Involvement of Two Specific Causes of Cell Mortality in Freeze-Thaw Cycles with Freezing to -196°C

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The purpose of this study was to examine cell viability after freezing. Two distinct ranges of temperature were identified as corresponding to stages at which yeast cell mortality occurred during freezing to -196°C . The upper temperature range was related to the temperature of crystallization of the medium, which was dependent on the solute concentration; in this range mortality was prevented by high solute concentrations, and the proportion of the medium in the vitreous state was greater than the proportion in the crystallized state. The lower temperature range was related to recrystallization that occurred during thawing. Mortality in this temperature range was increased by a high cooling rate and/or high solute concentration in the freezing medium and a low temperature (less than -70°C). However, a high rate of thawing prevented yeast mortality in this lower temperature range. Overall, it was found that cell viability could be conserved better under freezing conditions by increasing the osmotic pressure of the medium and by using an increased warming rate.

Cell freezing, which is commonly used in the food and pharmaceutical industries, allows preservation of microorganism with no loss of their functional characteristics or induction of genetic modifications. The freeze-thaw method, which is cheaper than the freeze-drying process, has been used industrially for several decades (2, 14). However, in spite of many studies, the exact mechanisms of cell injury by freezing are not known yet.

Most improvements in freezing have resulted from the use of cryoprotective agents (CPAs) (10). The purpose of CPAs is to lower the freezing point of the cellular cytoplasm by increasing the concentration of intracellular solutes. At the same time, addition of CPAs involves osmotic stress, which decreases the cellular volume before freezing (6). By these means, intracellular ice formation effects are reduced (1). However, the use of CPAs can lead to harmful toxicity due to excessive concentrations of intracellular electrolytes (16). CPAs should be used carefully; however, this may not solve the problem of hazardous osmotic stress effects on cells (10). Indeed, the osmotic stress induced by freezing could never be prevented completely except by using very concentrations of CPAs, which is not always possible for all cells.

The cooling rate is another parameter that has been used to optimize cell viability (2, 5, 6). Simultaneous management of the two parameters mentioned above, CPAs and the cooling rate, could be used. Indeed, the cooling rate determines ice crystal size during freezing. As a solution begins to freeze, water in the extracellular fluid turns to ice and hence increases the solute concentration in the liquid outside the cells. Subsequent osmosis then dehydrates cells as water diffuses from the cytoplasm into the more concentrated external solution (6, 14). When cooling rates are low (about $10^{\circ}\text{C min}^{-1}$), osmotically driven flow results in a reduction in volume, and all of the

intracellular water can flow out before intracellular crystallization (5, 6). When the cooling rates are in the middle range (200 to $5,000^{\circ}\text{C min}^{-1}$), the reduction in volume leads to irreversible damage to the cell, and significant mortality is observed. When cooling rates are very high, the cell may not have time to reduce its volume because of the rapid heat flow, and this may allow maintenance of significant viability (5, 6). Therefore, the kinetics of freezing have a great effect on cell viability; however, most of the time the cooling rate is not well controlled.

In numerous investigations workers have studied cell viability after freeze-thaw processes, and although many hypotheses have been proposed, the mechanisms of cell mortality are still not known. Two main mechanisms for damage associated with the cooling rate have been proposed. For low cooling rates, the extracellular solutes are concentrated in the remaining unfrozen extracellular water and cause cell dehydration by osmosis as water diffuses from the cytoplasm into the more concentrated external solution (6, 17). In contrast, high cooling rates result in intracellular ice formation during freezing, which is lethal for the cells (14, 19).

In previous studies, cell viability was related to the cooling rate, but all experiments were performed in liquid nitrogen and thus the final temperature was always -196°C (6). The purpose of this study was to observe the viability of the yeast *Saccharomyces cerevisiae* CBS 1171 after freezing to higher final temperatures.

MATERIALS AND METHODS

Biological materials. The yeast *S. cerevisiae* CBS 1171 was used in this study. Cultures were grown in 250-ml conical flasks containing 100 ml aerated modified Wickerham medium at 25°C and agitated at 250 rpm. This medium is composed of (per liter distilled water) 10 g glucose, 3 g pancreatic peptone, 3 g yeast extract, and 1.5 g Na_2HPO_4 . The pH of the medium was adjusted to 5.35 by adding orthophosphoric acid. Inoculation was carried out with 0.1 ml of a yeast suspension from a 48-h subculture grown under analogous conditions. Cells were harvested after 65 h of growth (stationary phase) and used immediately for experiments.

