Effect of Combined Physical Stresses on Cells: The Role of Water

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Abstract

The role of water in microorganism viability was assessed through the application of combined physical perturbations. The combination of different physical parameters could allow to balance the properties variations (especially water related) resulting from the increase of one parameter alone. Thus, it is possible to optimize the survival of cells in controlling these parameters. This was tested through two different examples.

The first example shows that combination of osmotic level and temperature can allow optimizing yeast cell survival in following membrane fluidity variation. Moreover this analysis has allowed a better comprehension of cell inactivation during rehydration and especially the impact of intracellular vesiculation during dehydration.

The second example deals with the effect of combination of high hydrostatic pressure, low temperature and water activity of the medium, on resistance *Escherichia coli* cells. These experiments show that synergetic effect of high pressure and low temperature was only observed at pressure lower

than 300 MPa and high water content. Otherwise, low temperature as well as low water activity protects the microorganisms from inactivation even at extreme pressure level (P > 600 MPa).

These two examples show the implication of water thermodynamical properties and their preservation on cell survival even after extreme treatment conditions. Preservations process would certainly benefit from extensions of this knowledge.

Introduction

The change of physical (hydrostatic pressure, temperature) or physicochemical (water activity, pH) environment would induce an important stress for eukaryotic and prokaryotic cells. Depending on the level of the perturbation and also on the kinetics of conditions change, this stress could lead to the inactivation of cells considered. Using high level and rapid perturbations in non-nutritive medium, cell can involve only few active adaptation systems. Cell response is in this case essentially passive. In these conditions, the cell resistance can be attributed to its constitution (robust cell wall, adaptability of cell membrane, cytoskeleton, …) and also on the repair systems that the cell can use after return to more favorable conditions.

Combination of physical treatment could modulate the effect of each stress in giving very interesting information on the mechanisms involved. The biological basis of these interactions is not clearly understood up to now. Numerous experiments have shown the role of cell osmotic balance and cell membrane passive and active permeability. Membrane structure and fluidity seem to play a great role during dehydration and rehydration processes and generally in all stress conditions.

The effects of these intense perturbations on cell survival are highly important considering food processes like drying, freezing, sterilization, pasteurization,… In these processes, very drastic perturbations are applied to food products and on microorganisms. This drastic change is necessary to

inactivate pathogens (food stabilization) or to preserve food and/or cell (drying, freezing). These industrial processes generally combine different drastic physical modifications including temperature, osmotic pressure, hydrostatic pressure, etc…

Combinations of physical perturbations have been experimented in model medium to understand the mechanisms leading to microbial inactivation. A better perception of such mechanisms would allow optimizing food processes but also other applications like the conservation of human cells and tissues at ambient temperature or in frozen state.

We will approach this research thematic through two examples of combined physical perturbations. The first example deals with hyperosmotic stress effect and the possibility to combine it with temperature. The second is centered on the effect of high hydrostatic pressure on cells and the possibility to combine it with low temperature and/or low water activity combination.

Example 1: Effects of combined hyperosmotic and temperature perturbations

Sequence of hyperosmotic perturbation on yeast cells

During the first part of dehydration, sudden exposure to a hyperosmotic stress causes rapid equilibration of the osmotic pressures of the cytoplasm and the external medium. During the transitional step of the passive osmotic response, water flows out of the cells, leading to cell shrinkage and permeant solutes, such as glycerol, penetrate into the cells. This exchange is very fast (Berner and Gervais 1994) and ends up in a stationary step, when osmotic pressures are equilibrated. As shown on Figure 1, cell volume decreased exponentially between aw of 0,99 and 0,8, before reaching a constant volume corresponding to 40% of the initial volume, generally called non-osmotic volume. Cell volume was evaluated from light microscopy images and thus took into account the total envelope of the yeast, i.e., cell wall and membrane. In contrast to plant cells and bacteria, in which the plasma membrane shrinks away from the cell wall, in yeast, the entire cell volume shrinks when cells are placed in hypertonic solutions (Morris *et al.* 1986). Considering the slight compressibility of

biological membranes, this strong cell shrinkage, must be associated to wrinkling of the membrane leading to shape modification as reported in the literature (Adya *et al.* 2006).

