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Osmotic destruction of *Saccharomyces cerevisiae* is not related to a high water flow rate across the membrane

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Abstract

Influence of the kinetics of osmotic pressure variation on yeast viability was related to the rate of water transfer across the yeast membrane. A very high value of membrane hydraulic permeability ($L_p > 6 \times 10^{-11}$ m s^{-1} Pa^{-1}) of the yeast *Saccharomyces cerevisiae* implies the presence of aqueous pores in the yeast membrane and explains the yeast resistance to a very high osmotic flow. The high water flow rate can not explain, by itself, the cell mortality following an osmotic shock. Experiments performed at different osmotic pressure levels show that the yeast death under osmotic shifts was related to the coupled effects of the kinetics of osmotic pressure variation and of the total osmotic pressure level of the medium.

Keywords: Kinetic parameters; Mass transfer; Membranes; Viability; Water activity; Yeast

1. Introduction

In a previous work [1], the decrease rate of the water potential was found to have a great effect on yeasts submitted to hypertonic shifts. The application of slow and linear decreases of water potential of the medium to cells of *Saccharomyces cerevisiae* demonstrated that the cells could survive, (90–100% of viability), at very high levels of osmotic pressure, (Π = 100 MPa), whilst faster kinetics of water potential variation were found to be associated with lower viability levels. Identical observations were also made on different bacterial species [2].

Numerous assumptions related to the effect of osmotic pressure level on the cells have been made. Some of these assumptions are only related to the final volume reached after the osmotic shock. For instance, the increase of the intracellular ionic concentration, especially for sodium ions, due to the water efflux, has been proposed to be involved in the denaturation of intracellular macromolecules (proteins and enzymes) [3]. Such an assumption could not be retained to explain the influence of the kinetics of the osmotic pressure increase, because it has been shown that this kinetics did not modify the final cell volume [1].

To explain this kinetics effect, the suggestion is made that, during the transient phase, the cell is injured by the water flow rate from the cell to the medium that is too large. The flow rate of the cell water is limited by the hydraulic membrane permeability (L_p) and the exchange surface area. A high and sudden water potential gradient between intra and extracellular media would put too much mechanical constraint on the membrane. Thus, the water potential gradient may be too large for the membrane resistance, which would be denaturated during the hydric shift, as proposed by previous workers during freezing [4,5], or during osmotic permeabilization [6]. Such damage, which is directly related to the membrane's ability to let water pass through, can involve an important mortality of the cell population.

The measurement of *S. cerevisiae* membrane water permeability has been investigated by numerous authors [7–9]. Table 1 summarizes the main data about the time response of cell volume variation following an osmotic shift obtained through the mixing of a cell suspension in a hypertonic solution and subsequent cell volume decrease measurement.

Analysis of these data for the same kind of cells, shows great discrepancy in the cell volume–time response. For *S. cerevisiae*, volume–time responses, (for 50% of the cell volume variation), were found to vary between 3.7 s and 15 min. Such variations must involve great variation in the estimation of L_p values for *S. cerevisiae*. Previous authors have already shown that L_p could be modified by mixing devices and unstirred layers, i.e. mixing time variations [10].

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Table 1
Time to reach 50% of the final cell volume variation of *S. cerevisiae* determined with different devices and different temperatures (Martínez de Marañón et al. [12])

Equipment for allowing cell volume variation measurement	Kind of cells	Time to reach 50% of the final cell volume variation	T (°C)
Diffusion chamber + microscopy [8]	*S. cerevisiae*	15–30 s	20
Centrifugation + microscopy [9]	*S. cerevisiae*	15 min	25
Mixing chamber + coulter counter [10]	Protoplasts of *S. cerevisiae*	3.7 s	23

This work is intended to study the previous hypothesis, which has been advanced to explain the influence of the kinetics of osmotic pressure variation on yeast viability, namely determining the mechanisms of water transfer across the yeast membrane in response to an osmotic stress.

In the first part, the measurement of the hydraulic membrane permeability (L_p) of *S. cerevisiae* CBS 1171, taking into account the mixing times of the devices used, has been achieved. A mathematical model has also been developed to calculate L_p from the kinetics of cells' volume variation and external osmotic pressure variation.

In the second part, the viability of *S. cerevisiae* has been estimated when submitted to different rates of osmotic pressure increase until different levels of osmotic pressure.