Freezing media. The binary media used for freezing are described in Table 1. These media were chosen for their water activities and the differences in their temperatures of crystallization. Nucleation of the medium was not induced, but

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TABLE 1. Compositions and characteristics of the media used

Medium	Water activity ^a	Osmotic pressure (MPa)	Mass of solute added to 1,000 g of water	Melting point (°C) calculated from equation 1
Water-glycerol	0.99	1.4	51	-1
Water-glycerol	0.91	14.5	461	-11
Water-glycerol	0.87	19.3	673	-14
Water-glycerol	0.83	30.0	892	-20
Water-sucrose	0.99	1.4	187	-1
Water-sorbitol	0.99	1.4	100	-1

^a Water activity was verified with a dew point osmometer.

the freezing temperature of each medium was calculated from the measured water activity (a_w) using equation 1 (4):

$$\ln(a_w) = -9.69 \times 10^{-3} \times \Delta T_m - 5.06 \times 10^{-6} \times \Delta T_m^2 \quad (1)$$

where $\Delta T_m = (T_o - T_p)$, where T_o (in K) is the freezing point of the solvent and T_p (in K) is the freezing point of the solution. The water activity can be expressed in terms of osmotic pressure (II) as follows:

$$\Pi = -\frac{RT \ln(a_w)}{V_w} \quad (2)$$

where Π is the osmotic pressure (in Pa), R is the gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature (in K), and V_w is the partial molar volume of water ($18 \times 10^{-6} \text{ m}^3 \text{ mol}^{-1}$).

Freezing and warming methods. Twenty milliliters of a yeast culture was centrifuged at $2,800 \times g$ for 5 min. The supernatant was then removed and replaced with 20 ml of the desired binary solution. Freezing was always initiated 15 min after osmotic shock.

Final temperatures were reached using liquid nitrogen for various times. Four distinct cooling rates were used in this study: 25, 115, 180, and $1,800^\circ\text{C min}^{-1}$. These rates were chosen because of their effects on cell viability. Indeed, previously (5, 6) it was shown that these cooling rates correspond to different levels of cell viability when cells are frozen in liquid nitrogen. These cooling rates were obtained using various containers and different volumes of cell suspension.

For the lowest cooling rate ($25^\circ\text{C min}^{-1}$), 0.5-ml samples in Nunc cryotubes (Vials, Germany) were placed in 50-ml plastic tubes (PolyLabo, France) for centrifugation so that they were protected from the cold gradient with a gas layer. The level of viability obtained with this cooling method was still significant after freezing to -196°C (about 30% mortality).

For cooling rates of $115^\circ\text{C min}^{-1}$ and $180^\circ\text{C min}^{-1}$, 1-ml samples were placed in Nunc and Sarstedt cryotubes (Sarstedt, Germany), respectively. These cooling rates resulted in significant cell mortality with freezing in liquid nitrogen.

A cooling rate of $1,800^\circ\text{C min}^{-1}$, which resulted in an increase in cell viability compared to that obtained with the other cooling rates, was obtained by introducing 0.1-ml samples into cylinders in glass disposable precalibrated Vitrex pipettes.

For all of the cooling rates studied, various final cooling temperatures were tested. Cell viability was determined after cultures were warmed to 25°C .

The temperature was monitored with a copper/copper-nickel thermocouple. This sensor was connected to an InstruNet 100 acquisition card (GW Instruments, Massachusetts). This system made it possible to obtain up to 60,000 measurements per s, which could then be examined using a spreadsheet. When the required temperature was reached in the middle of the sample, the thawing process was started.

Thawing was carried out by dropping a frozen sample into a controlled-temperature water bath. In the first experiments, the water bath temperature was controlled at 37°C , which allowed a warming rate of $40^\circ\text{C min}^{-1}$ to be achieved.

For warming rates of 55 and $170^\circ\text{C min}^{-1}$, the temperature of the water bath was kept at 50 and 90°C , respectively. An F81-MV ultracryostat (Julabo, France) was used to perform warming at a rate of $0.5^\circ\text{C min}^{-1}$.

Viability measurements. Viability measurements were obtained using the CFU method and plating cells on modified Wickerham medium supplemented with 15 g liter^{-1} Pastagar A. Petri dishes were then incubated for 2 days at 25°C . Viability was determined from the ratio of the number of colonies in experimental samples (i.e., after freezing at the appropriate temperature and thawing) to the number of colonies in control samples (without freeze-thawing). All the values were obtained in independent experiments. There were at least three repetitions of each experiment.

