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Increased xerotolerance of *Saccharomyces cerevisiae* during an osmotic pressure ramp over several generations

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Summary

Although mechanisms involved in response of *Saccharomyces cerevisiae* to osmotic challenge are well described for low and sudden stresses, little is known about how cells respond to a gradual increase of the osmotic pressure (reduced water activity; a_w) over several generations as it could encounter during drying in nature or in food processes. Using glycerol as a stressor, we propagated *S. cerevisiae* through a ramp of the osmotic pressure (up to high molar concentrations to achieve testing-to-destruction) at the rate of 1.5 MPa day^{-1} from 1.38 to 58.5 MPa (0.990–0.635 a_w). Cultivability (measured at 1.38 MPa and at the harvest osmotic pressure) and glucose consumption compared with the corresponding sudden stress showed that yeasts were able to grow until about 10.5 MPa (0.926 a_w) and to survive until about 58.5 MPa, whereas glucose consumption occurred until 13.5 MPa (about 0.915 a_w). Nevertheless, the ramp conferred an advantage since yeasts harvested at 10.5 and 34.5 MPa (0.778 a_w) showed a greater cultivability

than glycerol-shocked cells after a subsequent shock at 200 MPa (0.234 a_w) for 2 days. FTIR analysis revealed structural changes in wall and proteins in the range 1.38–10.5 MPa, which would be likely to be involved in the resistance at extreme osmotic pressure.

Introduction

In nature, yeasts have colonized different habitats and niches where they live at the interface between water and air (as soil and plants surfaces) meaning they have cope with fluctuating wet and dry conditions (Dupont *et al.*, 2014). Drying is also a widely used process in food industry to preserve yeasts of interest, such as *Saccharomyces cerevisiae*. When yeasts are exposed to drying, they encounter firstly mechanical stresses at the level of the plasma membrane and osmotic changes and then further water loss leading to the concentration of osmolytes and increases in cytoplasmic viscosity, and oxidative stress (arising from lipid peroxidation, desiccation, etc).

In a liquid environment, Martínez de Marañón *et al.* (1996) have reported that water potential (ψ) could be mainly related to the osmotic potential (ψ_s) and in a lesser extent to pressure potential (ψ_p) (Equation 1).

$$\psi = \psi_s + \psi_p \tag{1}$$

ψ_s results from the presence of solutes and could be related to the osmotic pressure (Π, Pa) and to the thermodynamic parameter water activity (a_w) as shown by Equation 2 where R is the universal gas constant (~ 8.31 J.mol^{-1}.K^{-1}), T is the temperature (K) and $\overline{V_m}$ the molar volume of water (m^3.mol^{-1}):

$$-\psi s = \Pi = -\frac{R.T.\ln a_w}{V_m} \tag{2}$$

ψ_p results from the hydrostatic pressure (P, Pa) as shown by Equation 3:

$$\psi_p = P \tag{3}$$

We know much about osmotic stress in microbial cells: (i) the optimal growth rate of many microbes occurs at osmotic pressures between 1 and 15 MPa (Marechal *et al.*, 1995), (ii) there is an osmotic pressure (or water

activity) value below which no more growth occurs (Stevenson *et al.*, 2015) and (iii) cells with a thick wall maintain their cytosol at a slightly higher osmotic pressure than that of the extracellular milieu. This difference in osmotic pressures, defined as turgor pressure ($\Delta P = \Pi_i - \Pi_e$, where *i* and *e* represent the intracellular and the extracellular medium respectively), is around 1.5 MPa in *Schizosaccharomyces pombe* in optimal conditions (Rojas and Huang, 2018).

Fluctuations in glycerol concentration do not induce permanent changes in cell volume because this solute passes relatively quickly through the plasma membrane (de Lima Alves *et al.*, 2015). However, glycerol does induce cellular stress by increasing osmotic pressure, that is reducing water activity (de Lima Alves *et al.*, 2015). When present at molar concentrations, this polyol can also induce stress by reducing non-covalent interactions within and between biomacromolecules and increasing entropy within their structures; an effect known as chaotropicity (Ball and Hallsworth, 2015). This phenomenon, known as chaotrope-induced stress, secondarily induces oxidative stress and also triggers a chaotrope-specific stress response (Hallsworth *et al.*, 2003; Cray *et al.*, 2015). In natural habitats, as well as within anthropogenic systems and foods, solutes can reach molar concentrations. However, studies of glycerol stress at high concentration are relatively scarce (Hallsworth, 2019), and relatively little attention has been given to extreme osmotic stresses up to 30 MPa which are often encountered in the nature (as in the case of high solute concentrations, air drying and desiccation induced by the sun) and in food processes as in the case of spray-drying and drying (Lemetais *et al.*, 2012; Zoz *et al.*, 2016). Most laboratory-based studies of solute-induced stresses have been conducted using salts (e.g. NaCl and KCl) or higher molecular mass polyols (e.g. sorbitol) to reach osmotic pressures lower than 5.5 MPa (Edgley and Brown, 1983; Klipp *et al.*, 2005; Mettetal *et al.*, 2008; Muzzey *et al.*, 2009; Parmar *et al.*, 2011; Miermont *et al.*, 2013; Araújo *et al.*, 2020).

In relation to microbial viability, we know that the slower the hyperosmotic kinetics, the higher the cultivability (or ability to grow) of yeasts and the slower the rehydration step, the higher the cultivability (Marechal *et al.*, 1999; Dupont *et al.*, 2010). In a batch culture, the final osmotic pressure influences the conformation of proteins and other macromolecules (Bellissent-Funel *et al.*, 2016) and also, therefore, cellular functions. Few data have been published on the precedent transitory phase during which water flows out of the cell more or less rapidly and could induce plasma membrane invagination or lateral reorganization, depending on the dehydration kinetics (Dupont *et al.*, 2010).

When a cell is exposed to a sudden hyperosmotic variation, two consecutive phases occur: (i) a transient phase (<1 s in the presence of a binary solution) characterized by water release from the cell, which may lead to alteration of plasma membrane integrity (water flow rate is proportional to the water potential gradient across the membrane) and the cell to reach a constant volume; and (ii) a steady-state phase during which cellular viability decreases and the integrity of the plasma membrane (and other cellular components) could change over time even if the cell volume remains constant (Berner and Gervais, 1994; Gervais *et al.*, 1996; Gervais and Beney, 2001; Dupont *et al.*, 2010; Dupont *et al.*, 2011). Although during the steady-state phase there is no difference in osmotic pressure between the cytosol and the extracellular medium, the amount of available water which participates in macromolecule hydration is reduced due to the increase of the osmotic pressure and so the decrease in water activity. Thus, during the stationary phase as soon as equilibrium is reached (i.e. there is no osmotic pressure gradient), cell death can occur and increase over time as it is the case during heat treatment (Dupont *et al.*, 2010).

If glycerol is added at low mM concentrations to a microbial culture cell, turgor changes transiently though glycerol rapidly equilibrates across the plasma membrane (de Lima Alves *et al.*, 2015). Nevertheless, it can also be involved in carbon metabolism as it can be used as a carbon source under aerobic conditions and it can be a by-product when fermentable sugars (as glucose) are converted to ethanol (Nevoigt and Stahl, 1997). Glycerol exhibits considerably chaotropicity in xerophilic fungi that can be exposed to high concentrations in the region of 6-7 M, but paradoxically retains some of its properties as a cellular protectant (Williams and Hallsworth, 2009; de Lima Alves *et al.*, 2015; Stevenson *et al.*, 2017b; Stevenson *et al.*, 2017a). During osmotic stress, the biotechnologically important cell-factory *S. cerevisiae* produces glycerol via the high-osmolarity glycerol response pathway. Furthermore, glycerol penetrates the plasma membrane freely (but slower than water), and so leaks into, and can accumulate in the extracellular medium membrane (de Lima Alves *et al.*, 2015). Glycerol can be present at high concentrations in both the cytosol and cellular environment of some microbes and microbial habitats [about 7 M equiv. to about 0.700 water activity and 40 MPa osmotic pressure].