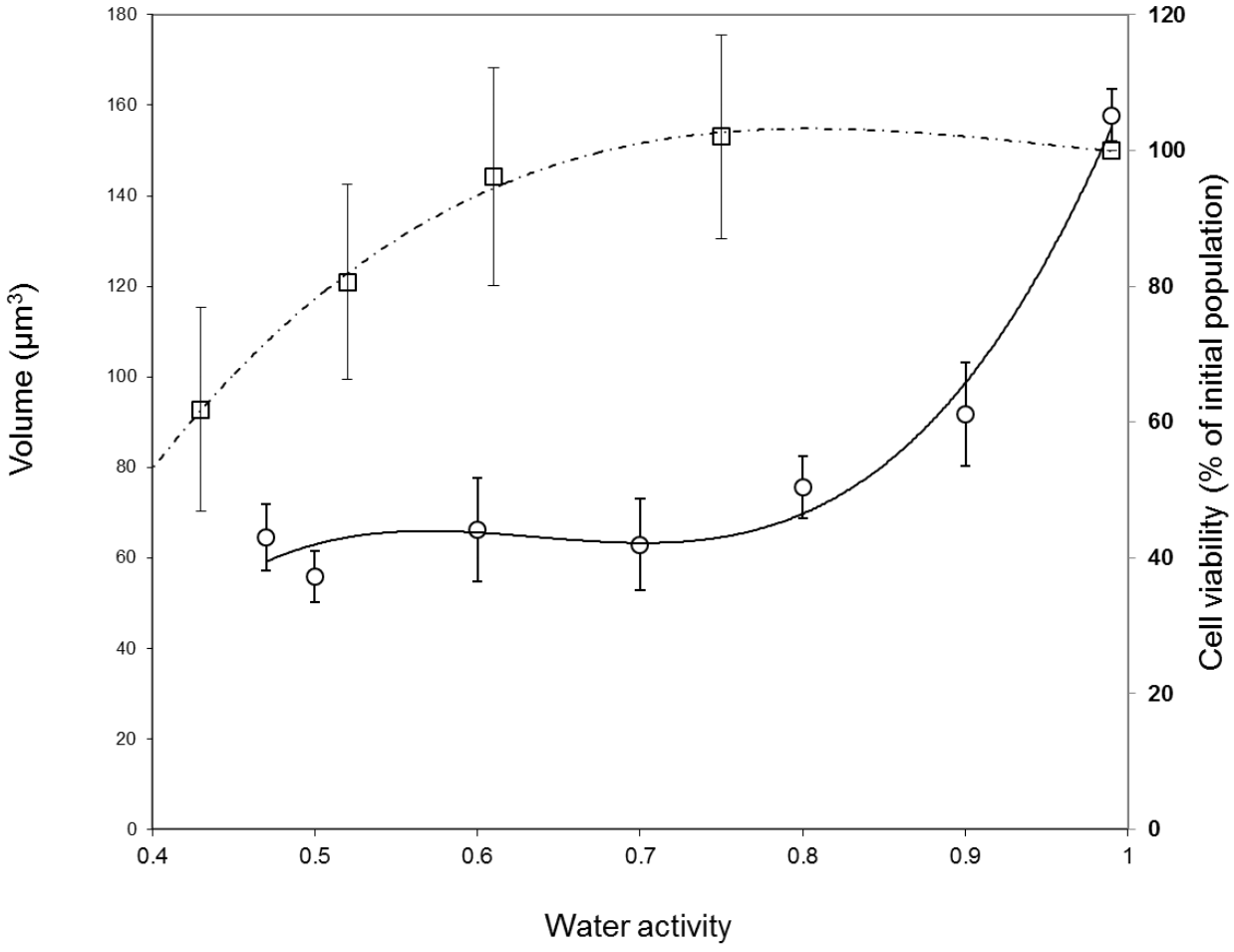

Figure 1: Variations of average cell volume (open circle) and cell viability (open square) of *Saccharomyces cerevisiae* after an osmotic shock from culture medium (Aw 0.99) to

binary medium (water/glycerol) at different water activity levels. Volume data are obtained from analysis of confocal images and viability from CFU method.

In decreasing water activity lower than 0.55, the cells became permeabilized as shown by the rate of propidium iodide (PI) stained cells on Figure 2. Therefore, this osmotic pressure interval appears to be critical for membrane permeability during dehydration. Phase transitions of phospholipids have been proposed as the main cause of the increase in membrane permeability in both phospholipid vesicles (Yamazaki *et al.* 1989) and yeasts (Laroche *et al.* 2001) under osmotic stress. Water loss from phospholipid head groups may lead to phase transitions in some lipids, resulting in a lateral phase separation (Lehtonen and Kinnunen 1995), which allows the leakage of intracellular contents (Crowe *et al.* 1992). The occurrence of a phase transition in yeast membrane lipids from aw 0.64 to 0.38 in glycerol solution at an average temperature of 22 °C as evidenced by Laroche et al. (2001) could explain the permeabilization observed.

