifugation. The changes of the permeability of the plasma membrane were monitored by spectrophotometric determination of the nucleotides content in the supernatant and of the total losses of dry substances determined by measurement of dry weight changes in the sample before and after dehydration. Gradual rehydration of the dry yeast was accomplished by 2-h incubation in the vapours of water in the chamber (over distilled water) at 37°C.

The T_m of dry cells were determined using a Fourier Transform Infrared (FTIR) microscope connected to a FTIR spectrometer (Perkin-Elmer 1620, United Kingdom). Dry samples were sandwiched between two CaF_2 windows, and the edges were sealed with high-vacuum silicon grease. The sealed windows were loaded into a liquid nitrogen-cooled temperature controller (Paige Instruments, Woodland, CA) and placed in the microscope. The sample chamber of the microscope was flushed with dry nitrogen for the length of each run. The sample temperature was increased in 2°C increments, held constant for 1 min before scanning, and 30 scans were taken at each temperature. T_m of the dry whole cells was determined by plotting the frequency of the PO_2 asymmetric vibration against temperature, as described earlier [12].

RESULTS AND DISCUSSION

We tried to reveal in our work the main mechanism of the phenomenon which we have found earlier. Incubation of yeast in sucrose or lactose solutions with higher osmotic pressure led to a significant increase in their survival after dehydration [14]. The highest increase of viability in these conditions was obtained for initially more sensitive yeast cultures. Both protective compounds gave practically similar effect. Taking into account multiple effects of sucrose upon the cell, including the possibility that sucrose can be used by yeast as a substrate, we chose lactose, which can not be metabolized by yeast *S. cerevisiae*, for further investigations in this work. The concentration of the lactose solution (0.75 M) and the average duration of incubation (2.5 h) were chosen based on our previous results [14]. Incubation of yeast suspensions in these conditions resulted in an increase of cell viability for 12–33% after dehydration [14]. So, if the viability of the initial (control) culture after dehydration in one of the experiments was 62%, the post-dehydration viability of the same culture after incubation in the lactose solution reached 78%. In another control experiment, the initial culture was very sensitive to dehydration and its viability after drying was only 31%. At the same time incubation of these cells in lactose solution before their dehydration resulted in a sharp increase of their resistance and their viability in the dehydrated state reached already 64%.

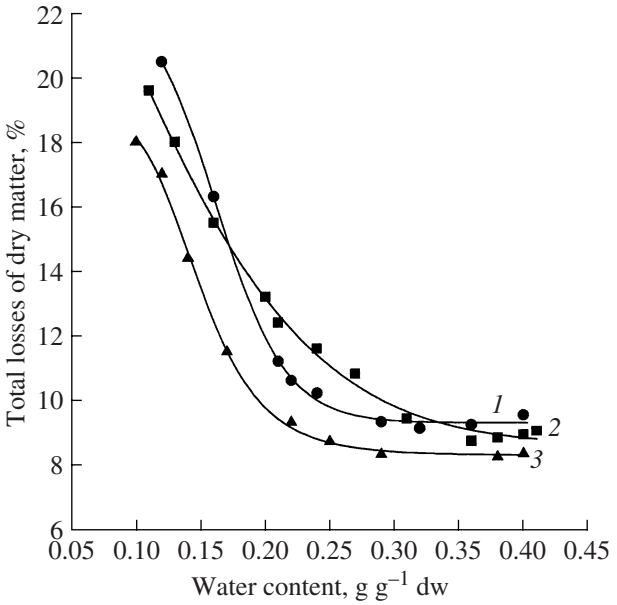

Fig. 1. Total losses of dry matter in dependence of humidity of rehydrated cells subjected to the incubation in 0.75 M lactose solution before drying procedure. *1*—control (without incubation); 2—cell incubation in water; 3—cell incubation in lactose.

An assay for stability of dry yeast cells that has long been used is leakage of the intracellular contents during rehydration. Using this simple procedure, we next studied the leakage of dry matter or nucleotides from the cells dried after incubation in lactose solutions. When the cells were placed in water at room temperature immediately after drying (with a residual water content of 8–10%), leakage was clearly higher in the control cells or those incubated in distilled water than in the cells incubated in lactose (Figs. 1 and 2). The differences in the control and experimental rates of leakage were highly significant (paired *t*-test, $p > 0.99$) when the initial water contents of the cells were smaller than 25%. However, as the water contents were increased by incubation over water vapour before the cells were placed in the water, the leakage steadily fell, and the differences between the control and experimental samples became smaller (Figs. 1 and 2). When the cells contained about 25% water before they were placed in water, leakage fell to minimal values, and the differences between the control and experimental samples became insignificant (paired *t*-test, $p < 0.1$). This effect of prehydration (slow gradual rehydration in vapours) upon the decrease of the permeability of the plasma membrane was shown earlier in the studies on yeasts [10], seeds [15], pollen [16–17], and anhydrobiotic nematodes [18]. It has been shown to be related to a phase transition in membrane phospholipids [12, 17, 19]. As membranes are dehydrated, the gel to liquid crystalline transition temperature (T_m) of their phospholipids rises. Thus, when such "dry" phospholipids are placed in water, they undergo a reverse phase transition,