Differential scanning calorimetry. A DSC-7 (Perkin-Elmer Corporation, Connecticut) calorimeter was used for differential scanning calorimetry (DSC) analysis. Samples (approximately 4 mg) were measured in a temperature range from -110 to 25°C at a scanning rate of $10^\circ\text{C min}^{-1}$, as described by Simatos and

FIG. 1. Viability of *S. cerevisiae* after freezing at different final temperatures with different cooling rates (25, 115, 180, and $1,800^\circ\text{C min}^{-1}$) in a 1.4-MPa water-glycerol mixture. The error bars indicate 90% confidence intervals. The two temperature ranges corresponding to cell mortality are indicated by gray and stippled bars.

Blond (18). The DSC experiments were carried out in the absence and presence of microorganisms with all the freezing media used in this study and for cooling rates in the range from 10 to $100^{\circ}\text{C min}^{-1}$.

RESULTS

Impact of the final freezing temperature on yeast cell viability. The results obtained for the four cooling rates are shown in Fig. 1. Each point in Fig. 1 indicates the level of cell viability obtained after freezing at one of the temperatures studied, followed by thawing at a rate of $40^{\circ}\text{C min}^{-1}$.

For the four cooling rates used, a common decrease in cell viability was observed at temperatures between 0°C and -5°C . The cell mortality observed in this temperature range was a function of the cooling rate. Small decreases in cell viability, 17% and 9%, were observed for cooling rates of 25 and $1,800^{\circ}\text{C min}^{-1}$, respectively. In contrast, for cooling rates of 115 and $180^{\circ}\text{C min}^{-1}$, decreases in cell viability of 60% and 44%, respectively, were seen.

At lower temperatures, the viability remained stable and for the two lowest cooling rates was equal to the cell viability observed at -196°C . However, the existence of a second stage of induction of cell mortality was distinguished at temperatures between -40°C and -80°C for the two highest cooling rates. When the cooling rate was $180^{\circ}\text{C min}^{-1}$, the viability decreased from 52% at -60°C to 15% at -85°C . When the cooling rate was $1,800^{\circ}\text{C min}^{-1}$, in spite of the small decrease in viability in the temperature range from 0 to -5°C , there was a 45% increase in cell mortality between -40 and -80°C .

Therefore, the data in Fig. 1 show that two temperature ranges were related to cell mortality at the cooling rates examined. The first temperature range for cell mortality, at about -5°C , was observed for the four cooling rates, but various values were obtained. The second cell mortality temperature range was observed with the two highest cooling rates, $180^{\circ}\text{C min}^{-1}$ and $1,800^{\circ}\text{C min}^{-1}$.

Influence of cell water content and final freezing temperature on cell viability. In order to determine the influence of the intracellular water volume on the cell mortality in the two temperature ranges described above, several concentrations of glycerol were used to obtain different osmotic pressures.

For this study, a cooling rate of $180^{\circ}\text{C min}^{-1}$ was used because it allowed us to study both temperature ranges in which cell mortality occurred. The cell viability after cooling, which was related to osmotic pressure, is shown in Fig. 2.

The results obtained at an osmotic pressure of 1.4 MPa are similar to those shown in Fig. 1. For the medium at an osmotic pressure of 14.5 MPa, the viability fell to 63% between -15°C and -20°C . The cell viability then remained stable until -70°C and fell from 57% to 31% at -100°C and slowly decreased to a nadir of 14% at -196°C .

The first temperature range for cell mortality was extended with the medium at an osmotic pressure of 19.2 MPa, and there was a decrease in cell viability from 100% at -10°C to 47% at -55°C . At lower temperatures, the viability fell from 48% at -65°C to 28% at -100°C and then slowly decreased until it was 15% at -196°C .

For the medium with an osmotic pressure of 25.2 MPa, the cell viability decreased progressively. Then the viability fell

FIG. 2. Viability of *S. cerevisiae* after freezing at different final temperatures with a cooling rate of $180^{\circ}\text{C min}^{-1}$ in water-glycerol media with different osmotic pressures. The error bars indicate 90% confidence intervals. The two temperature ranges corresponding to cell mortality are indicated by gray and stippled bars.

rapidly to 60% at -100°C and remained stable down to a temperature of -196°C .

