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Yeast Survival during Thermal and Osmotic Shocks Is Related to Membrane Phase Change

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The aim of this work was to study the survival of yeast cells exposed to a combination of thermal and osmotic treatments, as occurs during drying processes, and assess associated changes in fluidity of the plasma membrane, which have been studied previously in this laboratory. Cells that were maintained at a nonlethal dehydration temperature (5 or 30 °C) were rapidly dehydrated up to 120 MPa, and then thermal stress was suddenly applied between 5 and 30 °C. Cell viability was measured after a return to initial conditions (i.e., 1.38 MPa and 25 °C). Results showed that the viability of yeast cells exposed to identical combined thermal and osmotic treatments was dependent on the chronology of the stress application. Finally, the temperature at which the dehydration was conducted, up to 120 MPa, appeared to be the main factor involved in cell survival and could be related to fluidity variations of the plasma membrane.

KEYWORDS: ***Saccharomyces cerevisiae*; osmotic pressure; temperature; viability; plasma membrane phase; fluorescence anisotropy**

INTRODUCTION

Many studies on dehydration of the yeast *Saccharomyces cerevisiae* have been conducted because of the yeast's relevance to the food industry; however, the mechanisms involved in cell death and survival remain not well understood.

Several researchers have studied the effect of hyperosmotic stress on yeast viability. It appears that the main perturbation, which could explain cell death induced by the addition of nonpermeating compounds in the external medium, was cell shrinkage without plasmolysis (*1*). Moreover, Gervais et al. (*2*) showed that a transient phase followed the hyperosmotic shift. This was characterized by a transfer of water across the plasma membrane, the kinetics of which was related to yeast viability. Indeed, a low kinetics was found to maintain yeast viability even with a high level of osmotic pressure (*3*). These observations were recently confirmed by Beney et al. (*4*), who showed that yeast death was dependent on the combined effects of the kinetics of water across the plasma membrane and the level of osmotic pressure.

Although active responses could play a role in the cell response to osmotic stress (*5*−*7*), as reported by Morris et al. (*1*), the plasma membrane is considered to be the first site of osmotic injury. The yeast membrane phase-transition temperature increases with increasing osmotic pressure (along with the dehydration of the surrounding medium) and temperature (*8*) and varies with membrane composition, such as the presence (*9*) and type (*10*, *11*) of sterols or fatty acids (*9*, *12*). Furthermore, the transition to the liquid crystalline (Lα) phase is readily achieved by raising the temperature (*13*), and dehydrated lipids would be expected to enter the gel phase (Lβ) at which hydrated lipids are in the Lα phase (*14*).

Previous work carried out in our laboratory has demonstrated the importance of the plasma membrane in cell survival after heat (*15*) or osmotic stress (*2*, *4*). In both cases, plasma membrane permeabilization seemed to be a key factor in cell death. In further studies, we investigated the coupling effect of thermal and osmotic treatment on yeast (*16*) and bacterial cells (*17*). Laroche et al. (*8*) studied the plasma membrane phase-transition temperature of the yeast *S. cerevisiae* W303-1A over a wide range of temperatures (6−35 °C) and at seven levels of osmotic pressure (between 1.38 and 133.12 MPa) by measuring fluorescence polarization. The viability of *S. cerevisiae* W303-1A after combined thermal and osmotic treatments was associated with the mean phase-transition temperature of the plasma membrane (*16*). The authors suggested that cell death is related to a high water flow through an unstable membrane during the phase transition.

To confirm this observation and with the aim to improve microbial survival during drying, we have performed two different treatments on *S. cerevisiae* W303-1A that combined thermal and osmotic stress, which may or may not intersect with the plasma membrane phase-transition curve. After two different dehydration treatments of up to 120 MPa, *S. cerevisiae* W303-1A cells were subjected to different levels of thermal perturbations (between 5 and 30 °C) at high levels of osmotic pressure (120 MPa). The temperature variations were in the

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nonlethal range of 5−30 °C. Observed variations in viability were then related to phase changes of the plasma membrane.