Glycerol synthesis is central to the biochemistry of carbohydrate metabolism in *S. cerevisiae*, and glycerol stress in this yeast was identified some time ago. It is only recently, however, given the pressing need to optimize yeast cell performance during biofuel fermentations that research attention has begun to focus on glycerol as a stressor for *S. cerevisiae* (Cray *et al.*, 2015;

Mattenberger *et al.*, 2017). The current study focuses on the hypothesis that xerotolerance of *S. cerevisiae* increases during prolonged exposure to glycerol. We progressively increased glycerol concentration (1.38–58.5 MPa at the rate of 1.5 MPa day^{-1}) over several generations propagated during 40 days. Growing cells in the presence of glycerol at iso-osmotic pressure (1.38 MPa) and then exposing them to a higher osmotic pressure still in the presence of glycerol can be considered an osmotic stress with a non-permeating solute. High concentrations of glycerol in the cell imply a main osmotic water flow and in a lesser extend a minor glycerol and water Fickian diffusion flow through the plasma membrane. This is evidenced by the higher permeability coefficients (*P*) through an egg phosphatidylcholine planar membrane or a red blood cell membrane for water (3.4 × 10^{-3} cm s^{-1} and 1.2 × 10^{-3} cm s^{-1}) compared with those for glycerol (5.4 × 10^{-6} cm s^{-1} and 1.6 × 10^{-7} cm s^{-1}) meaning that water flow through a biological membrane is about 1000 fold higher than glycerol flow (Lieb and Stein, 1986; Walter and Gutknecht, 1986). Glucose consumption and yeast cultivability were then measured during the ramp of osmotic pressure. Cultivability was assessed after spreading colonies on solid medium at iso-osmotic pressure (1.38 MPa) and at the osmotic pressure at which yeasts were harvested during the osmotic pressure ramp. This approach revealed that growth occurred up to about 10.5 MPa and survival was possible up to 58.5 MPa. Although this ramp did not lead to increased xerotolerance between 1.38 and 58.5 MPa, xerotolerance was found to be increased when *S. cerevisiae* was subsequently exposed to an extreme osmotic pressure shock until 200 MPa (0.234 a$_w$). To understand the mechanisms involved in such xerotolerance, cell wall components and cell proteins were then analysed (using FTIR spectroscopy). Structural changes in wall and proteins found in the range 1.38–10.5 MPa would be involved in resistance at 200 MPa.

Results and discussion

We determined whether a slow increase of osmotic pressure (reduction of water activity) via a ramp of glycerol concentration confers any adaptive advantage for *S. cerevisiae* by comparison with cells exposed to a glycerol shock of the same magnitude. We started by determining the osmotic pressure window for growth and survival. Determinations of ability to grow were carried out on solid nutrient media at iso-osmotic pressure (1.38 MPa) and at osmotic pressures at which yeast cells of *S. cerevisiae* were harvested during the ramp. To determine the impact of this ramp on xerotolerance, cells collected at the high osmotic pressure phase of the ramp were then submitted to a glycerol shock at extreme osmotic pressure

(200 MPa) and survival (according to ability to grow) was compared to that of cells cultured at 1.38 MPa. FTIR analysis of cellular protein secondary structure and the cell wall was performed to better understand basis of any xerotolerance acquired during the ramp.

Osmotic pressure range for growth and survival of S. cerevisiae *by testing-to-destruction through the means of osmotic shocks*

The optimal osmotic pressure, at which *S. cerevisiae* is widely cultivated, is close to 1.38 MPa (Marechal *et al.*, 1995; Guyot *et al.*, 2006). In the present study, the initial growth osmotic pressure has been adjusted by addition of glycerol in the growth medium.

To determine the osmotic pressure window for growth and survival of the *S. cerevisiae* strain CBS1171, cultures initially grown at 1.38 MPa were subjected to 6 different levels of osmotic shocks ranging from 1.38 to 200 MPa and then maintained for 1, 24 and 48 h under aerobic conditions at 25°C (see *Experimental procedures* for details) at 25°C. The intensity of the glycerol shock is mitigated by pre-adaptation of cells (including high levels of glycerol already in the cytosol), and the passage of glycerol through the plasma membrane. So, there is a momentary hyperosmotic shock generated by high levels of glycerol, during which a decrease in cell volume occurs due to a transient water outflow (Gervais and Beney, 2001; Dupont *et al.*, 2011). This net outflow of water occurs because the movement of water across the plasma membrane is more rapid than that of glycerol. Substantial growth of *S. cerevisiae* occurred up to 10.5 MPa (0.926 water activity) and limit for survival was close to 34.5 MPa (0.778 water activity) (Table 1).

Studies have reported diverse values of osmotic pressure/water activity at which growth ceases, depending on solute stressor and the *S. cerevisiae* strain. King (1993) showed that growth of *S. cerevisiae* (strain not specified) is inhibited at water activity (a$_w$) of 0.94 (about 8.6 MPa) in the presence of glucose at 3.16 M and sorbic acid at 25 ppm (pH 4, 27°C). Edgley and Brown (1983) showed that it was possible to acclimate *S. cerevisiae* and its xerotolerant counterpart *Saccharomyces rouxii* from a basal medium with a high water activity 0.997 (about 0.42 MPa) to the same medium supplemented with 10% w/v NaCl at water activity 0.936 (about 9.26 MPa) both at 30 °C. Other studies record values at low water activity ranging from 0.900 (about 14.51 MPa) to 0.880 (about 17.60 MPa) (Cray *et al.*, 2015). Other yeasts, such as *Rhodotorula glutinis* and *Mestchnikowia pulcherrima*, can grow down to water activity 0.94 (about 8.5 MPa) and 0.85 (about 22.4 MPa), respectively, at 25°C with glucose as the stressor (Rousseau and Donèche, 2001). Although the ability of fungi to grow under solute-induced stresses

Table 1. Upper osmotic pressures (lower water activity) limits for *S. cerevisiae* CBS1171 growth and survival in the presence of glycerol by measuring cell number [Log(N/No)].

π (MPa)	Water activity (-)	Time (h)		
		1	24	48
1.38	0.990	0.05 ± 0.09	1.33 ± 0.10***	1.30 ± 0.15***
10.5	0.926	0.03 ± 0.03	0.69 ± 0.48	1.05 ± 0.09**
16.5	0.887	0.08 ± 0.05	0.02 ± 0.33	0.13 ± 0.09
34.5	0.778	-0.08 ± 0.11	-0.04 ± 0.11	-0.16 ± 0.10*
50	0.695	-0.07 ± 0.06	-0.30 ± 0.14*	-0.51 ± 0.11**
100	0.483	-0.46 ± 0.06***	-1.45 ± 0.09***	-1.94 ± 0.11***
200	0.234	-1.31 ± 0.17**	-3.36 ± 0.02***	-3.36 ± 0.02***

Growth

Death

Yeasts were glycerol-shocked to osmotic pressures ranging from 1.38 to 200 MPa at 25°C and then plated at 1.38 MPa. Results are presented as Log(N/No) where No is the initial CFU count and N the final CFU count after glycerol shock. CFU counting was performed after 1 h, 24 and 48 h maintenance at the indicated osmotic pressure. The means ± standard deviation of at least three independent measurements were calculated. The significance of difference between osmotic shocks and control (i.e. the yeast control grown at 1.38 MPa and subsequently glycerol-shocked: log(N/No) = 0.00) was determined by Student's unpaired *t*-test (see Experimental procedures section).

depends largely on osmotic pressure / water activity, other parameters such as the nature of the solute, life history and physiological condition of the cell, other environmental parameters (e.g. temperature) and nutrients available also act as determinants (Bubnová *et al.*, 2014; Stevenson *et al.*, 2015).

In the presence of glycerol, *S. cerevisiae* CBS1171 survived up to 34.5 MPa for 24 h and 50 MPa for only 1 h (Table 1). A value of 50 MPa (0.695 water activity) corresponds to the osmotic pressure level where yeast volume is close to the non-osmotic volume meaning that in glycerol solutions this osmotic pressure is a viability threshold (Gervais and Beney, 2001). Cell survival/death is known to depend on both the speed and magnitude of the solute stress imposed. In general, the more rapid and traumatic a stress event is, the more lethal it is. Conversely, the more gradual a stress condition is, the more time there is for adaptation (Hamill *et al.*, 2020). During a sudden hyperosmotic stress (osmotic shock), rapid outflow of water from the cell can be accompanied by changes in plasma membrane hydration that lead to cell death (Marechal *et al.*, 1995, 1999; Gervais and Beney, 2001). The plasma membrane condition, therefore, is closely implicated both in survival and death during an osmotic stress (Laroche and Gervais, 2003; Simonin *et al.*, 2007).

Membrane components such as sterols, for example, are key factors (Dupont *et al.*, 2011).

We considered whether *S. cerevisiae* could be acclimated or adapted to high level of osmotic pressure; more specifically whether this yeast can cope for more than 1 h at osmotic pressures, respectively, upper to 10.5 MPa (for growth) and 34.5 MPa (for survival). To grow at high osmotic pressure, *S. cerevisiae* must maintain plasma membrane structure (as well as those of other macromolecular systems), retain key metabolic activities, cell turgor (difference between intracellular and extracellular pressures, about 0.6 ± 0.2 MPa) (Schaber *et al.*, 2010). To test the ability of *S. cerevisiae* CBS1171 to acclimate or adapt to high osmotic pressure (low water activity), cells were subjected to a gradual ramp of glycerol concentration at a rate equivalent to 1.5 MPa.day^{-1} (from 1.38 to 58.5 MPa or from 0.990 to 0.654 water activity) over 39 days, at 25°C.