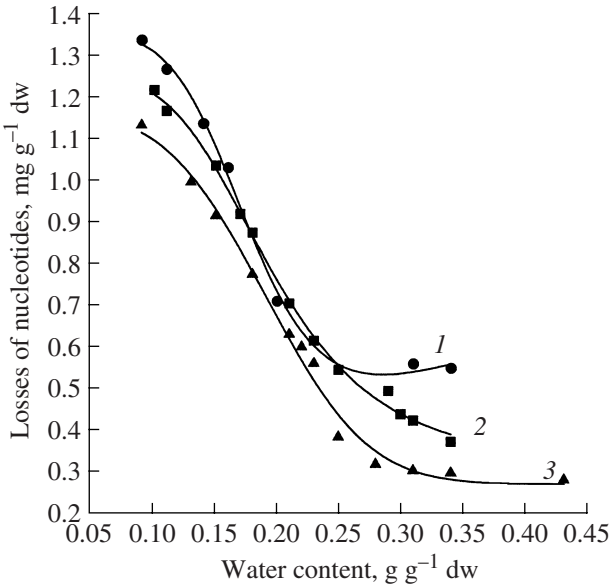

Fig. 2. Nucleotide release in the rehydration medium in dependence of humidity of rehydrated cells subjected to the incubation in 0.75 M lactose solution before drying procedure. *1*—control (without incubation); *2*—cell incubation in water; *3*—cell incubation in lactose.

from gel to the liquid crystalline phase. Phospholipid bilayers are known to become transiently leaky during such phase transitions [17, 20, 21]. Since T_m reaches minimal values at water contents of 20–25% [17, 20, 21], prehydration over water vapour would be expected to lead to minimal leakage, and that is indeed what is seen, with dry cells that have been studied previously [10], and in the present study as well (Figs. 1 and 2).

There is also strong evidence in the literature that disaccharides reduce T_m in dry membranes, thus reducing leakage during rehydration. In the present case, if incubation of the cells in lactose solutions reduced T_m value below room temperature, hydration of the dried yeast cells over water vapours should not improve their state and reduce the cell permeability. The results (Figs. 1 and 2) clearly show that this is not the case, but this is not a surprising finding. Leslie et al. [12] showed that trehalose produced endogenously by yeast cells prior to drying reduced T_m value from about 65°C to 35–40°C. In this case it was necessary to rehydrate the dry cells at temperatures >40°C to achieve maximal survival. Thus, addition of lactose would have to reduce T_m to <20°C in order for the membrane phospholipids to be in liquid crystalline phase at room temperature. Thus, we next resolved this issue by measuring T_m for membrane phospholipids in the dry cells.

Infrared spectroscopy has been used with good success in measuring lipid phase transitions in a variety of cells [17, 20, 22]. Basically, the method allows one to measure the vibrational frequency of CH_2 groups in lipids at various temperatures. A plot of frequency (wavenumber) as the function of temperature gives a distinct change in frequency that indicates T_m. Since the FTIR will record all hydrocarbons present in the cell, it is essential that the cell contain only small amounts of lipids other than those in the membranes or that phase transitions in the other lipids be sufficiently different from those in the membranes to permit one to distinguish between them. *S. cerevisiae* cells contain large stores of neutral lipids in the form of lipid bodies, which might make it impossible to use this method in this case. However, Leslie et al. [12] showed that the asymmetric phosphate stretch could also be used to determine lipid phase changes, a procedure that we have used here.