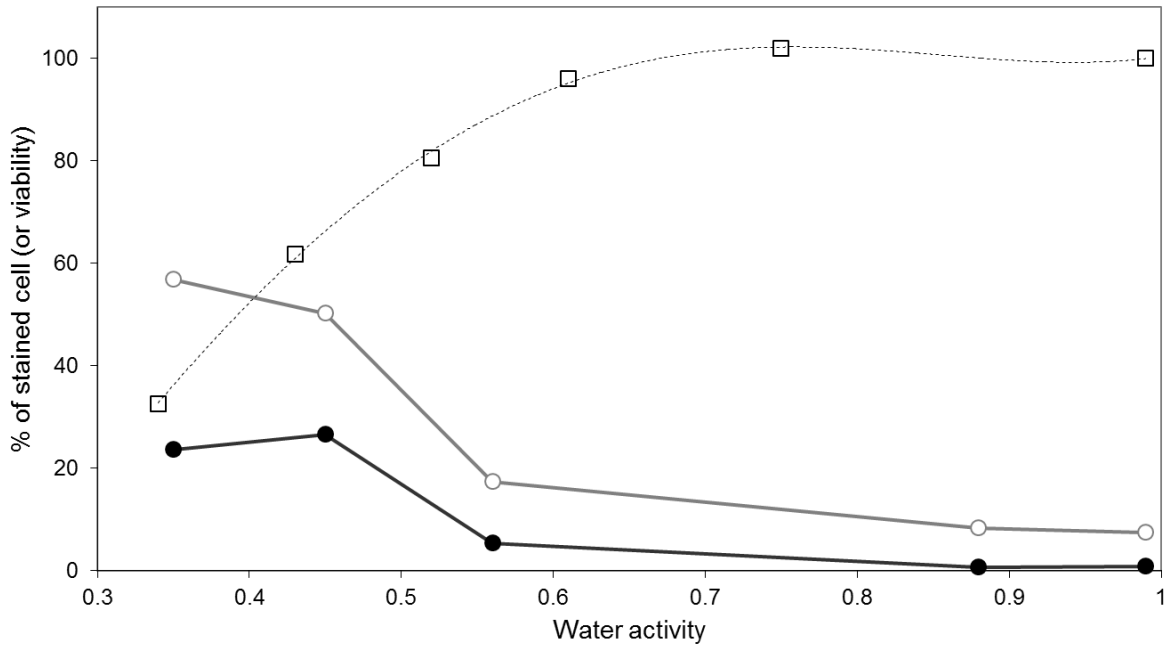

Figure 2: Staining rate of *Saccharomyces cerevisiae* versus water activity using 2 probes: Lucifer Yellow (LY) and Propidium Iodide (PI). Permealized cells (filled dot) are doubled marked, cells with endocytosis (open circle) are marked with LY and intact cells after rehydration (open square).

During hyperosmotic treatments, the number of Lucifer yellow (LY) stained cells also increased with increasing osmotic pressures (Figure 2). LY is a membrane-impermeant anionic dye. This polar tracer is usually loaded by microinjection, pinocytosis, or scrape loading. It has been used to characterize endocytosis in plant cells (Roszak and Rambour 1997) and yeasts (Wiederkehr *et al.* 2001) in which the presence of a cell wall prevents the access of high-molecular-weight molecules to the plasma membrane. In PI/LY double-stained cells, LY probably penetrated into the cells because the plasma membranes were permeabilized. In cells stained only with LY, the occurrence of plasma membrane endocytic vesiculation under hyperosmotic conditions seems possible. Endovesicules has already observed by Mille *et al.* (2002) with *E. coli*. Slaninova et al. (2000) reported the occurrence of deep plasma membrane invaginations filled from the periplasmic side with an amorphous cell wall material, when *S. cerevisiae* cells were transferred to hyperosmotic growth medium. Such invaginations, when associated with lipid phase separation induced by dehydration, could lead to the formation of endocytic vesicles. In fact, Liu *et al. (2006)* recently showed that the scission of membrane invaginations could be promoted by lipid phase separation to form endovesicles.

The percentage of permeabilized cells (PI-stained cells) was constant before water activity of 0.86 during rehydration and increased strongly at the upper levels of rehydration, showing that most of the cells that had reached a critical water activity of 0.35 could not recover their permeability. Therefore, the water activity interval between 0.86 and 0.99 appears to be critical for membrane permeability during rehydration. The existence of this critical step could be related to membrane events that occur during dehydration. Indeed, cells labelled with LY may have suffered from a reduction in surface area associated with the formation of endovesicles, as has already been proposed by Shalaev and Steponkus (1999) and is supported by our observations. Therefore, exposing these cells to rehydration levels that

impose significant increases in volume (cf. Figure 1) may result in their lysis during volume expansion. Okada and Rechsteiner (1982) reported that endovesicles that form under hyperosmotic conditions swell and burst upon rehydration of the cytosol.

In fact, we show that for water activity change lower than 0.6, the removal of a portion of water from the cells may lead to changes in the permeability of the cells resulting from the phase separation of phospholipids. In fact, lipid phase transition affects the resistance of membranes to shear forces (Sparr and Wennerstrom 2001) and volume contraction may thus be critical when this occurs.

Plasma membrane changes are strongly implicated in the mechanism leading to cell death during osmotic dehydration and rehydration. In particular, permeabilization resulting from lipidic phase transitions and severe volume contractions could explain the observed sequence of events. Moreover, the changes that occur during the steps of dehydration and rehydration are interdependent.

Effect of combined osmotic and thermal stresses

To show the link between yeast survival following combined osmotic and thermal treatments and membrane fluidity variations induced by such treatments, a cell viability diagram (a_w, temperature) and a membrane fluidity diagram (a_w, temperature) were presented in Figure 3 and Figure 4.