Osmotic pressure range for growth and survival of S. cerevisiae *changes induced by a gradual increase of the osmotic pressure*

Yeast cells of *S. cerevisiae* were subjected to a gradual increase of the osmotic pressure (ramp of glycerol

concentration) at the rate of 1.5 MPa day^{-1} from 1.38 MPa in daily renewed growth medium in the presence of glycerol (see *Experimental procedures*). To determine windows for growth and survival, *S. cerevisiae* was concomitantly spread on nutrient agar with osmotic pressures the same as those at the beginning of the glycerol ramp (1.38 MPa) and the osmotic pressure reached when the samples were collected during the ramp. Indeed, we checked that cells from colonies which formed on solid nutrient media at any given osmotic pressure were also able to grow in liquid nutrient media up to the osmotic pressure attained at the end of the ramp. In the liquid medium, during the glycerol ramp, *S. cerevisiae* that had formed colonies on solid media at 1.38 MPa was assumed to be alive. Control cultures maintained their ability to grow (Fig. 1A) whereas cultures that had been exposed to the glycerol ramp exhibited a reduced ability for subsequent growth (Fig. 1B). More specifically, cell suspensions exhibited different cultivability profiles depending on the osmotic pressure at which they were plated (Fig. 1B). Then, the number of cultivable cells remained constant for control (Fig. 1A) and cells that had been exposed to the glycerol ramp, up to an osmotic pressure of up to 10.5 MPa (Fig. 1B). Indeed, up to 10.5 MPa yeasts were able to form colonies when they were plated at the osmotic pressure attained by the end of the ramp and at 1.38 MPa (Fig. 1 B, Zone 1). Nevertheless, no colonies were detected on solid media with an osmotic pressure of between 10.5 and 58.5 MPa inclusive, whereas colonies were present when cell suspensions had been spread at 1.38 MPa meaning that growth occurred during the glycerol ramp from 1.38 to 10.5 MPa (Fig. 1B, Zone 1) and above 10.5 MPa only glycerol-adapted cells survived (Fig. 1B, Zone 2). Thus, *S. cerevisiae* (CBS1171) was able to undergo cell division from 1.38 to 10.5 MPa inclusive. Comparing these results (Fig. 1B) with those previously presented (Table 1), we assume that the glycerol ramp did not permit cells to extend the upper limit for growth, which was close to 10.5 MPa.

Regarding the decreased ability to grow at 1.38 MPa in Fig. 1B, Zone 2, the population detected at 10.5 MPa may have been composed of two or more phenotypically distinct subpopulations. The first one died between 10.5 and 13.5 MPa (Fig. 1B, Zone 2a) and the other(s) remained alive and active from 13.5 to 31.5 MPa (Fig. 1 B, Zone 2c). Beyond this osmotic pressure range, there was a continuous decrease of the number of cells able to form colonies up to 58.5 MPa (Fig. 1B, Zone 2d), so the remaining population was heterogeneous, including phenotypes with varying levels of adaptation to glycerol stress.

To determine whether cells were metabolically active at osmotic pressures above 10.5 MPa, residual glucose concentrations were determined. When *S. cerevisiae* was able of division, such as control cultures maintained at 1.38 MPa (Fig. 1A) and cells exposed the glycerol ramp from 1.38 to 13.5 MPa (Fig. 1B, Zones 1 and 2a), there was no residual glucose in the culture medium. Nevertheless, residual glucose increased from 13.5 (Fig. 1B, Zone 2b) to 18 MPa and then remained stable until the end of the glycerol ramp, meaning that yeasts consumed less and less glucose as the ramp continued to a point where the glucose consumption ceased. Experimental and modelling studies have demonstrated the importance of key factors as Hog1 and turgor pressure upon moderate hyperosmotic shocks by NaCl from 0.2 to 1.8 M (up to about 5.3 MPa and down to water activity 0.96) (Klipp *et al.*, 2005; Muzzey *et al.*, 2009; Parmar *et al.*, 2011; Petelenz-Kurdziel *et al.*, 2013; Tatebayashi *et al.*, 2020). Here, we show that glucose uptake occurred up to 13.5 MPa (down to 0.906 water activity) meaning that metabolism continued up to this limit. We hypothesized that glucose was the sole carbon source that was utilized up to 13.5 MPa since the genes involved in glycerol metabolism (GUT1, PCK1 and FBP1) could be repressed due to the presence of glucose (Weinhandl *et al.*, 2014); and water activity measurements performed after 24 h revealed that water activity (and so osmotic pressure) did not change, indicating that glycerol was not consumed. The upper limit of metabolic activity could be set at 13.5 MPa since a previous study showed that in the presence of 10% NaCl (w/v) at 0.936 water activity (about 9.26 MPa) some enzyme activities were maintained (Edgley and Brown, 1983).

In the light of these observations, one can ask whether the decrease in cultivability combined with the decline then the lack of glucose consumption is related to the appearance of dead and viable-but-non-culturable yeasts? To answer this question, permeabilized and non-permeabilized cells were assessed using the propidium iodide fluorescent probe combined with the use of a Malassez grid and then compared to colony-forming unit (CFU) determinations (Fig. 2). As expected, controls (samples maintained at 1.38 MPa) presented at least 10-fold less permeabilized cells (PI+) than non-permeabilized cells (PI-) and a slight proportion of supposed non-permeable but non-culturable cells (Fig. 2A). Unexpectedly, similar observations were made when *S. cerevisiae* was exposed to the glycerol ramp (Fig. 2B). The presence of non-permeable but non-culturable cells seemed not to be the main factors explaining the decrease of culturable cells during the gradual increase of the osmotic pressure. Such an approach has led us to employ the term 'supposed non-permeable but non-culturable cells' in the preference to 'viable-but-non-culturable cells'.
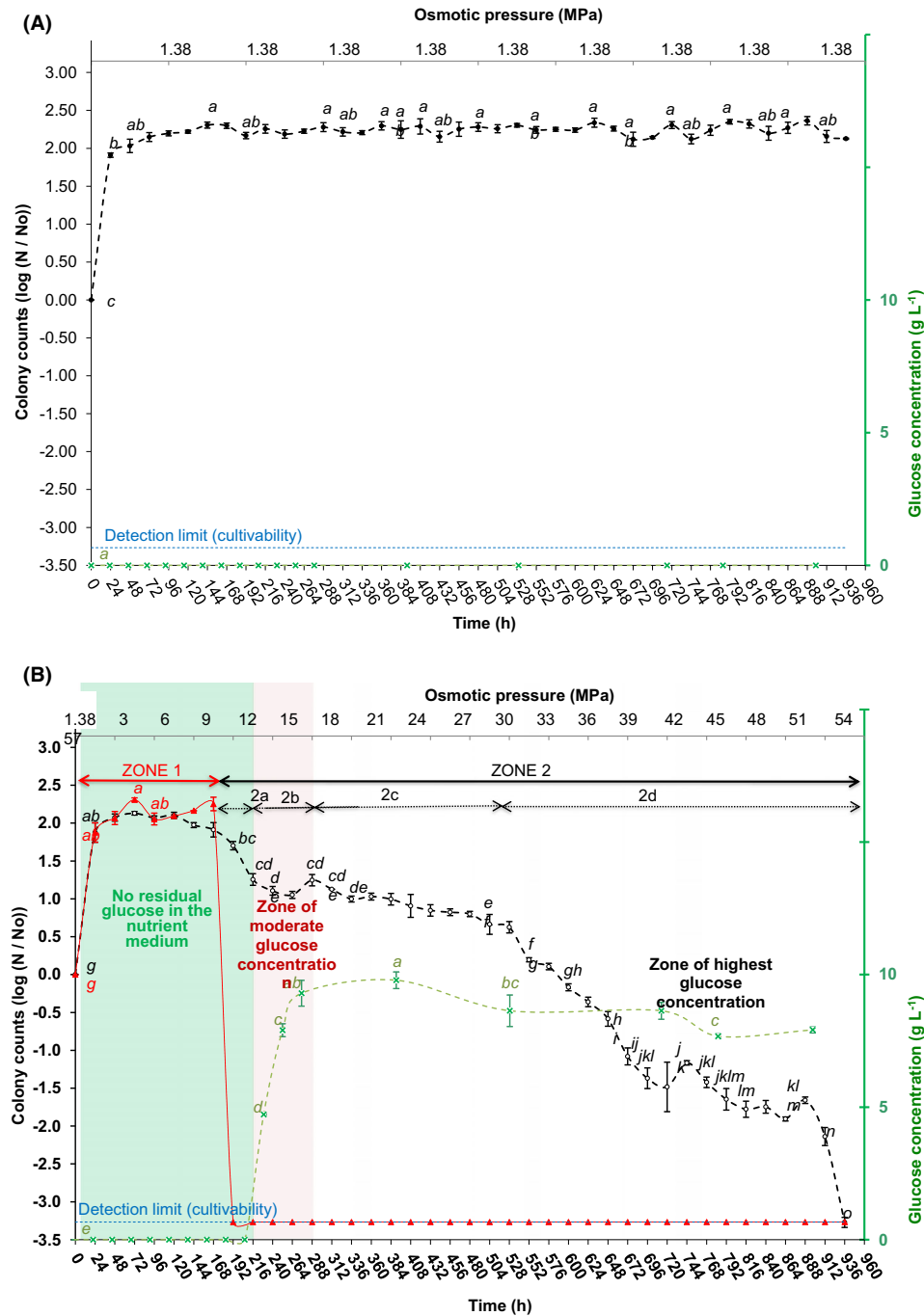
Fig. 1. *S. cerevisiae* cell cultivability and changes in residual glucose during the glycerol ramp for: (A) cultures maintained at 1.38 MPa over time (the nutrient medium was renewed every day), (●) CFUs were counted at 1.38 MPa; and (B) cells were exposed to the gradual increase of the osmotic pressure during the glycerol ramp at the rate of 1.5 MPa day^{-1} from 1.38 MPa to 58.5 MPa. CFUs were counted (▲) at the osmotic pressure attained by the end of the ramp and (○) at 1.38 MPa. (- - -). Colony counts were expressed as Log(N/No) where N corresponded to the number of colonies during the ramp and No to the counts before the start of the ramp. The detection limit was −3.27 log (with No = 1.85 x 10^6 CFU ml^{-1} and N_{limit} = 10^3 CFU ml^{-1}). In both cases, when specified (✗) residual glucose concentration was measured 24 h after refreshing the nutrient medium. *Post hoc* comparison of means was conducted by Scheffe's test (see Experimental procedures). Groups are denoted using a superscript letter, and the absence of a superscript letter indicates that no significant difference was found with the previous tagged point. Independent comparisons were performed between cultivability and glucose concentration. When *S. cerevisiae* was exposed to the gradual increase of the osmotic pressure, a global comparison of means recorded after spreading cells at 1.38 MPa and at the reached osmotic pressure attained by the end of the ramp was conducted.