In our experiments, we investigated resistant and sensitive cells of the same yeast strain. As explained above, incubation of the sensitive cells with lactose before drying resulted in a marked effect on the increase of survival, while in the case of initially resistant cells the effect of the similar treatment was very small or absent. Thus, the prediction of the phase transition hypothesis is that lactose should depress T_m in the sensitive cells. This hypothesis was tested in our further experiments. It was established that in the resistant cells' T_m in the fully hydrated cells is well below 0°C (data not shown), in agreement with the previous results on trehalose effects [12]. Then in the next series of experiments yeast cells were dried from exponential phase of growth. Yeast cells taken from the exponential growth phase are extremely sensitive to dehydration. The viability of such dehydrated cells seldom reaches 30%, and usually is significantly lower. In our experiments a prolonged transition is found in this case, stretching from about 20° to >50°C (Fig. 3). In similar experiments reported earlier [12] we saw a single, much sharper transition, and at even higher temperatures. Two factors may account for this difference in results: Leslie et al. [12] freeze-dried their cells, while ours were dried by convection (which is significantly more mild dehydration method). An even more likely explanation is that residual water contents in the freeze-dried cells were much lower than those obtained by convective drying used here (in our experiments the remaining humidity of the cells was at the level 8–10%). An elevated water content and heterogeneity in the distribution of that water in our experiments could lead to the broad transition seen here (Fig. 3).

Cells dried from stationary phase of growth which had rather high viability in dry state (about 70% and higher) gave very different results (Fig. 4). Such cells showed a single, sharp transition, between 25 and 35°C. This transition is about 10°C lower than that reported by Leslie et al. [12], probably due to the higher water contents of the cells dried by convection. The transition is clearly depressed relative to that seen in the exponential phase cells (cf. Figs. 3 and 4). Trehalose is synthesized in stationary growth phase [23], and it is likely that this endogenously produced sugar is responsible for this effect and protection of molecular organization of intracellular membranes. In our case, addition of lactose to

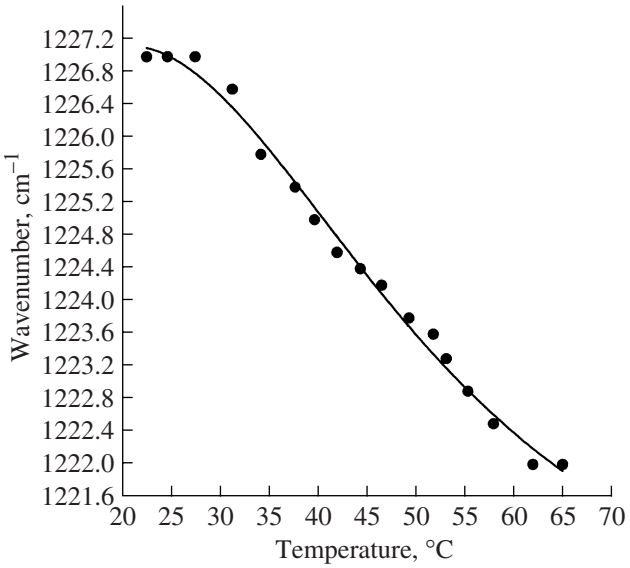

Fig. 3. Vibrational frequencies for the PO_2 asymmetric stretch as a function of temperature in the cells dried from the exponential phase of yeast culture growth.

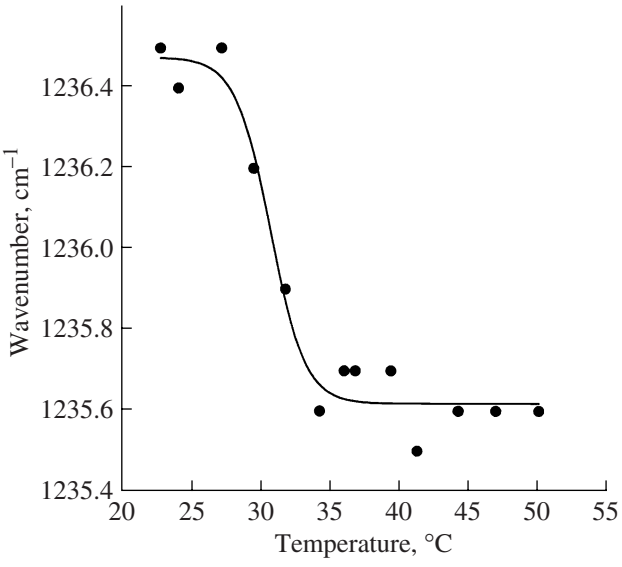

Fig. 4. Vibrational frequencies for PO_2 symmetric stretch as a function of temperature in stable cells dried from the stationary phase of yeast culture growth.