MATERIALS AND METHODS

Strain and Growth Conditions. *S. cerevisiae* W303-1A (*MATa leu2-3,112 his3-11.15 trp1-1 can1-100 ade2-1 ura3-1*) (*18*) was kindly donated by Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven. As reported by Thomas and Rosthein (*18*), this strain is derived from diploid W303, which was produced by transforming haploid strain W301-18A with a plasmid containing the HO gene and dissecting the subsequent diploid. In light of these observations, it appears that cell functions related to osmotic and heat stress responses of the studied strain are not affected by the transformation previously explained.

Yeast cells were maintained in Petri dishes in a modified Malt Wickerham medium supplemented with 20 g/L agar (VWR International, Limonest, France). The modified Malt Wickerham medium was composed of 10 g of glucose (Sigma, Saint Quentin Fallavier, France), 3 g of pancreatic peptone (VWR International), 3 g of yeast extract (Sigma), and 1.5 g of NaH_2PO_4 (VWR International) in 1 L of a water and glycerol solution, which had an osmotic pressure of 1.38 MPa (5.1 g of glycerol/100 g of water; Sigma); the pH was adjusted to 5.35 by the addition of orthophosphoric acid (Sigma). A single yeast colony was introduced into a 250 mL conical flask containing 100 mL of modified Malt Wickerham medium and shaken (250 rpm) for 48 h at 25 °C. Then, 100 μL of this culture was introduced into a 250 mL conical flask containing 100 mL of modified Malt Wickerham medium and shaken (250 rpm) for 48 h at 25 °C. Cultures were grown to the early stationary phase to obtain an important cell biomass which did not present a high level of stress sensibility as cells grown in log phase or a high level of stress resistance as cells well-entered in stationary phase.

The conical flasks were shaken on a rotary shaker (New Brunswick Scientific).

Preparation of Hyperosmotic Solutions. The hyperosmotic solutions used were binary water−glycerol solutions. To obtain different osmotic pressures, the mass of solute (i.e., glycerol) to be added to 1000 g of distilled water was determined using the Norrish equation (*19*). Osmotic pressure was verified with a dew point osmometer (Decagon Devices Inc.).

Application of Different Levels of Heat Stress at a High Hyperosmotic Pressure (120 MPa). *Sample Preparation.* Fifty milliliters of fresh yeast culture grown to the early stationary phase (i.e., initial conditions, 25 °C and 1.38 MPa) were centrifuged at 2880*g* for 5 min at 25 °C. The resulting pellet was then suspended in 10 mL of a binary water−glycerol solution corresponding to an osmotic pressure of 1.38 MPa at the desired temperature for 5 min (step 1).

Stress Treatments. Thermal and osmotic variations were conducted as follows: cell suspensions were centrifuged at 2880*g* at the sample temperature. Then, a thermal (5, 15, or 30 °C) or osmotic shock (120 MPa) was applied by rapidly suspending the cell pellet in a binary water−glycerol solution at the shock temperature and osmotic pressure level. Cell suspensions were shocked for 5 or 30 min as subsequently described.

Note that to realize osmotic shocks at a final osmotic pressure of 120 MPa, yeast cell pellets were suspended in a binary water−glycerol solution corresponding to an osmotic pressure of 145 MPa because of the exit of water from cells.

Shocked-cell suspensions were maintained for 5 or 30 min at 5 or 15 °C in a refrigerated bath (Huber, Bioblock Scientific, Illkirch, France) or at 25 and 30 °C in a heated bath (Memmert, Bioblock Scientific). During experiments, temperature was controlled using a type K thermocouple (TCSA, Dardilly, France).

Iso-osmotic Treatments. Iso-osmotic treatments consisted of 1.38 or 120 MPa for 30 min in thermally stressed yeast cells. To apply thermal treatments at 1.38 MPa, yeast cells were suspended in a water−glycerol solution at the desired temperature (5, 15, 30 °C) for 30 min. To thermally stress yeast cells at 120 MPa, each treatment was composed of six successive steps as described in the insets in **Figure 1**: step1, reaching dehydration temperature (5 or 30 °C) for 5 min; step 2, dehydration up to 120 MPa for 5 min; step 3, thermal treatment at 120 MPa for 30 min; and steps 4−6, return to initial conditions for 5 min. Note that samples thermally stressed at a temperature different from the dehydration temperature were dehydrated for 5 min (step 2) and thermally stressed for 30 min (step 3), whereas samples thermally stressed at the dehydration temperature were maintained at that thermal level for 30 min (step 2) without subsequent exposure to a higher temperature level (step 3).