This set of experiments led us to answer the initial question did a gradual increase of the osmotic pressure glycerol ramp modify xerotolerance in terms of growth limit and/or survival? The glycerol ramp did not permit *S. cerevisiae* to grow at osmotic pressures higher than 10.5 MPa, so did not confer any growth advantage from 10.5 MPa to 58.5 MPa. Moreover, from 10.5 MPa glucose consumption decreased and death occurred at osmotic pressure lower that this revealed by osmotic shocks (Table 1). Partial glucose consumption in the range 10.5–18 MPa and then absence of glucose consumption from 18 MPa allowed some yeast subpopulations to survive even when incapable of growth. Since such a ramp did not confer any advantage, one can ask whether it increased the resistance to extreme osmotic pressures?

Influence of a glycerol ramp on S. cerevisiae xerotolerance to extreme osmotic pressure

To answer this question, the behaviour of *S. cerevisiae* collected at 10.5 MPa or 34.5 MPa during the glycerol ramp was determined after exposure to a severe glycerol shock at 200 MPa for 1 h, 24 h and 48 h. In each case, the CFU were determined after incubating media (1.38 MPa) on which cells had been spread. Fig. 3 shows the log reduction of cells as a function of time by considering colony number reached at the beginning of the plateau phase at 200 MPa as the initial colony number (No). Except for cells propagated at 200 MPa (Fig. 3 A), a 1:20 dilution into 200 MPa MW was applied to achieve glycerol shocks at 200 MPa (Fig. 3B–G) leading to a corresponding decrease in colony number.

In this experiment, culture age influenced the behaviour of control cells since cultivability after the glycerol shock at 200 MPa of cells maintained 168 h (Fig. 3D) or 552 h (Fig. 3E) at 1.38 MPa was lower than that of cells propagated at 200 MPa after growing at 1.38 MPa (Fig. 3A). A recent study has revealed the extreme resistance of old *S. cerevisiae* cultures (post-diauxic), which were able to adapt to about 10.53 MPa with KCl used as the stressor (2.9 M) (Hirate *et al*., 2016, preprint). Interestingly, the authors found that genetically identical cells within each strains and culture were heterogeneous in term of size, shape and signalling behaviour. Nevertheless, it should be noted that aged stationary phase cultures frequently supplied with fresh medium are exposed to oxidative stress through the increase of reactive oxygen species and a concomitant decrease of antioxidant activity of superoxide dismutase (Jakubowski *et al*., 2000).

Two sets of control were studied: culture was grown for 48 h at 1.38 MPa and then immediately glycerol-shocked at 200 MPa (Fig. 3A); and cultures daily renewed and maintained at 1.38 MPa either for 168 h or 552 h before to being glycerol-shocked at 200 MPa; 168 h and 552 h were the times to reach 10.5 MPa and 34.5 MPa, respectively, during the glycerol ramp (Fig. 3 D, E). In all cases, CFUs were counted after incubation periods of 1 h, 2 h and 48 h for media with an osmotic pressure of 200 MPa. For cultures grown at 1.38 MPa, 1.9- and 4.5-log reductions were achieved after the glycerol shock at 200 MPa for 1 h and 48 h, respectively, when compared with the cell densities at 1.38 MPa for both of these time points (Fig. 3A).

Interestingly, when *S. cerevisiae* cells had been previously exposed to the glycerol ramp up to osmotic pressures of 10.5 MPa (Fig. 3B) or 34.5 MPa (Fig. 3C), decrease of cells able to form colonies after a subsequent glycerol shock at 200 MPa was significantly lower than that recorded for controls by considering CFUs reached after a 1:20 dilution step as the initial CFUs. Indeed, after 48 h at 200 MPa a reduction of 0.5-log was measured when cells were collected at the 10.5- or 34.5-MPa time point of the glycerol ramp, whereas there were reductions of more than 4 log for the respective controls (Fig. 3D, E). These results revealed unexpected beneficial effects conferred by the glycerol ramp. Indeed, cells harvested during early stages of the glycerol ramp at 10.5 MPa or 34.5 MPa had acquired a high level of xerotolerance leading them to survive at extreme osmotic pressure (200 MPa).

To better understand the nature of these beneficial effects of the glycerol ramp on survival of a severe glycerol shock, the ramp step was replaced by a moderate glycerol shock at 10.5 (Fig. 3F) or 34.5 MPa (Fig. 3G) for 10 min. This moderate glycerol shock did not predispose cells to better survive secondary subsequent glycerol shock of 200 MPa.

Although no competition experiment was performed, our results indicated that cells were acclimated to high osmotic pressures during the ramp (at 10.5 MPa and 34.5 MPa in view of our results) and have enhanced performance in term of cultivability relative to those maintained at 1.38 MPa. This finding is consistent with the beneficial acclimation hypothesis (enhancement of the physiological performance or fitness of an individual organism by the means of acclimation) reported by Wilson and Franklin (2002). However, it should be noted that the glycerol ramp did not confer any advantage up to about 50 MPa since a reduction of more than 1.7-log cells occurred at 51 MPa (816 h, Fig. 1B) during the glycerol ramp whereas there was only a 0.5-log reduction after 48 h at 50 MPa (Table 1). By considering the maximum cell density reached during the ramp, which was recorded at 4.5 MPa (72 h), as the initial colony number about 3.8-log reduction was achieved instead of a 1.7-log reduction. Collectively, these results indicate

(A)