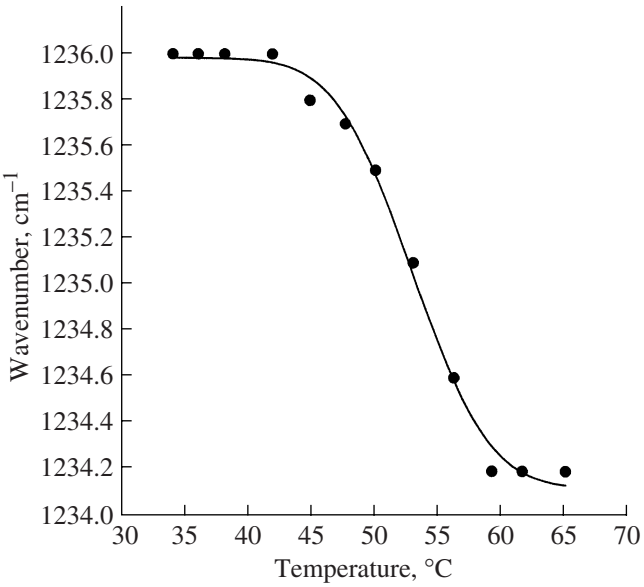

Fig. 5. Vibrational frequencies for PO_2 asymmetric stretch as a function of temperature in unstable cells dried from the stationary phase of yeast culture growth.

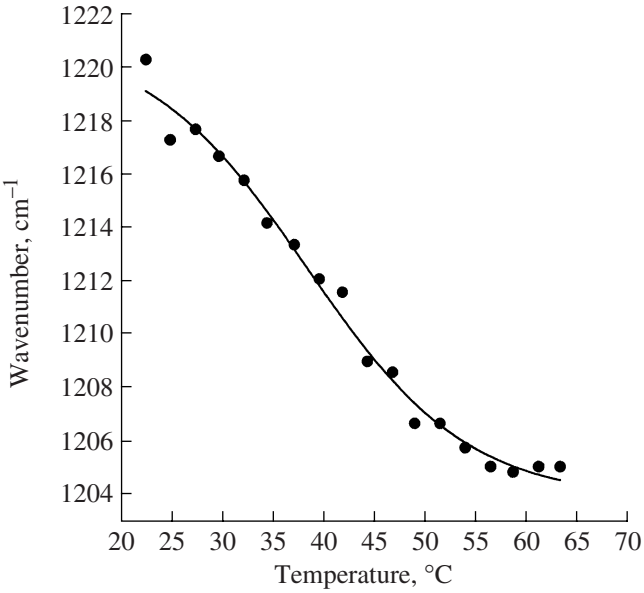

Fig. 6. Vibrational frequencies for PO_2 asymmetric stretch as a function of temperature in initially unstable cells from the stationary phase of yeast culture growth which were incubated before drying in 0.75 M lactose solution with the goal to increase their resistance to dehydration procedure.

the resistant cells from stationary growth phase before drying had no effect on the transition; it remained centred on about 30°C and did not change shape significantly (data not shown).

In the case of dehydration of sensitive cells from the stationary growth phase, a single phase transition, centred at about 50°C (Fig. 5), was revealed as it was seen for the cells from the exponential growth phase (Fig. 3). It is possible that in this non-resistant culture trehalose either was not produced, or it was synthesized in the amount insufficient to depress the phase transition temperature. By contrast with the resistant cells, incubation of these sensitive cells with 0.75 M lactose dramatically altered the phase transition temperature (Fig. 6). The transition was not as sharp as that seen in the resistant cells dried from the stationary phase, and it extended to

higher temperatures. Nevertheless, relative to the control (cf. Figs. 5 and 6), T_m was clearly depressed when the cells were incubated in lactose prior to drying.

Summarizing the results obtained in our investigation, we conclude that incubation of yeast cells sensitive to dehydration in the solution of disaccharide lactose can significantly improve their survival during drying. One of the effects of such incubation appears to be to reduce leakage across the plasma membrane during rehydration, probably due to depression of T_m in the membranes of dry cells. We are not suggesting that depression of T_m is the only effect of incubation of sensitive to dehydration yeast cells in lactose solution. For example, it is known that such sugars and other polyols can also stabilize labile proteins in the dry state [4]. Moreover, it was shown that incubation of yeast in lactose solutions leads to synthesis of polyols in their cells [24]. We suppose that in its turn they can substitute trehalose in the cells where it is absent or present in insufficient amounts. This assumption is supported also by our finding that the cells which can accumulate xylitol during their metabolism are much more resistant to dehydration than the cells of the relative strains which do not have such characteristic [25]. We do not suppose also that depression of T_m is the only factor which is sufficient for the enhanced resistance of the cells at their dehydration. This conclusion is in good agreement with more recent studies that small stress proteins can act synergistically with trehalose to confer desiccation tolerance [26]. Thus, the results received in our investigation make more clear the mechanisms which stabilize eukaryotic cells at dehydration procedure. We suggest also that the results presented here have both theoretical and applied importance and can be useful in biotechnological processes and especially in those which are linked with necessity to maintain the vitality of cells of microorganisms.

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