Measurement of Cell Viability. Cell viability was determined at different levels of the (T, Π) diagram (**Figure 1**). In each case, before measuring viability, samples were returned to initial conditions (i.e., 1.38 MPa and 25 °C). Yeast viability was determined by vital staining (methylene blue) as previously described (*16*). Briefly, the methylene blue stain was mixed with different nutrients and suspended in a water−glycerol solution at 1.38 MPa. The percentage of dead cells was determined and related to initial viability. Each experiment was repeated at least three times from three independent cultures. Results are given as averages, and standard deviations were calculated.

RESULTS

Two routes were studied in the (T, Π) diagram to thermally stress yeast cells at a high level of osmotic pressure (i.e., 120 MPa): cell suspensions were first thermally shocked at 5 or 30 °C, then dehydrated up to 120 MPa, and heat shocked for 30 min. Note that in all cases, cell viability was measured after cells were returned to initial conditions (1.38 MPa and 25 °C).

Controls, which were measured at the initial stage (1.38 MPa and 25 °C), presented viabilities close to 99 ± 0%. Moreover, viability that was measured at the end of step 1, at 1.38 MPa and 5 or 30 °C for 5 min, was also close to 99%. Interestingly, similar levels of viability were measured after 30 min in such conditions (**Figure 1**). Therefore, the range of temperature (5−30 °C) was not lethal for *S. cerevisiae* W303-1A.

Iso-osmotic Treatments. Cell suspensions were dehydrated up to 120 MPa at 5 and 30 °C. For cells dehydrated at 5 °C, thermal shock treatments were applied from 5 to 15 or 30 °C at 120 MPa, for 30 min. For cells dehydrated at 30 °C thermal shocks were applied from 30 to 15 or 5 °C for 30 min.

Note that cell suspensions were dehydrated up to 120 MPa at 5 or 30 °C (i) for 30 min in cell suspensions thermally stressed at the dehydration temperature or (ii) for 5 min in cell suspensions thermally stressed at a temperature different from the dehydration temperature, as explained under Materials and Methods. Dehydration for 5 min induced a low level of mortality. Indeed, on average 86 ± 4% of yeast cells survived at 5 °C and 120 MPa for 5 min and 71 ± 13% survived at 30 °C and 120 MPa for 5 min.

Cells Dehydrated at 5 °C. Yeast cells previously dehydrated at 120 MPa and 5 °C (step 2) were (i) maintained at such conditions for 30 min or (ii) thermally shocked from 5 to 15 °C and from 5 to 30 °C, at 120 MPa for 30 min. To appreciate the effects of thermal treatment alone on yeast survival, yeast cell mortality because of dehydration treatment for 5 min at 5 °C was deduced. Measured viabilities were corrected using eq 1, where V_c is the corrected viability, V_i is the viability measured

$$V_c = \frac{V_m \times 100}{V_i} \quad (1)$$

after the dehydration treatment for 5 min, and V_m is the viability measured after the iso-osmotic treatment (i.e., thermal stress). As previously explained, about 86% of yeast cells survived after an isotherm treatment of 5 min at 120 MPa and 5 °C. In