2. Material and methods

2.1. Description of the mixing systems

An osmotic shift involves the replacement of an isotonic extracellular solution by a hypertonic one. This shift was achieved in the present study by using two mixing systems that allow the cells' volume decrease to be continuously recorded during osmotic upshifts. These two devices were used since their mixing time constants were different. A microscopic chamber, previously described [11], with a high mixing-time constant, was used. Briefly, a syringe pump, joined to a three-way tap, was used to generate the osmotic pressure step changes in this microscopic chamber. The mixing of yeast and hyperosmotic solutions was achieved with two flow rates, by varying the syringe pump speed, and consequently with two mixing time constants. The second mixing device was a stopped-flow mixing apparatus, (SFA-20, Hi-Tech, UK), coupled to a spectrophotometer (Oriel, USA). The system is described elsewhere [12].

2.2. Osmotic solutions

2.2.1. L_p measurement

In this part of the study, only hypertonic variations of the medium have been investigated. Osmotic solutions of a fixed osmotic pressure value (5.20 or 5.62 MPa) were obtained by adding sorbitol to water, as sorbitol is considered to be a non-permeable solute [9,13].

2.2.2. Influence of the level of osmotic pressure on the yeast viability

For high osmotic pressure values, (up to 15 MPa), sorbitol can not be used any more, due to a weak solubility of the molecule in water. Instead, glycerol solutions were prepared by dissolving glycerol in distilled water. Cells were quickly injected into the glycerol solution for sudden increase in osmotic pressure.

In all experiments, the required mass of depressors to be added to 1000 g of water has been described in the previous work [11].

2.3. Cells treatments

Osmotic shifts intended for L_p measurement were performed on the yeast *S. cerevisiae* CBS 1171. The yeast was kept on a gelose slant [14]. Cells were grown on a modified Wickerham medium, (osmotic pressure of 0.96 or 1.38 MPa obtained by the addition of glycerol), in 250 ml conical flasks at 250 rpm and 25°C. Cells were harvested after 48 h of growth (stationary phase) and immobilized on microscopic chambers. A cationic polymer, chitosan (Fluka, Biochemie, no. 22741, USA), was used to improve the immobilization of the cells [11,15]. The chitosan solution was removed from the chamber and was dried for 1 h before putting the yeast in the bottom of the microscopic chamber for immobilization. The cell suspension, (200 μl), with a concentration of approximately 8×10^6 cells ml^{-1}, was placed in the centre of the chitosan-coated microscopic chamber and was subjected to a final osmotic solution corresponding to an osmotic pressure increase of 4.24 MPa, (from 0.96 to 5.20 MPa, or from 1.38 to 5.62 MPa). At high concentrations, chitosan is known to have toxic effects on cells [16,17]. However, at the low concentration used in this study, control cultivation verified the concentration to be nondetrimental.

2.4. Cell viability

Cell viability measurement, which was followed by methylene blue dying for yeast, was performed at 24°C, just before and after all osmotic perturbations, through counting with a Malassez's chamber. The viability was expressed in a relative way, as a percentage of the initial viable population. There were at least three repetitions for all experiments.

2.5. Cell volume measurement

The microscopic chamber was placed on an inverted microscope, (Leitz-Labovert, Germany). An image analysis system (series 151, Imaging Technology Inc., USA) allowed images to be recorded via a camera CCD (model 6710, Cohu, USA) during the osmotic shift, and subsequently permitted their analysis using Visilog software, (Noesis, France). A detailed description of the system has been given previously [11,18].

In the stopped-flow chamber, the measurement of the kinetics of cell volume variation was achieved by measuring the kinetics of absorbance evolution, at 700 nm, which has been correlated to cell volume variation [19].

2.6. Evaluation of the cell membrane hydraulic permeability (L_p)

Previous considerations [12] showed that on-line measurement of the cell volume, during osmotic shift, must take into account the time constant for two successive systems, which are: the mixing time constant and the cell volume variation time constant. By considering the mixing chamber as a linear first order system and by using the Kedem–Kaltchasky model and the Boyle Van't Hoff law for the cell, previous work [12] allows the following differential equation (Eq. (1)), which relates the intracellular osmotic pressure to the time.

$$\frac{d\Pi_i}{dt} = \frac{A\,L_p\,\sigma}{RTn_s}[\Pi_i^2(\Pi_e - \Pi_i)] \quad (1)$$

where t is the time following the osmotic shift, Π_i the cell osmotic pressure (Pa) at time t, Π_e the external osmotic pressure (Pa) at time t, A the cell area (m^2), σ the reflection coefficient for a solute ($\sigma = 1$ for semi-permeable membrane), L_p the hydraulic membrane permeability ($m\,s^{-1}\,Pa^{-1}$), R the ideal gas constant ($J\,K^{-1}\,mol^{-1}$), T the temperature (K) and n_s the number of intracellular solute moles considered as constant during the osmotic stress.