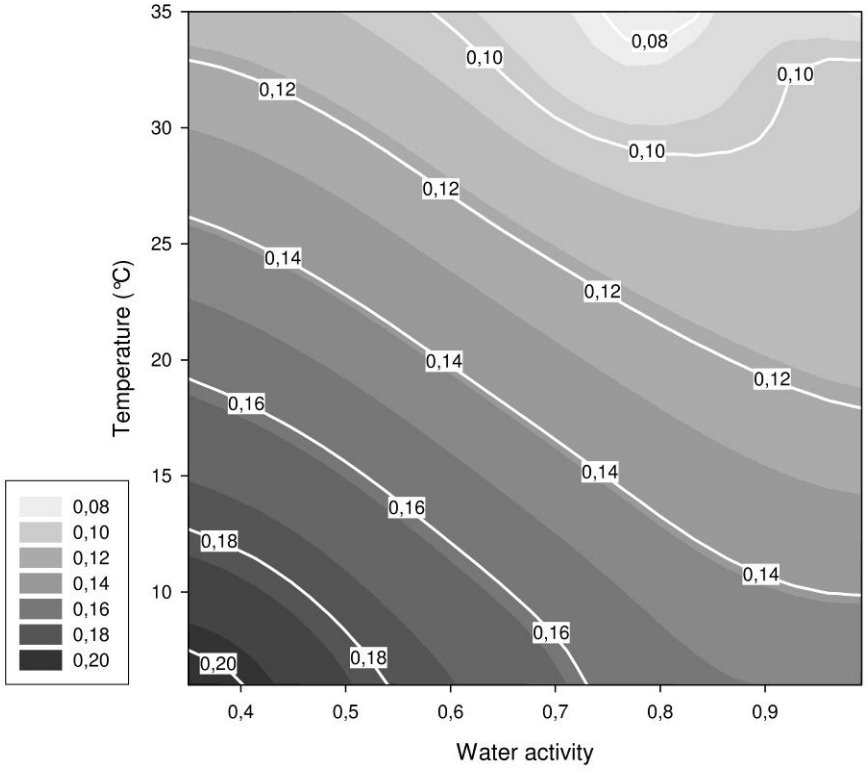

Figure 3: Iso-viability diagram of *Saccharomyces cerevisiae* versus temperature (4 °C to 40°C) and water activity. The viability data were obtained after 1 hour at indicated physical conditions and a rehydration to optimum conditions.

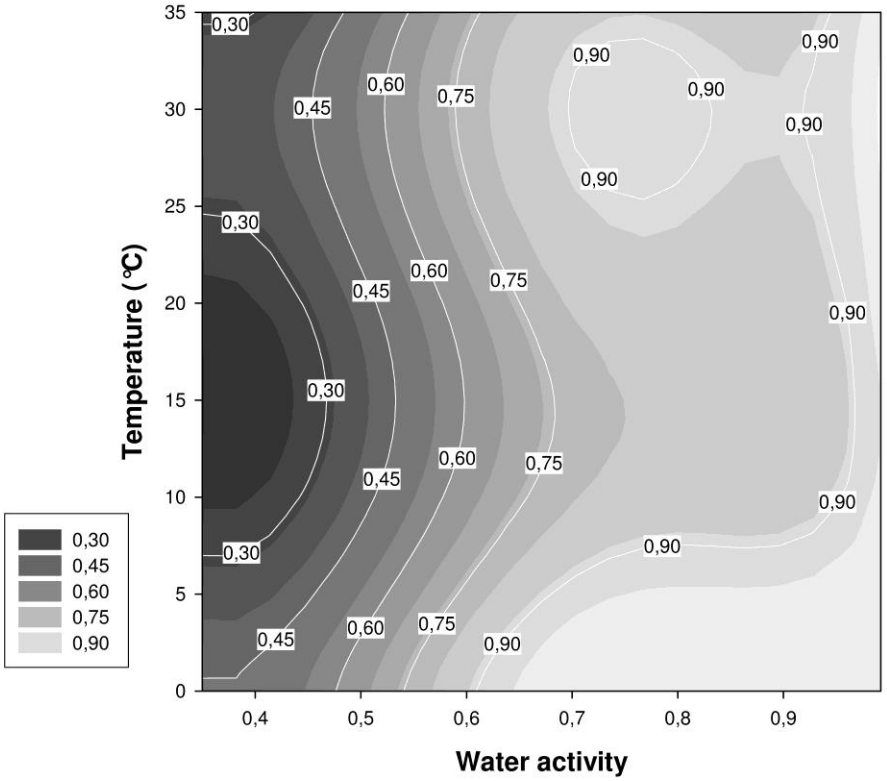

Figure 4: Iso-anisotropy of *Saccharomyces cerevisiae* membrane versus temperature and water activity. The anisotropy was measured with Diphenyl Hexatriene (DPH) fluorescence polarization.

According to these figures, we see that without phase change during dehydration, we can expect to have a greater survival. Thus, the fluidity diagram appears to be a potential tool for controlling membrane fluidity during cell dehydration and rehydration by simultaneously and independently managing a_w and temperature over time.

The fact that cell death provoked by osmotic shocks depends on temperature is well established. Dried yeast recovery is optimal if rehydration is performed at 38–40 C or at 50 C (Poirier *et al.* 1999). Furthermore, the temperature at which dehydration shock occurs in liquid medium has been shown to affect cell viability greatly (Laroche and Gervais 2003). Figure 4 shows enhanced resistance of

yeast cells to osmotic shocks at temperatures lower than 10 C and higher than 22°C. However, resistance to osmotic shock according to temperature is strain dependent, and each strain may have a specific behavior. A strain-dependent response to glycerol osmotic stresses has also been reported by Blomberg (1997).