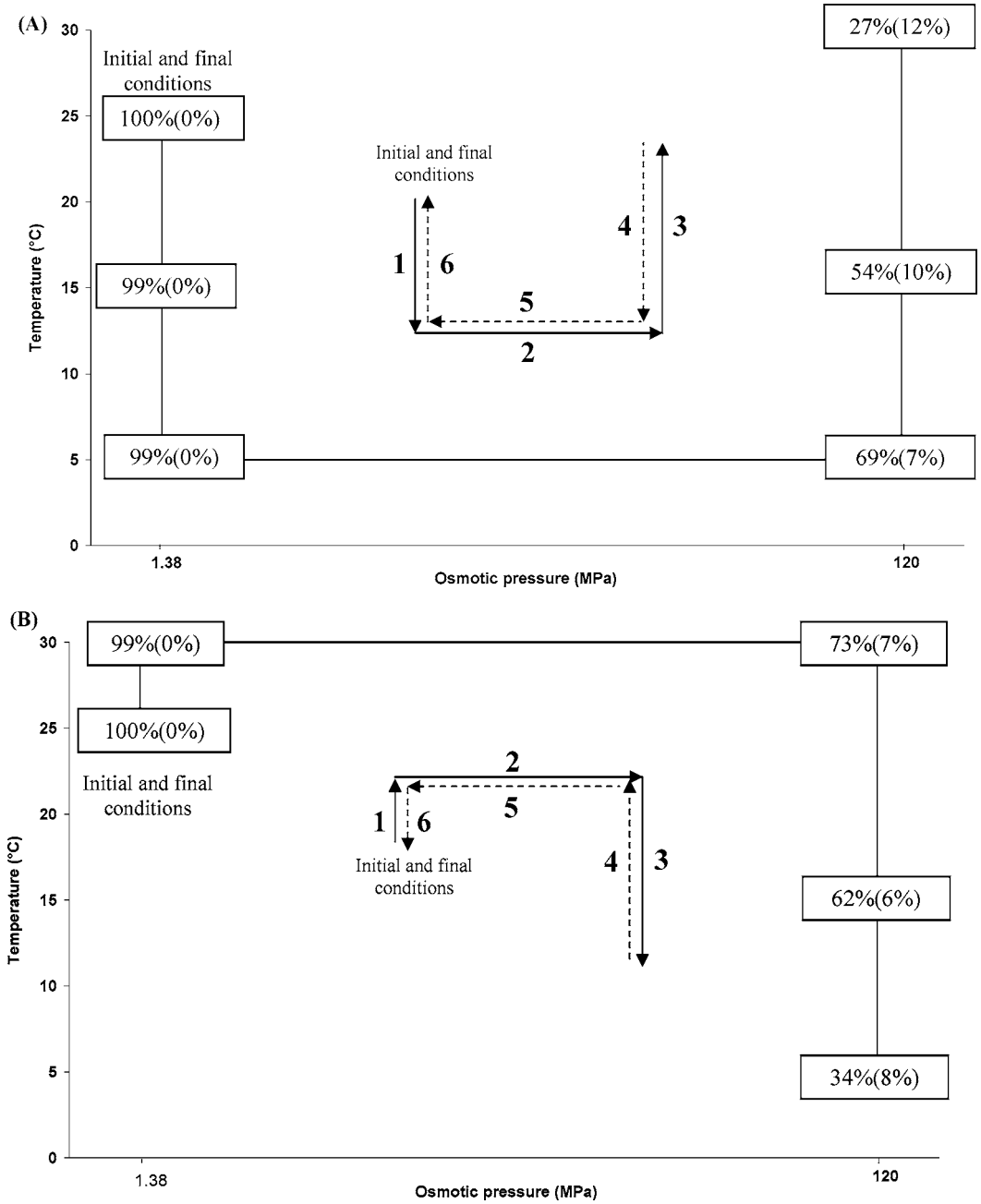

Figure 1. Viability of *S. cerevisiae* W303-1A as a function of temperature and osmotic pressure. Cells were grown at 25 °C. To thermally stress yeast cells at 1.38 MPa between 5 and 30 °C, samples were maintained for 30 min at the desired temperature and then returned to the growth temperature. To thermally stress samples at 120 MPa, samples were dehydrated at 5 or 30 °C before the stress was applied. Each experiment was repeated at least three times, and means for viabilities were determined. Standard deviations of the means are included in parentheses. As described in the insets, each treatment was composed of six steps: step 1, yeast cells were maintained at the dehydration temperature, i.e., cooled from 25 to 5 °C (see panel **A**) or warmed from 25 to 30 °C (see panel **B**); step 2, cells were dehydrated from 1.38 to 120 MPa; step 3, cells were heat stressed at different temperatures, i.e., 5, 15, or 30 °C for 30 min; and steps 4–6, return to initial conditions via the reverse pathways.

accordance with eq 1, and as shown in **Figure 1A**, yeast viability decreased with increasing temperature. Indeed, at 5 °C for 30 min 69 ± 7% of cells survived, whereas at 30 °C for 30 min about 27 ± 12% of cells survived.