From Eq. (1), the calculation of the L_p value clearly needs knowledge of the real osmotic shift intensity, i.e. $\Pi_e - \Pi_i(\Delta\Pi)$.

As the realization of a perfect step change of the extracellular osmotic pressure is not possible, Π_e and then $\Delta\Pi$ are the functions of time. Indeed, there is an obligatory response time of the system which depends upon the size of the mixing chamber and on the rheological properties of the solutions. Therefore, as shown in Table 1, the discrepancies between results of cell volume time response following an osmotic shift should be mainly attributed to phenomena such as the different mixing properties of the equipment used. Hence, the cell's hydraulic permeability (L_p) value of 1.97×10^{-15} given by Gélinas et al. [10] must be associated to the corresponding mixing time, i.e. 3.7 s (see Table 1).

Nevertheless, and from the previous theoretical consideration, exact L_p estimation would be possible only if an experimental difference between Π_i, (estimated through the cell volume evolution, i.e. Boyle Van't Hoff law), and Π_e, (estimated through spectrophotometric measurement of a dye) is detected for each time of the osmotic perturbation.

If such a difference can not be estimated, then the cell time response is much shorter than the mixing time response, and only an approximate value by default of L_p can be given.

These mixing phenomena have been taken into account in this work by using three different mixing systems. Osmotic shifts were carried out on the yeast *S. cerevisiae* and estimations of L_p values (membrane hydraulic water permeability) have been obtained by numerical fitting of Eq. (1) to experimental data of cell volume variation (i.e. Π_i) and of external osmotic pressure (i.e. Π_e). The Matlab software (version 4.2c, MathWorks, USA) running under AIX (version 4.2.5) on an IBM RS/6000 550 computer was used in order to perform the numerical computations and the graphics. The subroutines used were ODE45 for the solution of the differential equations.

3. Results and discussion

3.1. Characterization of the mixing systems: Π_e measurement

All the mixing devices used were considered as linear first-order systems. So, the evolution of the external osmotic pressure (Π_e) during the mixing time is directly related to the time constant value (λ_m).

The mixing characteristics of the microscopic chamber were determined using methylene blue as a mixing tracer which was analysed through the microscope and image analysis system. The mixing time constants (λ_m) corresponding to 63% of the response of these first-order systems are $\lambda_m =$ 3.2 and 1 s, when the osmolyte flow rates in the chamber were fixed at 2 and 7 ml min^{-1}, respectively (Table 2).

With the stopped-flow system, also considered as a first-order system, the mixing time constant could be measured by the evolution of the absorbance due to the increase of methylene blue concentration. The mixing of two sorbitol solutions (e.g. one at 0.98 MPa and the other at an osmotic pressure of 9.42 MPa) with one containing methylene blue, was performed and the mixing time constant was found to be $\lambda_m = 3 \times 10^{-2}$ s.

Table 2
Estimated L_p values of *S. cerevisiae* obtained from the three devices described in this paper

	Mixing time constant (λ_m)	L_p ($m\,s^{-1}\,Pa^{-1}$)
Stopped flow	0.03	$>6 \times 10^{-11}$
Microscopic chamber, osmotic flow rate: 7 ml min^{-1}	1	$>2 \times 10^{-13}$
Microscopic chamber, osmotic flow rate: 2 ml min^{-1}	3.2	$>6 \times 10^{-13}$