Laroche and Gervais (2003) proposed that mortality following rapid dehydration or rehydration was related to water flow through an unstable membrane. In recent work, Guyot et al. (2006) completed this assumption and hypothesized that change in the fluidity of the plasma membrane was the critical event leading to cell death and that water flow was not necessarily involved in the cell death mechanism. Our present work confirms this latest assumption. However, here, if water outflow is not sufficient to provoke cell death, variation of fluidity in the case of thermal stress alone in the range 4–40 °C, i.e., without osmotic stress, did not provoke cell death. Thus, change in the fluidity of membranes is the critical event, but it must be accompanied by an osmotic stress and certainly the subsequent volume contraction. In the case of hyperosmotic shock, not only is the membrane in phase transition but also the cells are contracted. It is well known that cells shrink in response to osmotic stress. Such conditions of shrinkage associated with lipid phase separation occurring before and/or during the dehydration/rehydration step (Δr_2 provoked by the osmotic stress) could probably lead to plasma membrane permeabilization and leakage of cellular components. Our hypotheses concerning the mechanism leading to cell death during dehydration and rehydration is developed in two recent works (Simonin *et al.* 2007a; Simonin *et al.* 2007b).

Conclusions on first example: osmotic and temperature combination

There is a link between membrane state and survival of osmotic stresses. Particularly, changes in membrane fluidity before and/or during an osmotic treatment have an influence on yeast survival and lipid phase transitions in membranes are disadvantageous for cells submitted to osmotic shocks. The

use of the membrane fluidity diagram permitted control of the membrane fluidity of cells during dehydration and rehydration.

In order to understand the plasma membrane changes occurring during dehydration and rehydration, complementary techniques of membrane study should now be used. Actually, it must be taken into account that membranes are complex organelles composed of a variety of lipids structured in membrane domains and a global coefficient related to membrane fluidity is not sufficient to appreciate all the changes occurring in it. In fact, complex lipid phase behavior is known to occur at low water content (Milhaud 2004). Particularly, non-lamellar phases are suspected to arise at low water concentrations, as observed in model biomembranes (Shalaev and Steponkus 2001). We show that such a diagram like figure 4 should be a useful tool for improving yeast survival in dehydration/rehydration processes. Such process are involved when drying food or ferment, but also in freezing process. In fact freezing process at moderate temperature rate ($\Delta T<1000°C/min$) consists essentially for microorganisms in a hyperosmotic perturbation at low temperature (near 0°C, during water crystallization). Cell inactivation in this process could be mainly attributed to the combination of osmotic and temperature perturbation (Dumont *et al.* 2006).

Example 2: Effects of high hydrostatic pressure, low temperature and hyperosmotic combined perturbations

Combination of high hydrostatic pressure and low temperature on *E. coli* survival.

Numerous studies have demonstrated the temperature dependence of the antimicrobial effects of high pressure (Sonoike *et al.* 1992). Moreover, the efficiency of high-pressure treatments is controlled by other process parameters such as the applied pressure and the kinetics of pressurization (Palou *et al.* 1998) as well as by the physicochemical properties of the medium being treated, such as pH (Alpas *et al.* 2000) and water activity (Van Opstal *et al.* 2003). Precise control of these parameters is necessary

to ensure efficient treatment. With appropriate combinations of these parameters, a synergistic effect could be achieved, reducing the pressures and treatment times required.

The combined effects of high pressure and low or subzero temperatures on microbial inactivation have been studied by some authors. A synergistic effect between these parameters has generally been reported in the inactivation of microorganisms in the vegetative state (Hashizume *et al.* 1995; Perrier-Cornet *et al.* 2005). In some cases, the initial microbial populations were completely inactivated with a combined treatment of high pressure and low or subzero temperature, whereas only a slight microbial inactivation was achieved under the same pressure conditions at room temperature (Perrier-Cornet et al. 2005). The magnitude of this synergistic effect is strongly dependent on the type of microorganism (Takahashi 1992).

The interaction of high pressures and subzero temperatures in microbial inactivation is complex, and possible phase-transition phenomena must be taken into account. Some authors have recently demonstrated that freezing under hyperbaric conditions is an effective way to reduce microbial contamination (Luscher *et al.* 2004). In addition to the antimicrobial effects of combining high pressure and subzero temperature treatments, these treatments when combined offer various processing advantages such as rapid freezing and thawing and cold storage of foods under liquid conditions (Cheftel *et al.* 2002).