(B)
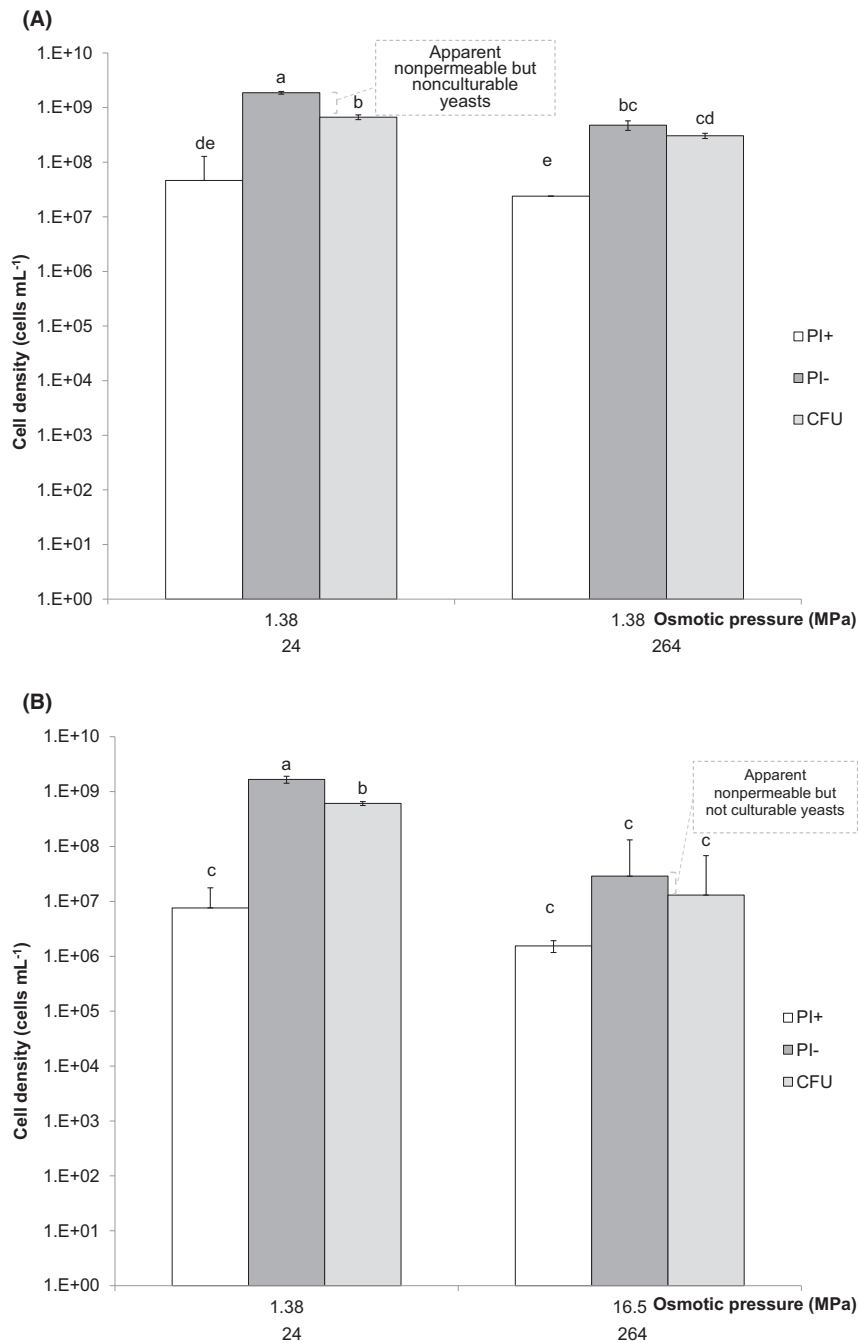
Fig. 2. Cultivability and plasma membrane permeability of *S. cerevisiae* exposed or not exposed to a glycerol ramp. Cells were stained with the propidium iodide fluorescent probe to assess changes in plasma membrane permeability. Cell density was evaluated using a Malassez Grid. (A). Cultures were maintained at 1.38 MPa over time (control), and (B) cells were collected during the ramp of osmotic pressure. Error bars represent SD, a Post hoc comparison of means conducted by Scheffe's test has been applied to determine significant groups (identified by a superscript letter).

that the main benefits acquired during the glycerol ramp and leading to a great survival at 200 MPa have been revealed during the stationary phase (200 MPa) and not during the subsequent glycerol shock. Indeed, during the subsequent stationary phase at 200 MPa, the cultivability remained high if yeasts were previously exposed to a

glycerol ramp up to osmotic pressures of 10.5 MPa (Fig. 3B) or 34.5 MPa (Fig. 3C) whereas it decreased over time if cells have been previously maintained at 1.38 MPa (Fig. 3D, E) or glycerol-shocked at either 10.5 MPa (Fig. 3F) or 34.5 MPa (Fig. 3G). This observation implies that water transfer occurring across the
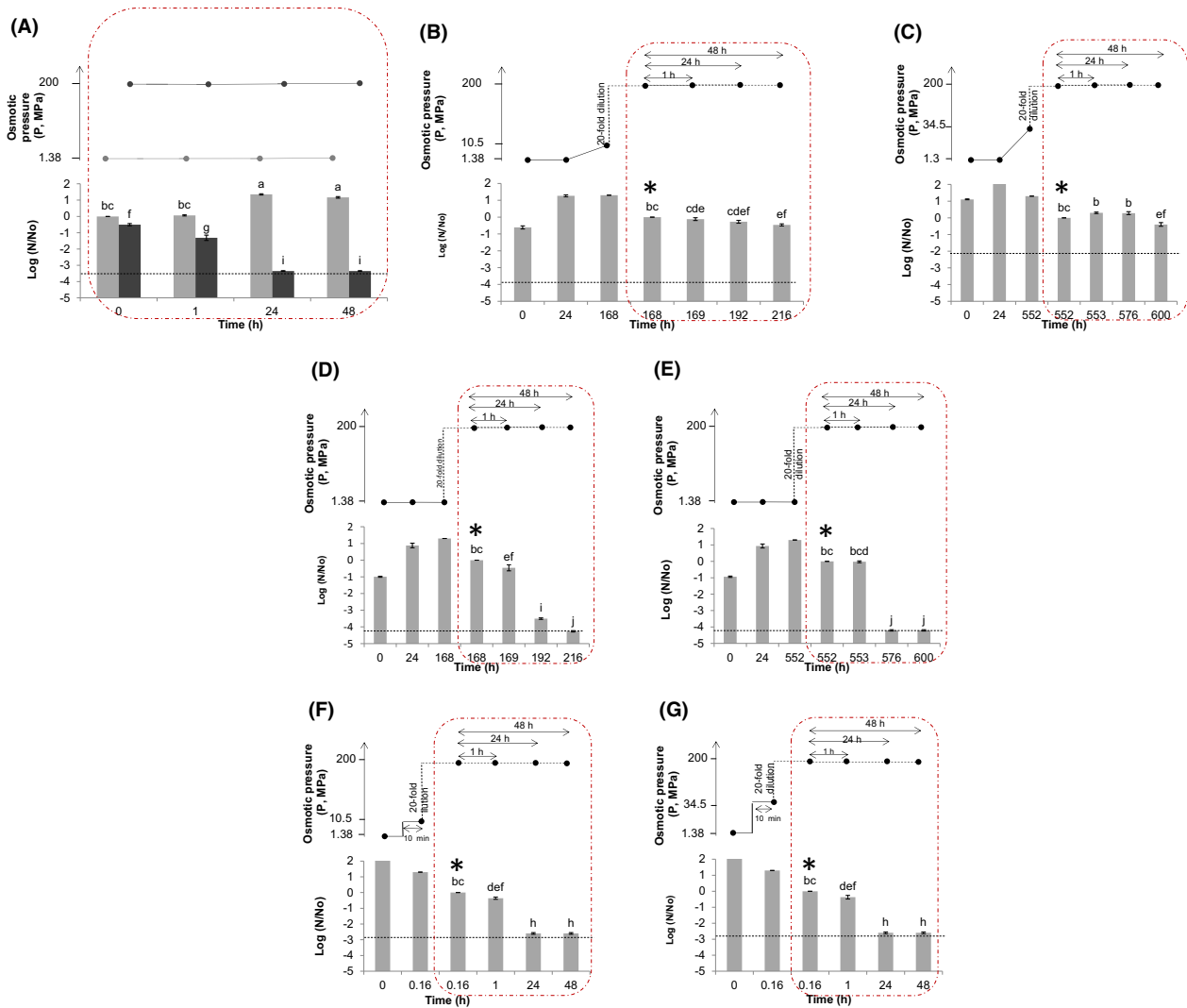
Fig. 3. Cultivability of *S. cerevisiae* exposed a to glycerol shock (200 MPa) during the glycerol ramp. Cells were collected during the glycerol ramp at either 10.5 or 34.5 MPa and then exposed to 200 MPa for 1, 24 or 48 h. (●) indicates time when CFUs were counted.

A–G. *S. cerevisiae* was grown at 1.38 MPa for 48 h before exposure to a glycerol treatment which starts at t = 0 h on each graph. Three sets of experiments were performed. (✱) Initial cell concentration (No) was set at the beginning of the stationary phase after the 20-fold dilution step (see *Experimental procedures*). Colony counts were expressed as Log(N/No). One statistical test was run by considering only the stationary phase at 200 MPa for 48 h (indicated by a dashed rectangle) to allow appropriate comparisons. (- - -) Detection limit of panels A–G was −3.36 log (with No = 2.28 x 10^6 CFU ml^-1), −3.88 log (with No = 7.63 x 10^6 CFU ml^-1), −2.16 log (with No = 1.45 x 10^5 CFU ml^-1), −4.26 log (with No = 1.84 x 10^7 CFU ml^-1), −4.21 log (with No = 1.62 x 10^7 CFU ml^-1), −2.60 log (with No = 3.99 x 10^5 CFU ml^-1) and −2.60 log (with No = 3.99x10^5 CFU ml^-1), respectively (in any case N_{limit} = 10^3 CFU ml^-1). In any case, CFU were determined after incubation of 1.38-MPa media on which cells had been spread.

First set. There were three controls: (A) cells grown at 1.38 MPa and then propagated at either (●) 1.38 MPa or (●) 200 MPa for 48 h. Yeasts were propagated at 1.38 MPa for 24 h then exposed to a glycerol ramp at either (B) 10.5 MPa (reached at t = 168 h) or (C) 34.5 MPa (reached at t = 552 h) and finally exposed to a glycerol shock at 200 MPa.

Second set. (D) Cells grown at 10.5 MPa for 168 h (time to reach 10.5 MPa during the ramp of osmotic pressure see (B) and then glycerol-shocked at 200 MPa (through a 20-fold dilution step), (E) cells grown at 34.5 MPa for 552 h (time to reach 34.5 MPa during the glycerol ramp see C) and then glycerol-shocked at 200 MPa. Third set. Cells were collected at 1.38 MPa then, exposed to a glycerol shock at either (F) 10.5 MPa or (G) 34.5 MPa for 10 min and finally exposed to a glycerol shock at 200 MPa.

plasma membrane during a sudden glycerol shock damaged the membrane, leading to cell death. We believe that the plasma membrane becomes more robust due to changes in lipid composition, increases sterol synthesis and accumulation of compatible solute(s).