The decreases in cell viability related to the final freezing temperature were not equal with respect to the osmotic pressure of the medium. The first temperature range for cell mortality could be identified at osmotic pressures of 1.4, 14.5, and

FIG. 3. Viability of *S. cerevisiae* after freezing at different final temperatures with a cooling rate of $180^{\circ}\text{C min}^{-1}$ in three water-solute media with an osmotic pressure of 1.4 MPa and a thawing rate of $40^{\circ}\text{C min}^{-1}$. The error bars indicate 90% confidence intervals.

19.2 MPa but not when the osmotic pressure of the medium was 25.2 MPa. With this exception, the higher the osmotic pressure, the lower the temperature for the first stage of mortality. The second range of cell mortality was observed at all osmotic pressures of the medium and was always observed at temperatures close to -80°C .

To study the influence of glycerol on the second temperature range for mortality, three solutes with the same osmotic pressure were used (Table 1), and sucrose and sorbitol were used to replace glycerol as the solute in the freezing media.

Figure 3 shows that the curve for cell viability after freezing in water-sucrose or water-sorbitol medium was similar to the curves obtained with glycerol. A decrease in cell viability of 55% was observed at about -5°C , and the viability then remained stable at temperatures down to -70°C . The viability then fell to 22% at -90°C .

With a water-sorbitol medium, the first decrease in viability, to 70% viability, was seen at -5°C . The viability then remained stable at temperatures down to -60°C , before it fell to 20% at -100°C . Therefore, with a cooling rate of $180^{\circ}\text{C min}^{-1}$, an increase in the osmotic pressure of the medium decreased the temperature of the first stage of mortality, until it disappeared when the osmotic pressure of the medium was 25.2 MPa. Conversely, the second stage of mortality was always observed at this cooling rate, regardless of the osmotic pressure and solute used.

Influence of the thawing rate on cell viability related to the temperature of freezing. The aim of part of the study was to determine the influence of the warming rate on viability in each temperature range. Several warming rates were used with suspensions of *S. cerevisiae* CBS 1171 previously frozen to various temperatures at a cooling rate of $180^{\circ}\text{C min}^{-1}$. This cooling rate is not favorable for cell viability (Fig. 1). The first temperature studied was 40°C , which corresponded to a temperature higher than the temperature for the second stage of mortality. The second temperature used was -100°C , which is lower than the temperature for the second stage of cell mortality.

Warming rates of 0.5, 40, 55, and $170^{\circ}\text{C min}^{-1}$ were studied. The results showed that for all warming rates studied, the cell

viability after freezing to -40°C was between 58% and 72%. Therefore, the warming rate had no significant influence in this case. In contrast, for specimens cooled to less than -100°C , the higher the warming rate, the higher the viability; the viabilities were 0.6% for a warming rate of $0.5^{\circ}\text{C min}^{-1}$, 14% for a warming rate of $40^{\circ}\text{C min}^{-1}$, 44% for a warming rate of $55^{\circ}\text{C min}^{-1}$, and 66.2% for a warming rate of $170^{\circ}\text{C min}^{-1}$. Thus, the warming rate had an impact only when the cooling temperature was less than -40°C .

DISCUSSION

In previous studies, it was shown that the cell mortality observed for the cooling rates used in this study results from competition between water and heat efflux from the cell (5, 6). Indeed, because the cytoplasm is more concentrated than the growth medium, the external water freezes before the cell contents, which increases the osmotic pressure of the medium. The extracellular solutes are concentrated in the remaining unfrozen extracellular water, and this dehydrates the cell by osmosis as water diffuses from the cytoplasm into the more concentrated external solution. The lower the temperature of the medium, the greater the concentration of the unfrozen solution and the greater the loss of intracellular water.

Cell death was proposed to occur due to the massive water outflow, which was related to an increase in the extracellular osmotic pressure and the membrane-lipid phase transition, or from crystallization during water outflow from the cell, which involved lethal membrane damage.

Based on this assumption, cell death must occur during the lag time determined by the crystallization of the medium. Figure 1 shows that there was a stage of cell mortality that corresponded to temperatures between 0°C and -5°C . However, there was a second stage of cell mortality at lower temperatures, as shown in several of the graphs in Fig. 1 and 2. Based on our results, some explanations about these two stages of cell mortality can be proposed.

First range: impact of crystallization. For solutions mainly composed of water, the range of temperatures at which almost all crystallization takes place is reduced to a few degrees after the onset of freezing (8), and the crystallization temperature is dependent on the concentration of the solute. The theoretical temperatures for the onset of freezing in the water-glycerol media used in this study at osmotic pressures of 1.4 MPa, 4.5 MPa, 19.2 MPa, and 25.2 MPa were -1°C , -11°C , -14°C , and -20°C , respectively, as calculated with equation 1. If the cellular mortality observed in the first temperature range is truly related to the crystallization of the medium, when the concentration of the solute increases, the mortality must occur at a lower temperature. This hypothesis was verified by the data shown in Fig. 2: the higher the glycerol concentration, the lower the temperature of the first range of cell mortality. The relationship between the temperature of crystallization and the temperature at which mortality occurs is shown in Table 2.