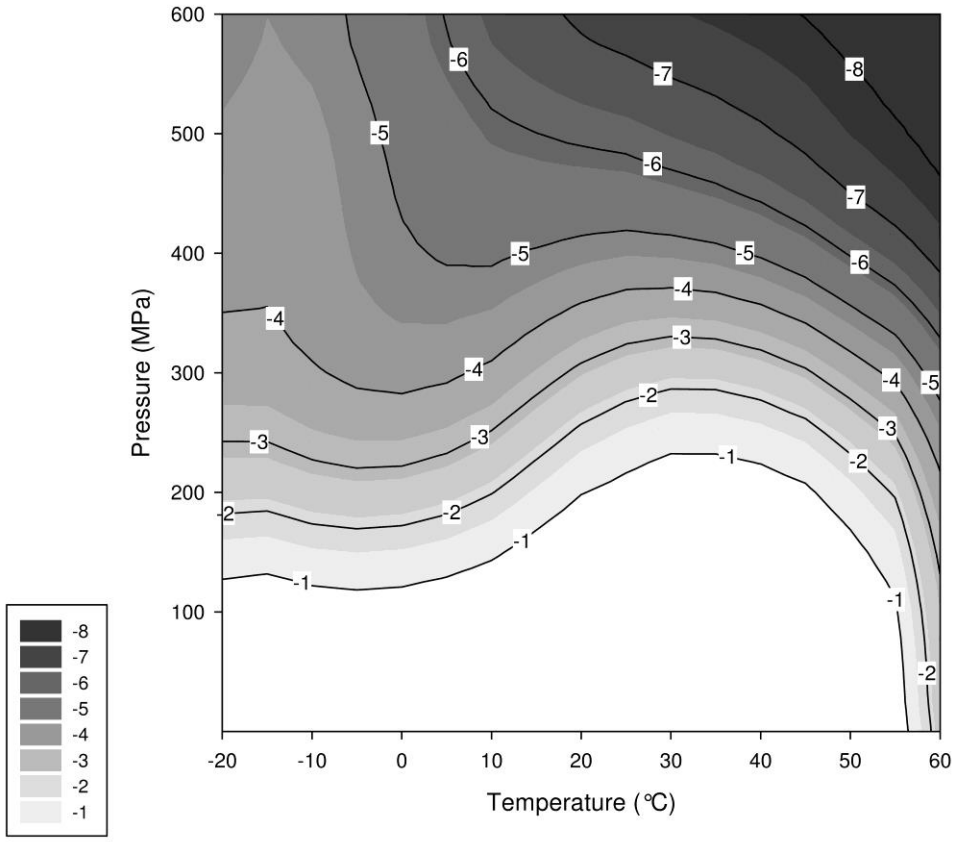

Figure 5: Iso-inactivation (log N_0/N) of *Escherichia coli* versus pressure and temperature in binary medium (water/glycerol) at water activity of 0.99. The sample was maintained 10 min at indicated conditions before growing in optimum conditions

The figure 5 shows the effect of a 10 min treatment at different pressure and temperature levels on the logarithmic inactivation of *E. coli* K12TG1. At –20 °C, in the supercooled region, the pressure sensitivity was greater than at 25 °C for pressure lower than 350 MPa. This synergism between high pressure and subzero temperature made it possible to reduce the pressure and/or improve the pressure-mediated inactivation. Irrespective of the inactivation rate, our findings corroborate the observations of Takahashi (41) who examined the inactivation of *E. coli* after pressure treatment (200 MPa, 20 min) at –20 °C and at room temperature. More recently, we reported that at a fixed

pressure of 150 MPa, an initial population of *S. cerevisiae* was completely inactivated at –20 °C (more than 8 log cycles under liquid conditions), whereas it was only slightly inactivated at 25 °C (less than 0.5 log cycles)(Perrier-Cornet et al. 2005). The viability of *E. coli* cells was less affected by the synergism between high pressure and subzero temperature than were the viabilities of *L. plantarum* and *S. cerevisiae* cells.

Above 350 MPa, the synergistic effect was completely neutralized by an antagonistic effect of subzero temperature. Accordingly, *E. coli* K12TG1 cells were more resistant at subzero temperature than at room temperature. A similar observation was described by Pagán and Mackey (2000) for *E. coli* H1071 cells in stationary phase of growth after pressure treatments at room temperature. The unusual pattern of survival of *E. coli* K12TG1 cells after combined high pressure and subzero temperature treatments was observed consistently in many experiments. These observations reflected a baroprotective effect at subzero temperature and very high pressure levels (>300 MPa). This effect has never been observed before and could be brought together with the atypical behavior of water molecule under pressure. At pressure lower than 300 MPa, due to hydrogen bonds, water exhibits atypical properties especially at low temperature (maximum of density, phase change, viscosity, …). At higher pressure (P> 400 MPa) water behavior become more regular. The water activity of the medium has been modulated in order to better understand the relationship between thermodynamic properties of water and the inactivation of microorganisms by combined high pressure and subzero temperature.