Unexpectedly, this set of experiments revealed that tolerance to extreme osmotic pressure (200 MPa) could be acquired through a glycerol ramp applied up to 10.5 MPa or 34.5 MPa over few days. Whereas we observed xerotolerance up to 200 MPa when

determined by cell survival, we did not know whether cells were able to grow at osmotic pressures greater than 10.5 MPa. In the current study, CFU determinations were made at 1.38 MPa, but not at 200 MPa because media with corresponding amounts of glycerol do not solidify. One can ask whether xerotolerance mechanisms were exclusively induced between 1.38 MPa and 10.5 MPa and so were then maintained up to 34.5 MPa given that growth occurred during the ramp up to 10.5 MPa and glucose consumption was observed up to 13.5 MPa (Fig. 1B). To better appreciate the nature of the mechanisms involved in this type of xerotolerance, the impact of removal of water through the plasma membrane (during the glycerol ramp) and the cell wall and macromolecules such as proteins were assessed using FTIR spectroscopy.

### Impact of the glycerol ramp on the S. cerevisiae *cell wall and proteins*

To study the evolution of protein and cell wall structures during the glycerol ramp, controls were maintained at 1.38 MPa, cells were collected at 1.38, 9, 10.5 and 19.5 MPa during the glycerol ramp. We then considered whether these structures changed around the limit of growth (at 10.5 MPa) during the ramp of osmotic pressure (Fig. 1B).

Results presented in Fig. 4 indicate changes in the secondary structure of proteins from 9 MPa. Indeed, a relative inversion of intensities of the amide I and amide II bands could be observed on underived spectra (Fig. 4A). Looking at the second derivatives (Fig. 4 B), one can observe the disappearance of the β-form C = O stretching/β-sheets in aggregates peaks (amide I) from 9 MPa and changes in C-N and N-H vibrations (amide II) from 10.5 MPa. In a complementary way, Fig. 4C revealed dramatic changes in the amide I / amide II area ratio (dashed line) from 9 MPa whereas cultivability was close to that measured at the beginning of the ramp of osmotic pressure (1.38 MPa) and inversion in the α-helical structure (1650 cm^{-1})/condensed β-sheets in aggregates (1635 cm^{-1}) intensity peak ratio from 19.5 MPa. Changes in the aqueous environment that modify protein hydration can reduce stability and/or functionality of the macromolecule. Other studies have provided evidence of the relationship between water-structure and protein stability through water H-bond networking at protein surface (see for review Bellissent-Funel *et al.*, 2016). Moreover, changes in the amide I and amide II spectra could certainly be explained by the presence of glycerol which can interact for instance with aromatic C, amide N and cationic N (Knowles *et al.*, 2015). It should be noted that glycerol partakes in hydrogen-bonded networks

with water and proteins, and it is this behaviour (along with the polarity that is slight less than that of water) that is the basis of its chaotropic activity (Hallsworth, 1998; Williams and Hallsworth, 2009; Cray *et al.*, 2013; Ball and Hallsworth, 2015).

Changes in cell wall structure were also observed from an osmotic pressure of 9 MPa (Fig. 5). Mainly, glucan peaks could be well identified on underived spectra (Fig. 5A) and second derivatives (Fig. 5B) from 9 to 19.5 MPa. As reported by Rapoport *et al.* (2019), the rigidity of the cell wall and cell shape could be determined by glucans and chitin components of the wall. Moreover, Kulikova-Borovikova *et al.* (2018) reported that survival during dehydration/rehydration steps could be strongly correlated to changes in the cell wall structure/composition as damages of cell wall proteins (more particularly of proteins non-covalently anchored to the glucan matrix). Such changes may be in part related to a decrease in cell volume given that a previous study showed a 30% volume reduction at 14.5 MPa for cells previously grown in the presence of glycerol at 1.38 MPa (Marechal *et al.*, 1995). Changes in plasma membrane permeability in relation with changes in cell wall structure could be explained by formation of membrane invaginations (Dupont *et al.*, 2010) leading to a decrease in plasma membrane surface and so to an excess of the wall surface compared to the membrane. Because of the presence of many anchorage sites between the cell wall and the membrane, such a change in wall / membrane surface and any change in wall structure ratio could lead to alteration of plasma membrane properties (as permeability).

Taken together, these results indicated that during the glycerol ramp some structural changes occurred in the range 9 and 10.5 MPa which more-or-less corresponded to the limit for growth (Fig. 1B). Unexpectedly, changes in protein secondary structure and cell wall occurred between 1.38 MPa and 10.5 MPa, so likely contributed to the increased of xerotolerance at extreme osmotic pressures (up to 200 MPa, Fig. 3). In light of these observations, this xerotolerance was likely acquired during the increased residence time in the range of osmotic pressure where glucose consumption (Fig. 1B: zones 1 and 2a) was possible and could be related to the impact of the osmotic pressure on macromolecule conformation meaning that physico-chemical mechanisms instead of phenotypic plasticity could be involved in this type of acclimation. Proteins and lipids can be stabilized by trehalose, a well-known protectant involved in dehydration resistance in yeast, able to stabilize macromolecules by the means of hydrogen bonding (water replacement hypothesis) and glass formation (Crowe and Crowe, 1984; Crowe *et al.*, 1998; Jain and Roy, 2009; Olgenblum *et al.*, 2020). The role of trehalose should be
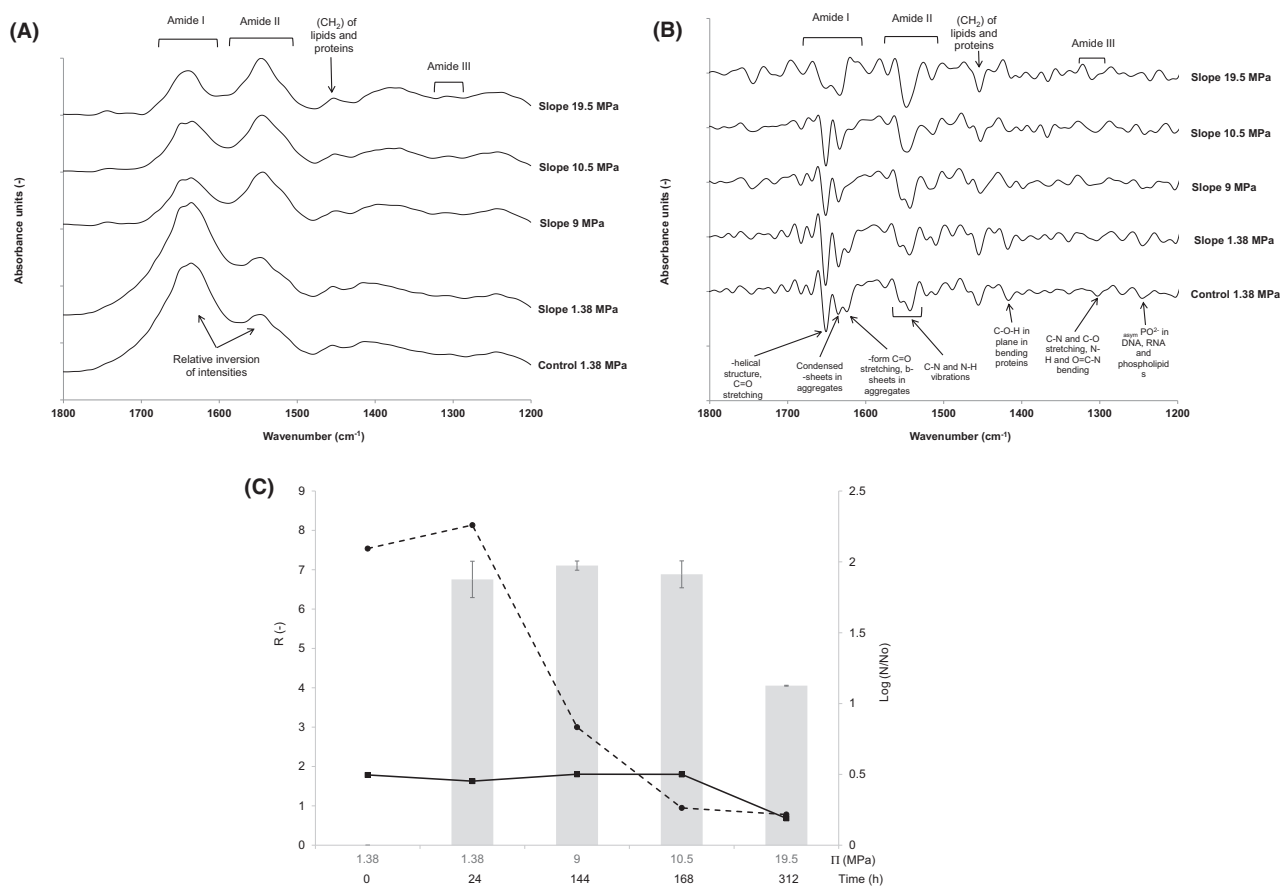
Fig. 4. FTIR spectra of *S. cerevisiae* grown at 1.38 MPa (control) or collected during a glycerol ramp in the spectral window of proteins (1200 to 1800 cm⁻¹). During the ramp, cells were collected at 1.38, 9, 10.5 and 19.5 MPa.
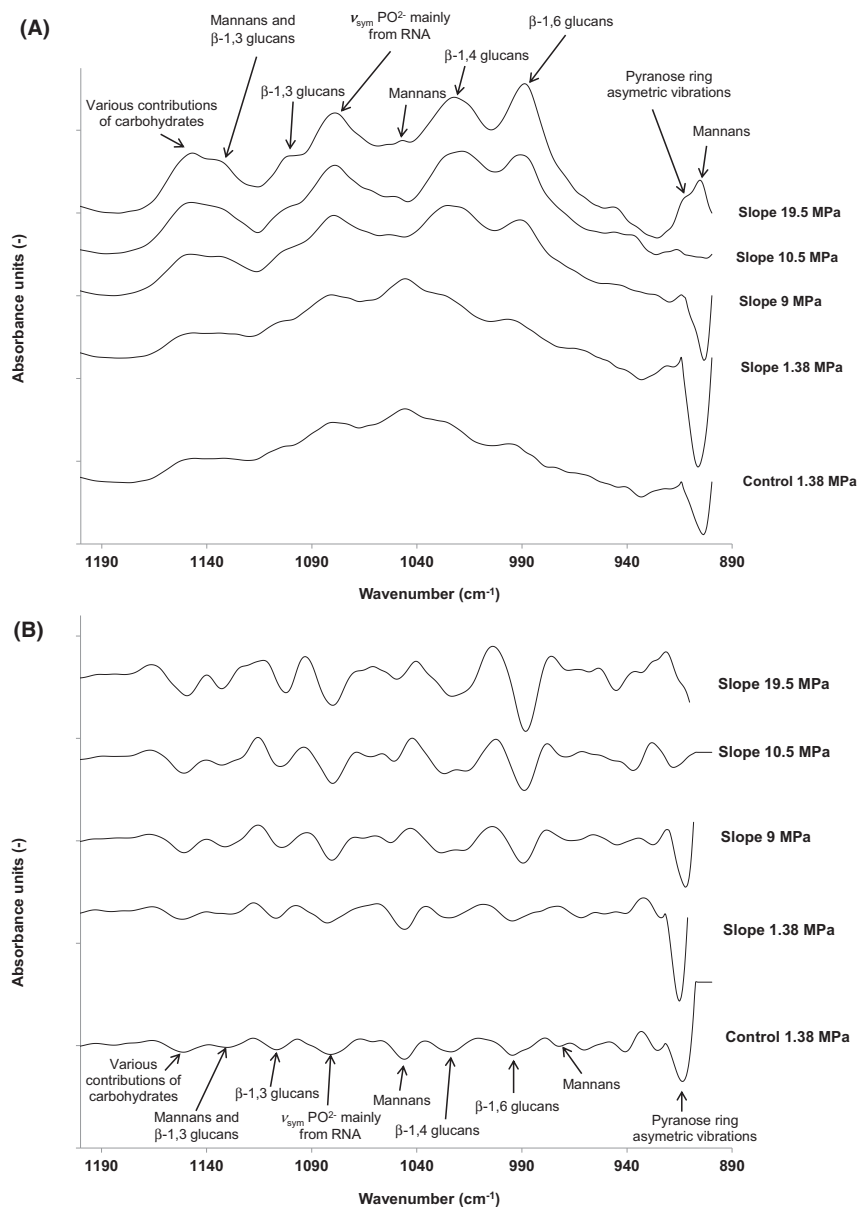A. Means of processed raw spectra.
B. Second derivatives were calculated from means of values obtained from processed raw spectra. (C) *R* represents (i) (- - • - -) fractional areas of the amide I/amide II bands from raw spectra and (ii) (−•−) α-helical structure (1650 cm⁻¹)/condensed β-sheets in aggregates (1635 cm⁻¹) intensity peak ratio (calculated from the second derivative, (B) compared to (▨) CFU counting during the glycerol ramp as a function of the osmotic pressure and time.