For the medium with an osmotic pressure of 30 MPa, no crystallization was observed. However, this medium contained 47% (wt/wt) glycerol, the freezing rate was $180^{\circ}\text{C min}^{-1}$, and high glycerol concentrations and rapid cooling rates promoted transition to a vitreous state. Thus, the higher the concentration of glycerol, the lower the cooling rate required for vitrification. A

TABLE 2. Comparison of experimentally determined and calculated crystallization temperatures of the media used and decreases in cell viability observed in the range of temperatures used

Osmotic pressure of medium (MPa)	Theoretical temp of crystallization (°C)	Observed temp of crystallization (°C)	% Mortality in the temp range corresponding to the crystallization temp
1.4	-1	-5	45
14.5	-11	-15 to -19	40
19.3	-14	-15 to -55	50
30.0	-20	None	20

solution containing 45% (wt/wt) glycerol should vitrify completely at cooling rates higher than $70^{\circ}\text{C min}^{-1}$, whereas a solution containing 50% glycerol vitrifies with a cooling rate of $38^{\circ}\text{C min}^{-1}$ (3). Furthermore, results obtained using DSC have confirmed that even in the presence of yeast cells, significant vitrification could be observed in this medium at cooling rates higher than $100^{\circ}\text{C min}^{-1}$ (data not shown). In this case, no shift in the temperature corresponding to external crystallization was observed for the temperature kinetics. Therefore, cell mortality in the first range of temperatures is related to crystallization of the medium and, consequently, to the cooling rate. This observation is in agreement with the assumed mechanism for cell mortality during freezing. Indeed, crystallization of the medium is the origin of the osmotic phenomena that induce cell dehydration, and rapid water outflow can explain the mortality seen with the cooling rates used in this study (5). Nevertheless, there is no explanation for cell mortality at temperatures lower than those in the zone of crystallization. Thus, the second stage of cell mortality requires other explanations.

Second range: influence of the warming rate. (i) Mortality occurs simultaneously with vitrification of the medium. The presence of a second range of temperatures corresponding to cell mortality is shown in Fig. 1 and 2. The existence of this range cannot be explained by crystallization of the medium.

As the onset of crystallization corresponds to the first temperature range for cell mortality, a role for crystallization in the second temperature range is not possible. Neither supercooling of the medium nor the gap of more than 40°C can be physically explained. However, the second range for mortality always occurs in the same range of temperatures (i.e., between -50°C and -100°C), regardless to the cooling rate (Fig. 1) or the osmotic pressure of the medium (Fig. 2).

The level of cell mortality in the second stage depends on the cooling rate (Fig. 3). The data show that mortality is reduced with lower cooling rates (25 and $115^{\circ}\text{C min}^{-1}$). Therefore, a minimal cooling rate is necessary to observe the mortality in the second range. The increased cooling rate promotes a decrease in the size of the crystals and the occurrence of vitrification, which are not considered harmful to cells (7, 15, 20). Indeed, the vitreous state involves no variation in the volume and thus no mechanical stress to the cells. Moreover, it does not induce any changes in the solute concentration, which prevents osmotic phenomena and intracellular solute toxicity. The vitreous state is also known to protect the cellular membrane and to conserve protein structure (11).

As shown in Fig. 2, the use of glycerol-water solutions containing glycerol at several concentrations influenced cell viability. With regard to the glycerol concentration, the second range of cell mortality was always observed at a cooling rate of $180^{\circ}\text{C min}^{-1}$, in contrast to the first stage of cell mortality, in which an increased glycerol concentration resulted in loss of the lethal effect. Therefore, for the second range, the presence of glycerol seems to be important, regardless of its concentration. It has been noted that -80°C , which is always included in the second range of cell mortality, corresponds to the vitreous-phase transition temperature of glycerol (1).

Experiments with different solutes were performed in order to determine the role of vitrification in the media in cell mortality. However, the curves obtained with glycerol, sorbitol, and sucrose, solutes that are characterized by different glass transition temperatures (-80°C [1], -52°C [21], and -7°C [12], respectively), are very similar (Fig. 3).