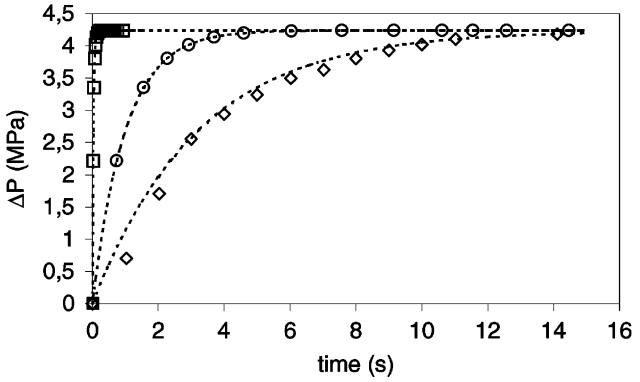

Fig. 1. Simultaneous evolution of the intracellular osmotic pressure (Π_i) of *S. cerevisiae* and of the extracellular osmotic pressure (Π_e) (dashed lines) after an osmotic shift ($\Delta\Pi$) of 4.24 MPa. The osmotic shift was achieved with three different mixing devices: the microscopic chamber with two flow rates: 2 ml min^{-1} ((◇), $\lambda_m = 3.2$ s) and 7 ml min^{-1} ((○), $\lambda_m = 1$ s) and the stopped-flow mixing apparatus ((□) $\lambda_m = 0.03$ s).

3.2. Cell volume response to an osmotic stress: Π_i measurement

Data concerning the yeast cell volume transient evolution during the osmotic shift were collected. These volume data were converted to the intracellular osmotic pressure data (Π_i) through the Boyle Van't Hoff law (see Eq. (2)), with the hypothesis that only water was flowing through the membrane, (i.e. n_s is constant).

$$\Pi_i = \frac{RTn_s}{V - b} \quad (2)$$

where Π_i is the osmotic pressure of the cell (Pa), V the cell volume (m^3), b the non-osmotic volume (m^3), R the ideal gas constant (J K^{-1} mol^{-1}), T the temperature (K) and n_s is the apparent number of osmotically active moles into the cell.

From these results and Π_e measurements, the L_p value was estimated through the fitting of Eq. (1) to experimental results. If the Π_i evolution could not be experimentally differentiated from the Π_e evolution, the fitting of Eq. (1) will only give an approximate value of L_p (by default).

For *S. cerevisiae*, as shown on Fig. 1, for the three mixing time devices (i.e. stopped-flow with $\lambda_m = 0.03$ s and microscopic chambers with $\lambda_m = 1$ and 3.2 s, respectively) the water exit from the cell was always limited by the mixing time as for the three experiments $\Pi_e = f(t)$ (measured by the dye concentration evolution during the mixing) and $\Pi_i = f(t)$ (measured through the cell volume evolution during the mixing) are merged. So, for the three devices the estimated L_p value for this yeast (from Eq. (1)) can only be approximated by default. Therefore, the real L_p value would be probably greater to the estimated value as proposed in Table 2. These results show how far the L_p value is influenced by the mixing device used for the experiment.

The high osmotic permeability of *S. cerevisiae* found using the stopped-flow mixing system linked to a spectrophotometer ($L_p > 6 \times 10^{-11}$ m s^{-1} Pa^{-1}) implies that this yeast may contain water channels in the plasma membrane. Indeed, red blood cells which have a L_p around 1.5×10^{-11} m s^{-1} Pa^{-1} possess water channels [20], and on the basis of sequence similarities, André [21] has speculated that two *S. cerevisiae* aquaporin-like proteins are water-channel proteins.

3.3. Influence of fast osmotic shifts on yeast viability

Viability measurements of *S. cerevisiae* have been made after the realization of very fast osmotic shocks with the three devices used in this study. A faster device with a mixing time constant of about 1 ms has also been tested, (SF61-DX2, Hi-Tech, UK) between 1.38 and 3.29 MPa (sorbitol addition).

Yeasts viability results, after different osmotic shocks in the previous different devices, are shown in Table 3. These results clearly demonstrate that even for very high water flow rates, corresponding to a very high variation rate in osmotic pressure, (1209 MPa s^{-1}) the yeasts stayed totally viable. So, high water flow rate across the cell membrane is not detrimental for *S. cerevisiae*.

As the osmotic perturbations generated in the stopped-flow apparatus were optimised, in order to reach fast mixing times, the viscosities of the solutions to be mixed (yeast suspension and glycerol solutions) were close to each other, and close to the water viscosity. So, the higher osmotic gradient generated in such systems did not exceed 5 MPa, whereas the negative influence of the kinetics of hydric potential variation on yeast viability was previously observed through osmotic upshifts to 100 MPa (in glycerol) [1]. So, in a second step, experiments with osmotic gradients of higher intensities, by injecting quickly 1 ml of cell suspension in 100 ml of a glycerol solution, have been carried out. The final hydric potential reached by such a