Cells Dehydrated at 30 °C. We also measured yeast viability after dehydration at 30 °C (i) for 30 min or (ii) followed by thermal stress at 15 and 5 °C. Results are presented in **Figure 1B**. As previously explained, viabilities were corrected so that thermal effects alone could be appreciated. In accordance with eq 1, 73 ± 7 and 34 ± 8% of cells survived at 30 and 5 °C, respectively. Interestingly, at 15 °C cell viability appeared to be slightly higher than that at 50%, independent of the dehydration temperature, as shown in **Figure 1**.

These results showed that the history of cells plays a major role in survival. Indeed, at the same temperature, yeast viability varied in accordance with the dehydration temperature. For example, when cells were dehydrated at 120 MPa and 5 °C for 30 min, on average, 69% of the initial population survived, whereas only about 34% survived after a thermal stress from 30 to 5 °C and 120 MPa for 30 min. Similarly, about 73% of cells survived after dehydration at 120 MPa and 30 °C for 30 min, whereas about 27% survived after exposure to a thermal

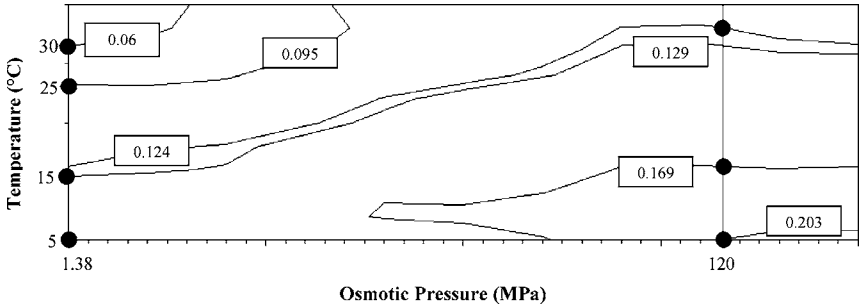

Figure 2. Iso-anisotropy curves of the plasma membrane of *S. cerevisiae* W303-1A as a function of temperature and osmotic pressure, deduced from results obtained by Laroche et al. (*8*) using fluorescence polarization. Iso-anisotropy curves were obtained using SAS 9.1. (●) represents different points of viability measurement at 1.38 and 120 MPa (vertical bars) described in **Figure 1**.