Thus, viability in the second range of mortality appears to be independent of the solute used but to be strongly related to a high cooling rate.

(ii) Mortality is linked to the thawing rate. As the glass-forming tendency increases, water recrystallization during warming can be observed. This well-known recrystallization during devitrification is identified on a DSC thermogram as an exothermic peak close to the vitreous transition temperature (generally for a higher temperature), with the position related to the quantity of water in the medium analyzed (8, 13). This crystallization peak appears during warming, particularly if the water did not crystallize during freezing because of a high cooling rate or a high solute concentration, as shown in Fig. 4. Based on the DSC results, the cause of the cell mortality in the second temperature range appears to have been clarified. We have not observed this phenomenon with cooling rates lower than $10^{\circ}\text{C min}^{-1}$, but it was always found with cooling rates higher than $100^{\circ}\text{C min}^{-1}$. Furthermore, the freezing process requires temperatures lower than -70°C for the appearance of the second mortality stage. Thus, high cooling rates and consequent vitrification are important in allowing recrystallization to occur during warming, and as cooling rates and temperatures below

FIG. 4. Heat flow thermograms obtained during the DSC warming protocol at $10^{\circ}\text{C min}^{-1}$ for a water-glycerol medium with an osmotic pressure of 1.4 MPa and a cooling rate of $100^{\circ}\text{C min}^{-1}$. The positive values for heat flow imply that there was endothermic heat release in the sample.

–70°C are known to enhance recrystallization, we propose that such a phenomenon is the origin of the observed cell death.

Mortality in the second critical temperature range is not directly linked to cooling but appears to be related to the warming process. Indeed, the results obtained for the different warming rates confirm this hypothesis. When the final temperature is above the critical temperature range, the warming rate does not influence cell viability. However, when a sample is cooled to a temperature below the critical temperature range, the warming rate becomes a major parameter in the determination of cell viability. Again, the cell mortality is related to the crystallization of the medium, although in the second temperature range, the role of crystallization is different. Indeed, as previously described, crystallization in the first mortality stage induces osmotic disturbances, which for the cooling rates used in this study are the cause of cell death. In the second mortality stage, crystallization of the medium induces only small osmotic perturbations because of the low hydration of the medium at the very low temperatures. Intracellular recrystallization during warming can be anticipated, and the mechanical effects induced can be lethal to cells. Cell injury may be a consequence of internal freezing in critical organelles, such as mitochondria, rather than a consequence of ice formation in the whole cytoplasm, as proposed by Gao et al. (9).

This study enabled us to better understand the freeze-thaw process, particularly the management of the rate of temperature change. If the cooling rate is low, cell viability is a function of the osmotic tolerance of the microorganism (5), regardless of the thawing rate. In contrast, if the cooling rate is ultrahigh (e.g., about 30,000°C min⁻¹, as described in previous studies [5, 6]), osmotic perturbations in the cell are prevented and cell viability can be maintained if the warming rate is high enough to prevent intracellular crystallization.

Conclusions. This study of cell viability during freezing allowed us to identify two distinct temperature ranges that correspond to increases in yeast cell mortality after freezing to –196°C.

The effects in the first mortality range are related to the temperature of crystallization of the medium. This temperature range depends on the solute concentration, and mortality does not occur at high solute concentrations. In this case, the cell mortality is due to the consequences of external crystallization (i.e., a large osmotic water efflux from the cell through a weakened membrane) or to water crystallization in the membrane during water efflux.

The second mortality range is related to crystallization that occurs during warming after the cooling stage. Cell mortality due to a high cooling rate and/or a high concentration of the freezing medium requires temperatures lower than –70°C and can be avoided by using a high warming rate. If a high warming rate is not used, the glass-forming tendency induced by these conditions permits the formation of intracellular and extracellular water crystals during the warming stage, which result in mechanical injuries to cells.

Therefore, the two causes of cell mortality described here (one extracellular and involving osmotic perturbations, and the other intracellular and occurring during warming) are linked to crystallization and depend on the cooling rate. Indeed, if the cooling rate is low, cell mortality occurs during the cooling

phase and is observed particularly in the first temperature range. In comparison, if the cooling rate is high enough to promote vitrification, cell death is more prominent in the second temperature range. Thus, we found that conservation of cell viability following freezing could be improved by increasing the osmotic pressure of the medium and the warming rate should be optimized.

This study showed the importance of optimization of warming methods, particularly when the cooling protocol leads to significant vitrification of the medium (that is, with a high cooling rate, a high solute concentration, and a low temperature).

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