clarified in future work. Indeed, it could play a role in xerotolerance acquisition occurring during the gradual increase of the osmotic pressure as it could be strongly accumulated during the osmoregulation step in glucose growing during the stationary growth phase in *S. cerevisiae* (Gadd *et al.*, 1987). Trehalose can reduce water activity down to 0.970 (see figure 3 of de Lima Alves *et al.*, 2015), implying that it could partly assume the role of glycerol as a compatible solute (Nevoigt and Stahl, 1997). However, given that the high-glycerol medium provided a source of glycerol for the cell, we doubt that de novo synthesis of compatible solutes occurs.

Conclusion

Bulk movement of water and glycerol through the plasma membrane did not prevent *S. cerevisiae* growth up to 10.5 MPa, so whatever the kinetics of the osmotic pressure meaning that the glycerol ramp did not confer any advantage compared to the glycerol shock regarding ability to grow at high osmotic pressure (low water activity). Indeed, the glycerol ramp applied over several cell generations during 39 days did not allow *S. cerevisiae* to increase xerotolerance in the range 1.38-58.5 MPa. Nevertheless, the glycerol ramp allowed *S. cerevisiae* to survive at extreme osmotic pressure (200 MPa). This increased xerotolerance was likely due to increased residence time in the range of osmotic pressure where glucose was consumed. Our study also revealed that membrane structural changes occurred between 9 and 10.5 MPa during the glycerol ramp thereby altering the ability to grow at higher osmotic pressure. Although the glycerol ramp did not improve ability to grow up to 50 MPa, it improved ability to survive at extreme osmotic pressure (200 MPa) over 48 h. The implementation of such a glycerol ramp might therefore enhance preservation processes of cells of *S. cerevisiae* or other organisms in liquid media over long periods. The risk of

Fig. 5. FTIR spectra of *S. cerevisiae* grown at 1.38 MPa (control) or collected during a glycerol ramp in the spectral window of the cell wall (890 to 1,190 cm⁻¹). During the ramp, cell pellets were collected at 1.38, 9, 10.5 and 19.5 MPa.
A. Means of values obtained from processed raw spectra.
B. Second derivatives were calculated from the means of processed raw spectra.

potential bacterial contaminants would be limited by the high level of osmotic pressure. Compared to classical methods of cell storage (as freezing at −80 or −196°C), the advantage of such an approach lies in the possibility of maintaining samples at room temperature.

Experimental procedures

Yeast strain and physiological methods

Saccharomyces cerevisiae strain CBS 1171 (kindly donated by Laboratorium voor Moleculaire Celbiologie,

Katholieke Universiteit Leuven) was used in this study. Colonies were maintained on a solid Malt Wickerham (MW) medium composed of 10 g glucose (Sigma-Aldrich, Saint Quentin Fallavier, France), 3 g yeast extract (Sigma-Aldrich), 3 g pancreatic peptone (VWR International, Limonest, France) and 1.5 g NaH_2PO_4 (VWR International) in 1 l of distilled water supplemented with 20 g l^{-1} agar (VWR International) at 25°C.

For the experimental studies, a range of modified MW media composed of 10 g of glucose (Sigma-Aldrich), 3 g of pancreatic peptone (VWR International, Limonest,

France), 3 g of yeast extract (Sigma-Aldrich) and 1.5 g of NaH_2PO_4 (VWR International) in 1 l of a binary solution of water and glycerol (Sigma-Aldrich) to increase the osmotic pressure and so to reduce water activity. The modified MW media were made up by adding the dry components to binary water-glycerol solutions (instead of distilled water) which were made up to give final glycerol concentrations of 20.44 g l^{-1} (0.22 M, 1.38 MPa equivalent to 0.990 water activity) to 4343.72 g l^{-1} (47.17 M, 200 MPa equivalent to 0.234 water activity). According to the desired water activities, the mass of glycerol to be added to 1000 g of distilled water was determined using the Norrish equation (Norrish, 1966). Osmotic pressure was determined with a dew point osmometer (Decagon device Inc., Pullman, WA, USA).

Determination of the osmotic pressure (or water activity) range for growth and survival

Yeasts were grown on a modified MW in 1 l of a binary solution of water and glycerol with an osmotic pressure of 1.38 MPa (5.1 g of glycerol (Sigma-Aldrich) per 100 g of distilled water). A pre-culture was prepared by introducing a single colony [grown at 25°C on a solid MW medium with an osmotic pressure of 1.38 MPa and supplemented with 20 g l^{-1} agar (VWR International)] into a 250 ml conical flask containing 100 ml of MW and shaken at 250 r.p.m. (New Brunswick Scientific, C24KC refrigerated benchtop incubator shaker, Edison, NJ, USA) for 48 h at 25°C. Then, cultures were prepared by inoculating 1 ml of the pre-culture into a 250 ml conical flask containing 100 ml of MW at 1.38 MPa and shaken at 250 rpm for 48 h at 25 °C to reach the stationary growth phase.