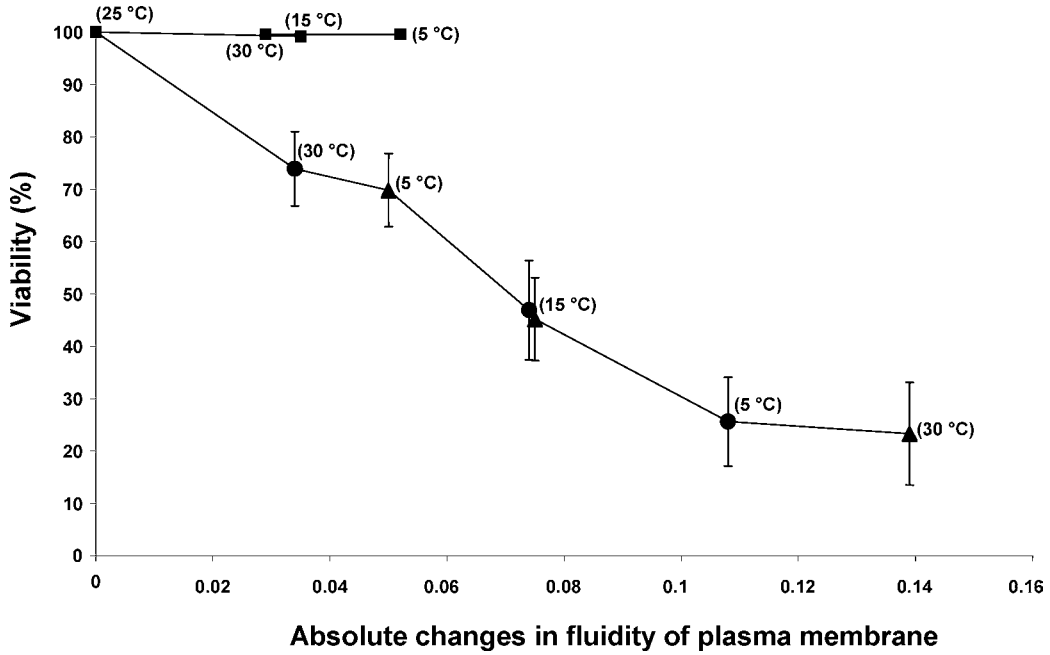

Figure 3. Viability of *S. cerevisiae* W303-1A as a function of absolute changes in fluidity of the plasma membrane (described in **Figure 2**). Viabilities were reported for six thermal stresses between 5 and 30 °C at 1.38 (■) or 120 MPa (●, ▲). In thermal treatments applied at 120 MPa, yeast cells were previously dehydrated at 5 °C (▲) or at 30 °C (●).

stress from 5 to 30 °C at 120 MPa for 30 min. These observations were not confirmed at 15 °C, at which cell survival was independent of dehydration temperature and, therefore, independent of cell history. In light of these observations, it appeared that differences in yeast viability could generally be obtained at the same level of heat shock at a high osmotic pressure across a large range of nonlethal temperatures.

Effect of Changes in Plasma Membrane Fluidity on Cell Survival. Previous studies have shown that variations in cell viability during isothermal treatment at high osmotic pressure are related to changes in plasma membrane fluidity (*8*, *16*). Such measurements were conducted using the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), and anisotropy values (*r*) were given. Note that the *r* value decreases with increasing plasma membrane fluidity.

Isofluidity curves were deduced from these previous data using statistical analysis (Statistical Analysis System [SAS/GRAPH] v 9.1 software, G3GRID, GCONTOUR, and G3D procedures) and are presented in **Figure 2**. The results showed that important variations in *r* values occurred between 5 and 30 °C at 1.38 and 120 MPa. Indeed, the *r* value varied from 0.147 to 0.060 at 1.38 MPa and from 0.203 to 0.124 at 120 MPa at 5 and 30 °C respectively. In light of these results, absolute changes in *r* values ($\Sigma |\Delta r|$) were calculated for each treatment (back routes were not considered). These absolute changes were related to cell viability measurements obtained after 30 min at 1.38 MPa (which corresponded to thermal stress without osmotic treatment) and after 30 min at 120 MPa; iso-osmotic curves as a function of yeast viability and absolute changes in *r* values are presented **Figure 3**. No variation in cell viability was recorded at 1.38 MPa as a function of absolute changes in anisotropy. Indeed, yeast viability was not affected by the range of temperatures studied (between 5 and 30 °C). At 120 MPa, cell viability was highly related to absolute changes in *r* values, whatever the dehydration temperature (5 or 30 °C). Indeed, whatever the dehydration temperature, cell survival decreased with increasing absolute changes in *r* values. These observations strongly suggest that changes in plasma membrane fluidity could be used to predict cell viability, although such variations seem to increase with osmotic pressure.

DISCUSSION

The aim of this work was to better understand the combined effects of osmotic and heat stresses on *S. cerevisiae* survival. In particular, this study assessed the effects of applying thermal stress at a high level of osmotic pressure (i.e., 120 MPa) within a range of nonlethal temperatures. High levels of viability

(around 70%) were observed after complete dehydration between 5 and 30 °C for 30 min (**Figure 1**). Our results showed that after a thermal shift at 120 MPa for 30 min, cell survival was dependent on the dehydration temperature. For example, after a thermal shift at 30 °C, yeast survival was around 27% when the dehydration was performed at 5 °C and around 73% when the dehydration was performed at 30 °C. This demonstrates that cell viability is highly dependent on the dehydration temperature and, therefore, on cell history. However, after isoosmotic treatment at 15 °C, yeast viability was around 50% and was independent of the dehydration temperature.