Table 3
Influence of the variation rate of osmotic pressure on the viability of *S. cerevisiae* with regard to different mixing systems used

Mixing system	Mixing time constant (λ_m, s)	Osmotic pressure shift (MPa)	Variation rate in osmotic pressure (MPa s^{-1})	Viability (percentage of the initial population)
Microscopic	3.2	4.24	0.73	100
Chamber	1	4.24	2.67	100
Stopped-flow	0.03	4.24	89.07	100
	0.001	1.91	1209	100

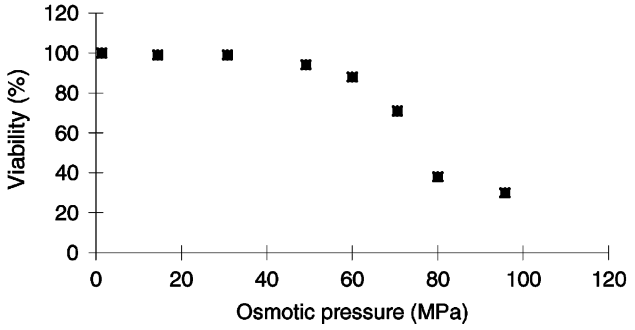

Fig. 2. Viability of *S. cerevisiae* after sudden osmotic shifts of increasing final osmotic pressure. Depressor used: glycerol; initial osmotic pressure: 1.38 MPa; mixing time constant: 1 s.

mixing method was contained between 15 and 100 MPa and the mixing time constant was approximately 1 s. So, the corresponding variation rates in osmotic pressure were between 15 and 100 MPa s^{-1}, values which have been found not to be detrimental in the previous results (see Table 3).

3.4. Yeast viability after sudden osmotic shifts ranging from 15 to 100 MPa

Yeast viability was measured by methylene blue dying after return to initial osmotic pressure (water addition). Viability results after instantaneous osmotic shifts of increasing gradients are presented in Fig. 2. Cell death was not linearly related to the osmotic gradient, since it only occurred for osmotic shifts higher than 50 MPa. This first observation indicates that the kinetics of osmotic pressure variation do not influence cell viability in the osmotic pressure range 0.96–50 MPa. For higher osmotic gradients, cell viability was drastically decreased after an instantaneous shift, whereas progressive increases to such levels greatly enhance the cell viability [1]. An osmotic gradient of 50 MPa could correspond to the maximal water flow that the yeast cell can support. However, the intensity of such an osmotic gradient (50 MPa s^{-1}) is largely inferior to the intensity generated in the previous stopped-flow systems (see Table 3) and the resulting water flow is less intensive than in the stopped-flow systems.

By comparing these results to cell volume evolution proposed in previous work [1], cell death appears to occur after the water has totally flown out of the cell, because cell non-osmotic volume was reached from an osmotic pressure level of 50 MPa. So water exit flow rate from the cell could not be involved at all in cell death.

The assumption could then be made that a fast water exit from the cell could induce a modification of the area to volume ratio of the cell, which implied a folding or a vesiculation of the membrane, accompanied with a loss of membrane material. Successive rehydration of such cells could then imply a necessary burst of the membrane that could not support the global water entry.

4. Conclusion

This work has allowed a precision in the range of values for the time constant of water transfer across the cell membrane of the yeast *S. cerevisiae*, but an exact L_p value of the hydraulic permeability of the cell membrane could not be estimated. However, the minimal water permeability of *S. cerevisiae* obtained in this study is greater than all the previous data determined for *S. cerevisiae* (see Table 1) and greater than the permeability estimated in the literature for red blood cells, implying that this yeast may contain aqueous pores in the membrane. As yeast cells were found to be viable after a cell volume decrease achieved within a few milliseconds, the conclusion was made that the water efflux intensity was not the sole parameter influencing yeast viability under osmotic shifts. The kinetics of increase in the osmotic pressure has been found to influence yeast viability in the osmotic pressure range 50–100 MPa in glycerol solutions, where the yeast approaches its non-osmotic volume. The hypothesis of a membrane structural change, caused by instantaneous osmotic pressure variation between 50 and 100 MPa is advanced to explain the difference in yeast viability following slow or rapid increase in medium osmotic pressure. Further works will focus on the following two points:

1. A more accurate determination of yeast hydraulic permeability (L_p) by use of faster mixing devices.
2. A physical study of yeast membrane under osmotic stress based on the spectrometric measurements.

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