A 2.5-ml aliquot of culture was inoculated into 250 ml conical flask containing 50 ml of MW at the desired osmotic pressure (1.38, 10.5, 16.5, 34.5, 50, 100 or 200 MPa equivalent to, respectively, 0.990, 0.926, 0.887, 0.778, 0.695, 0.483 or 0.234 water activity) and shaken at 250 rpm for 48 h at 25°C. Colony-forming units (CFU) were counted after 1, 24 and 48 h.

Use of glycerol ramp to stress S. cerevisiae

Cells were exposed to a gradual increase of the osmotic pressure at the rate of 1.5 MPa day^{-1} from 1.38 MPa (0.990 water activity) to 58.5 MPa (0.635 water activity) over 39 days at 25°C. A pre-culture was prepared by introducing a single colony [grown at 25°C on a solid MW medium with an osmotic pressure of 1.38 MPa and supplemented with 20 g l^{-1} agar] into a 250 ml conical flask containing 50 ml of MW and shaken at 250 rpm for 48 h at 25°C. Two cultures were prepared by inoculating 500 μl of the pre-culture into two 250 ml conical flasks

containing 50 ml of MW at 1.38 MPa and shaken at 250 rpm for 24 h at 25°C. Every 24 h, cultures were centrifuged at 2880 g for 5 min at 25°C to replace the medium with fresh one with an osmotic pressure that was either 1.5 MPa higher (gradual increase of the osmotic pressure) or 1.38 MPa (control). Cultures were maintained at 25°C and shaken at 250 rpm. When osmotic pressure reached 16.5 MPa, cultures were centrifuged 10 min instead of 5 min to collect the cells.

Determination of colony-forming units to assess ability to grow

Cell suspensions were used to make a series of 10-fold dilutions in fresh MW at 25°C at 1.38 MPa and/or at a higher osmotic pressure as specified in Results and Discussion section. A 10-μl volume of the appropriate dilution was spread on solid MW medium (in Petri dish) at the same osmotic pressure than this applied during the dilution step. Petri dishes were incubated 48 h at 25°C under aerobic conditions before counting colonies to determine CFU. The detection limit was 10^3 CFU ml^{-1}.

Assessment of plasma membrane permeability

For microbial cells under extreme stress, the plasma membrane is known to be a failure point of the system, so we assessed plasma membrane permeability as a measure of cellular vitality. Cells were enumerated using a Malassez counting chamber and stained with the non-permeant propidium iodide fluorescent probe (Sigma-Aldrich); 2 μl taken from a 1 mg ml^{-1} was added to 500 μl of cell suspension. Samples were incubated in the dark at 25°C for 10 min, placed in a Malassez counting chamber (100 μl) and observed under a light microscope (Axioplan 2 imaging, Zeiss, Marly le Roi, France) equipped with a halogen lamp (HAL 100, white light illumination), a 120 W mercury vapour short arc lamp (X-Cite® 120Q excitation light source used for fluorescence illumination) a black and white camera (AxioCam MR, Zeiss) driven by the AxioVision Rel 4.8.0.0 software (Zeiss) and a Plan Apochromat DIC X 63 oil NA 1.4 objective. Cells were allowed to sediment (3 min) prior to inspection. Permeabilized cells were quantified by using fluorescence illumination combined with filter cube BP 560/40-FT 585-BP630/75. Total cell number and concentration were determined under white light illumination in order to calculate the ratio of permeabilized to non-permeabilized cells. Because of the presence of glycerol (i.e. at high osmotic pressure), blurry or out-of-focus (where cells and Malassez grid were not always in the same focal plane) could not be recorded. To avoid potential counting errors due to refraction of light by glycerol, images were analysed using a counting algorithm

based on Fourier transform filtering and the Hough transform and allowing complete erasing of the Malassez grid without altering yeast (Marin *et al.*, 2014; Denimal *et al.*, 2015).

Assessment of xerotolerance during the glycerol ramp

S. cerevisiae was first grown at 1.38 MPa for 48 h and exposed to a gradual increase of osmotic pressure were then exposed to a severe glycerol shock at 200 MPa for 1, 24 and 48 h. Three sets of experiments based on three different osmotic pressure historiesy were performed: glycerol-shocked cells at 200 MPa were previously either maintained at 1.38 or 10.5 or 34.5 MPa, collected during the gradual increase of the osmotic pressure at 10.5 or 34.5 MPa, glycerol-shocked from 1.38 MPa to either 10.5 or 34.5 MPa for 10 min by centrifuging a volume of yeast suspension (2880 g for 5 min at 25°C) and then mixing the pellet with a volume of fresh MW medium at the appropriate osmotic pressure (10.5 or 34.5 MPa). To apply the final glycerol shock at 200 MPa, 0.5 ml of cell suspension was diluted 20-fold by adding 9.5 ml of 200-MPa MW into a 40 ml plastic tube maintained at 25°C and shaken at 250 rpm. Cell suspensions were used to make a series of 10-fold dilutions in fresh MW at 25°C at 1.38 MPa. A 10 μl aliquot of the appropriate dilution was spread on solid MW medium (in Petri dish) at 1.38 MPa. Petri dishes were incubated 48 h at 25°C before counting colonies to determine CFU.

Quantification of residual glucose in the culture medium

To evaluate glucose uptake during the pressure glycerol ramp, the concentration of glucose remaining in the growth medium was determined by high-performance liquid chromatography Aminex HPX 87-H column (Bio-Rad, Marnes-la-Coquette, France), after elution with filtered (Durapore® membrane, 0.45 μm, Merck Millipore, Fontenay sous Bois, France) H_2SO_4 (5 mM, Sigma-Aldrich) at a flow rate of 0.6 ml min^{-1} at 65°C.

Evaluation of structural changes in the cell wall and proteins using ATR-FTIR spectroscopy

The changes in the cell wall, the secondary structure of proteins and variations of the ν_{sym} PO^{2-} band were determined using ATR-FTIR spectroscopy. The spectra were measured using samples that were still hydrated to maintain their osmotic pressure. Cell suspensions (10 ml) were centrifuged at 2880 g for 5 min at 25°C. Note that when osmotic pressure reached 16.5 MPa, cultures were centrifuged 10 min instead of 5 min to collect cells. The cell pellets were then placed on an infrared-transparent ZnSe window and allowed to equilibrate

for 2 min at 25°C. The temperature was regulated by water circulation in a double envelope surrounding the ZnSe window. All the attenuated total reflectance FTIR spectra were recorded between 4000 and 900 cm^{-1} (wavenumbers) on a Vector 22 FTIR spectrometer from Bruker (Karlsruhe, Germany) with a Bio-ATR II unit and equipped with a mercury–cadmium–telluride (MCT) detector. The spectral resolution was 4 cm^{-1}. To obtain the spectrum for each sample, 10 scans were recorded and pre-processed and analysed using the OPUS 6.5 software (Bruker).

For data analysis, raw spectra were baseline-corrected via the Rubberband method and normalized with respect to the corresponding amide II band (around 1545 cm^{-1}). Normalization against a specific band meant that the intensity of this band could be used as the internal standard and was set to unity. Second derivatives of the mean of at least three baseline-corrected/normalized spectra were calculated after smoothing (17 data points). Note that for second derivative spectra only peaks with negative intensity were considered as true peaks.

Statistical analysis

All experiments were carried out at least three times using three independent replicate cultures. To compare results, a one-way ANOVA followed by Scheffe's test or a Student *t*-test was performed. Values for means and the standard deviation of the means of the three independent measurements were calculated. One-way ANOVA and Scheffe's test were performed using R software [version 3.2.4 Revised (2016-03-16 r70336)] whereas group variances and means were compared using, respectively the one-sample test of variance '*vartest2*' and then the two-tailed unpaired Student's *t*-test '*ttest2*' performed by Matlab® software (R2011b; 7.13.0.564 version). In every case (Scheffe's test, ttest2 and vartest2) α risk was 0.1.

Student's *t*-test was conducted taking into account the variance type (i.e. variances of the populations were equal or unequal). The null hypothesis (means were equal) was initially tested, then if means were unequal: 'right' (mean of the population x > mean of the population y) and 'left' (mean of the population x < mean of the population y) alternative hypotheses were subsequently tested. In all cases, the *P*-value (*P*) was calculated. Significance was accepted at $P < 0.05$.

Relative cell area was analysed using R software [version 3.5.1 (2018-07-02)] to draw box plots. Medians and probability distribution were compared between all samples by, respectively, running a median test (median.test function, agricolae library) and Kolmogorov–Smirnov test (ks.test function, dgof library) both with a α-risk of 0.05. Non-parametric tests were applied because many samples displayed a non-Normal distribution.

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Conflict of interest

The authors have no conflict of interest to declare.

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