To explain the effect of combined osmotic and thermal treatment on yeast cells, a study previously carried out in our laboratory (*16*) showed that osmotically stressed yeast cells at different temperatures presented a viability that could be related to the plasma membrane phase-transition temperature. Such an event, which is also known as a phase separation state, is characterized by the coexistence of different lipid phases in the membrane (*20*) that lead to modification of membrane properties such as permeability (*21*). As previously shown by Laroche et al. (*8*), the mean membrane phase transition temperature of *S. cerevisiae* W303-1A increased with temperature and osmotic pressure. Therefore, in this study, cell viability could be related to changes in plasma membrane fluidity, as shown in **Figure 2**. This assumption, which relates cell survival to passive mechanisms, such as physical changes in the plasma membrane, is strongly supported by the absence of metabolism at high osmotic pressure. The results presented in **Figure 2** show that at 1.38 and 120 MPa, *r* values decrease with increasing temperature. Similar observations have been published by several authors such as Los and Murata (*22*). Moreover, as shown in **Figure 3**, cell viability appears to be highly related to absolute changes in plasma membrane fluidity at 120 MPa. Thus, yeast cells record changes that could be used to predict yeast survival at high osmotic pressure. In addition, yeast cell survival highly depends on cell history. However, this does not occur at 1.38 MPa because of the lack of temperature effect on yeast viability.

Although changes in the fluidity of the plasma membrane could play an important role in osmotic- and thermal-stressed yeast cells, some authors, such as Slaninovà et al. (*23*), have shown that plasma membrane invaginations could appear during dehydration. This study, along with our observations, suggests a hypothesis that may explain cell death during combined osmotic and thermal treatments. This would first involve the induction of plasma membrane vesicle formation when changes in fluidity of the plasma membrane occur during the heat shift at 120 MPa (this would need to be confirmed by subsequent microscopic observations). Such an event would lead to a decrease in the area of the plasma membrane.

The final steps consist of yeast rehydration to measure cell viability. During the return to initial osmotic conditions, when the membrane phase-transition temperature could occur a second time, yeast cell volume increases could be limited by a possible reduction in plasma membrane area, as previously explained. Such increases in cell volume could lead to disruption of the plasma membrane because of the presence of cell wall, as shown by Gervais et al. (*15*) in heat-shocked cells. These authors suggest that such an event leads to membrane permeabilization, then to intracellular leakage, and finally to cell death. Interestingly, as shown by several authors such as Leslie et al. (*24*), yeast cell death could also be related to the rehydration temperature. Indeed, if they are to remain viable, yeast cells must be rehydrated with warm water. In 1987, Beker and Rapoport (*25*) established that a minimal loss of intracellular components (such as nucleotides or potassium ions) occurred when dried yeast cells were rehydrated at 35−45 °C. In our study, the release of intracellular components could have occurred in yeast cells rehydrated at 5 °C and, in part, could explain the cell mortality we observed.

Moreover, our study led us to review the assumption, previously proposed by Laroche and Gervais in 2003 (*16*), that relates death of yeast cells dehydrated at nonlethal temperatures (5−30 °C) to water leakage through an unstable plasma membrane during the phase-transition temperature. To revise this assumption, it is important to note that (i) in accordance with the method presented in **Figure 1**, water flow across the cell membrane occurred during the previous dehydration step and not during the following heat shift and (ii) whatever was the dehydration temperature, yeast viability was always higher at the end of the dehydration step (i.e., step 2) than after the heat shift (i.e., step 3). Such observations led us to conclude that, contrary to the previous hypothesis, cell death could not be related to water flow, although plasma membrane integrity could play a major role in cell survival.

In conclusion, this study clearly shows that at the same level of nonlethal temperature, dehydrated yeast cells present different levels of survival that are a function of the dehydration temperature. In accordance with previous studies in our laboratory, the plasma membrane and, more particularly, changes in the fluidity of the plasma membrane seem to be involved in the survival and death of yeast cells exposed to these types of treatment, although other factors could be involved. Such an approach could be very helpful in improving the destruction or preservation of dried microorganisms.

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