Effect of low temperature and hyperosmotic perturbation on E. coli baroresistance

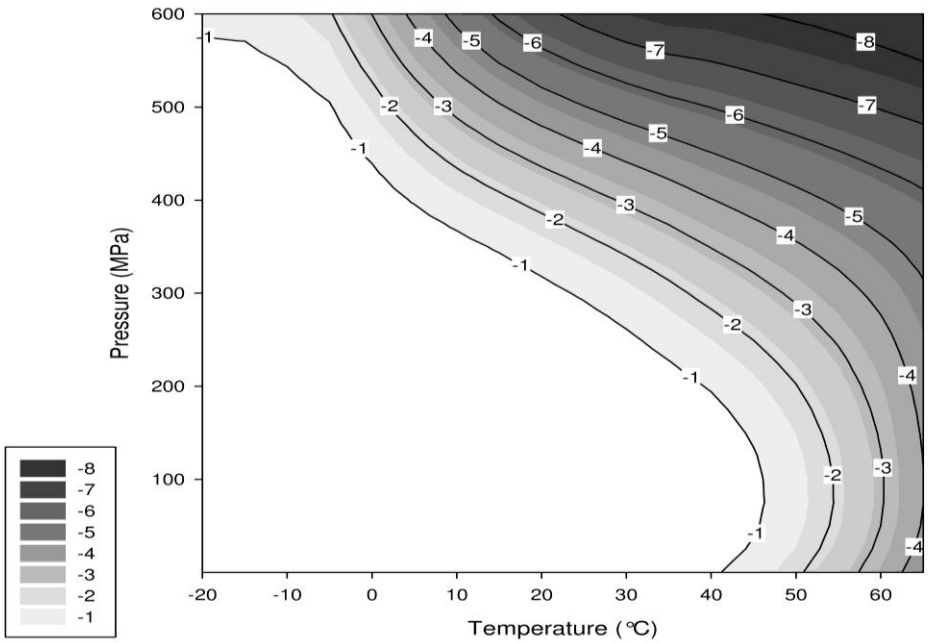

Figure 6: Iso-inactivation (log N_0/N) of *Escherichia coli* versus pressure and temperature in binary medium (water/glycerol) at water activity of 0.85. The sample was maintained 10 min at indicated conditions before growing in optimum conditions

As shown on figure 6, the pressure sensitivity of *E. coli* K12TG1 was highly dependent on the water activity of the system. When the bacterium was suspended in a water/glycerol solution with an a_w of 0.85, it appeared to be more pressure resistant than at an a_w of 0.99. This finding underscores the baroprotective effect of solutes, previously described for *E. coli* (Satomi *et al.* 1995; Van Opstal et al. 2003), *Rhodotorula rubra (Oxen and Knorr 1993)*, and *Zygosaccharomyces bailii* (Palou *et al.* 1997). The combination of subzero temperature and high pressure at an a_w of 0.85 caused a cumulative protective effect of solute and subzero temperature against pressure-induced inactivation. Only the protective effect of low temperature appears on figure 6 at medium with a a_w

0.85. Owing to the the protection conferred by the solute, higher pressure levels are necessary to inactivate *E. coli* cells. Moreover, inactivation occurs only in the P-T domain where synergistic effect was dominant. When pressurized in distilled water (a_w of approximately 1), *E. coli* K12TG1 showed a much higher pressure sensitivity than at lower water activities, especially at –20 °C (Figure 7). In this case only the synergistic effect of low temperature is observed probably because all the population is inactivated at a pressure lower than 400 MPa.

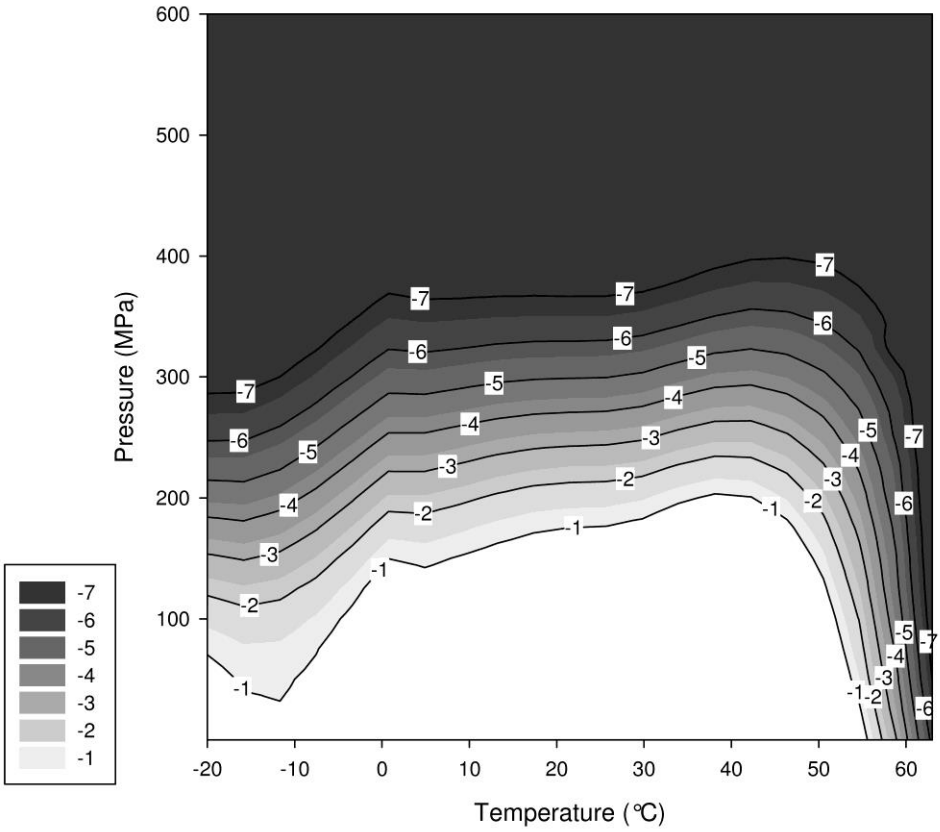

Figure 7: Iso-inactivation (log N_0/N) of *Escherichia coli* versus pressure and temperature in distilled water. The sample was maintained 10 min at indicated conditions before growing in optimum conditions

Parallel change with pressure and temperature of protein behavior, microbial inactivation and water structure

Several studies have highlighted the crucial role of water in the pressure-induced denaturation of biological systems. Oliveira *et al.* (1994) reported that protein denaturation decreased linearly with a decrease in water concentration. Similarly, Kinsho *et al.* (2002) observed that the removal of water by the addition of polyols or small cationic ions had an efficient protective effect against enzyme inactivation at high pressures and subzero temperatures. These latter authors also reported that cold-inactivation mechanisms were pressure dependent and differed at pressures below 200 MPa from those at pressures above 200 MPa. Moreover, a maximum stability temperature was evidenced for different proteins and a bell-shaped dependence of protein stability on temperature was observed (Smeller 2002). A parallel has been proposed between the structure of water and the thermal denaturation of proteins (Klotz 1999). In fact, among other similarities, the graph of liquid water density follows a bell-shaped curve at atmospheric pressure with a maximum at 4 °C. Some authors emphasized the effect of pressure on water density as a key for understanding cold denaturation of proteins at high pressure (Marques *et al.* 2003).

The properties of water under pressure vary and are largely a function of the pressure range (Cavaille *et al.* 1996). Indeed, the effect of increasing pressure on the behavior of cold water is to systematically push the temperature of maximum density to lower and lower temperatures. The so-called atypical properties are observed for pressures below 200 MPa. However, above 400 MPa pressure, water loses its particular characteristics and behaves like a classic hydrogen-bonded liquid. The addition of solutes causes the formation of hydration shells, leading to a new organization of water molecules. This phenomenon is strongly enhanced when the pressure is increased and, accordingly, it cancels out the particular properties of pure water in the pressure range 0.1–200 MPa (Kanno and Angell 1979).

The variation in water properties with pressure, temperature, and the presence of solutes reflects changes in the arrangement of water molecules. From a biological point of view, this could explain the baroprotective effects of solutes on proteins and microorganisms under denaturing conditions. The mechanisms of pressure-induced microbial inactivation may involve denaturation of some critical life processes such as enzyme reactions as suggested by some authors (Hashizume et al. 1995; Perrier-Cornet et al. 2005). Also, a parallel between water properties and microbial inactivation can be identified. For a known set of hydration conditions, a synergistic effect was observed at pressures up to a critical level (250 MPa for an a_w of 0.992), whereas antagonism occurred at pressures higher than this critical level. The consequence of increasing the hydration rate at a fixed pressure was to enhance the synergism and increase the pressure threshold that marked the crossover between synergism and antagonism. Below this threshold, pressure and temperature affect microbial viability in a similar manner and, in the same way, water behaves as a singular liquid. Above this threshold, pressure and temperature have roughly opposite effects on microbial viability and, at the same time, water behaves as a classic hydrogen-bonded liquid.

Conclusions on second example: high pressure, temperature and osmotic combination

This work shows that combined high-pressure and subzero temperature treatment is a promising way to optimize high-hydrostatic-pressure processes, since such a combination made it possible to reduce the pressure magnitude and/or improve the pressure-mediated inactivation. Nevertheless, the interaction between high pressure and subzero temperature appears to be complex. Indeed, it was pointed out that, depending on pressure level and a_w of the medium being treated, subzero temperature counteracted the inactivation caused by high pressure. This unexpected phenomenon leads to the necessity to take into account the process parameters to ensure efficient treatment. The structure of water versus the stability of proteins and the microbial inactivation allowed to suspect the crucial role of water in such phenomenon. Further work should be undertaken with a view to better elucidate this phenomenon.

General conclusions

These two examples show the critical importance of thermodynamic properties of water in the survival of microorganisms. Maintenance of living structures by water could only be effective if water keeps its specific properties. Modifying molecule properties by pressure, temperature or osmotic solutes would change cell equilibrium. This change of thermodynamical conditions is also accompanied by mechanical constraints (water efflux with hyperosmotic stress, hydrostatic compression with pressure) which will destabilize the cell and especially the cell membrane. In minimizing such perturbation and choosing correct thermodynamical properties, it is possible to maintain the viability of living cell even under very drastic conditions. The osmotic dehydration at controlled temperature can allow obtaining viable dehydrated cells even on sensible organisms. Appropriate combinations of high pressure processing, low temperature could also allow to preserve the cell viability at very high pressure and without adding solutes. This process would be interesting to maintain cell at low temperature and high pressure in liquid conditions. Thus, the understanding of the role of water in the mechanisms of cell sensitivity to pressure and temperature perturbations would allow developing promising processes combining different thermodynamic parameter to preserve or inactivate organisms and microorganisms.

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