



Morphological and physiological changes in *Saccharomyces cerevisiae* by oxidative stress from hyperbaric air

I. Belo*, R. Pinheiro, M. Mota

Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

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Abstract

Increase in air or oxygen pressure in microbial cell cultures can cause oxidative stress and consequently affect cell physiology and morphology. The behaviour of *Saccharomyces cerevisiae* grown under hyperbaric atmospheres of air and pure oxygen was studied. A limit of 1.0 MPa for the air pressure increase (i.e. 0.21 MPa of oxygen partial pressure) in a fed-batch culture of *S. cerevisiae* was established. Values of 1.5 MPa air pressure and 0.32 MPa pure oxygen pressure strongly inhibited the metabolic activity and the viability of the cells. Also, morphological changes were observed, especially cell-size distribution and the genealogical age profile. Pressure caused cell compression and an increase in number of aged cells. These effects were attributed to oxygen toxicity since similar results were obtained using air or oxygen, if oxygen partial pressure was equal to or higher than 0.32 MPa. The activity of the antioxidant enzymes, catalase and superoxide dismutase (SOD) (cytosolic and mitochondrial isoforms) indicated that the enzymes have different roles in oxidative stress cell protection, depending on other factors that affect the cell physiological state.

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1. Introduction

Many enzymes, antibiotics, biochemicals, diagnostics and therapeutics are produced using aerated bioreactors. Supplying oxygen to aerobic cells has always represented a significant challenge to fermentation technologists due to the low oxygen solubility in cul-

ture media. In order to overcome this problem, air pressure increases have been proposed by many authors (Yang and Wang, 1992; Wendlandt et al., 1993; Belo and Mota, 1998; Pinheiro et al., 2000).

Total air pressure is an important operating factor because it affects local dissolved oxygen tension, and may influence cell morphology and physiology.

Despite the fact that oxygen is an essential nutrient for aerobic organisms, it can also be a toxic agent that can damage cells by the action of reactive oxygen

* Corresponding author. Tel.: +351 253 604400;

fax: +351 253 678986.

E-mail address: ibelo@deb.uminho.pt (I. Belo).

species (ROS). ROS are by-products of the normal cellular metabolism but generation is stimulated by many different stress conditions, including oxygen pressure increase (Moradas-Ferreira et al., 1996). However, aerobic cells have evolved several defence mechanisms. Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), constitute the primary defences of the cells because they are responsible for the degradation of some ROS. Oxidative stress is a well-known phenomenon that results whenever the ROS concentrations exceed the antioxidant capacity of the cells (Santoro and Thiele, 1997). The cellular damage caused by oxidative stress is due to the chain reactions that are established between the ROS and the biomolecules of the cells, such as DNA, proteins and lipids. Thus, oxidative stress affects cellular physiology and morphology. The present report investigates the effects of increased air and oxygen pressure on the oxidative stress response of *Saccharomyces cerevisiae* in a fed-batch bioreactor. This is the most used mode of cultivation for the production of yeast biomass used in many important industrial processes, such as baker's yeast (Van Hoek et al., 2000) and recombinant proteins production (Koo et al., 1998). Cellular behaviour under different air and oxygen pressures was followed by the analysis of several aspects: metabolic activity of the cells, cellular viability, intracellular antioxidant enzymes activity, genealogical profile of the cells and cell-size distribution. Cell size was evaluated by computer-aided image analysis techniques. This methodology has been proven to be very accurate and rapid, and it has been applied for decades in the measurement of cell viability by epi-fluorescence (Raynal et al., 1994), in the assessment of the physiological state of cells by the measurement of vacuoles size (Zalewski and Buchholz, 1996) and of cell volume (Gervais et al., 1992).

2. Materials and methods

2.1. Yeast strain and medium

S. cerevisiae ATCC 32167 was used in this study and was preserved at -80°C in 20% (v/v) glycerol. Agar plates (20 g L^{-1} agar) were inoculated from stock cultures and cells were grown by incubation overnight at 30°C . The culture medium consisted of $5\text{ g KH}_2\text{PO}_4$,

$2\text{ g (NH}_4)_2\text{SO}_4$, $0.4\text{ g MgSO}_4\cdot 7\text{H}_2\text{O}$, 1 g yeast extract (DIFCO), 5 g glucose and 1 L of distilled water. The pH was adjusted to 4.0 prior to autoclaving. Colonies from an agar plate were grown in 200 mL of culture medium, at 150 rpm and 30°C , for 16 h . Cells from this pre-culture were harvested by centrifugation, re-suspended in 130 mL of fresh medium and fed into the bioreactor.

2.2. Fed-batch cultivation

Fed-batch experiments were carried out in a 600 mL stainless steel bioreactor (Parr 4563) at a temperature of 30°C , stirring rate of 400 rpm and a gas flow rate of 1 L min^{-1} (measured at 0°C and 0.1 MPa). The operating pressure was set by compressed gas pressure (inlet gas) and the regulatory valve position in the exit gas line.

The medium was similar to that used in inoculum preparation, with the exception that 2 g L^{-1} glucose and 1 mL of antifoam (Merck 7743) were used. The feed medium contained per litre: $7.5\text{ g KH}_2\text{PO}_4\text{ L}^{-1}$, $10\text{ g (NH}_4)_2\text{SO}_4\text{ L}^{-1}$, $1\text{ g MgSO}_4\cdot 7\text{H}_2\text{O L}^{-1}$, 5 g yeast extract L^{-1} and 60 g glucose L^{-1} .

An exponentially feeding profile was used according with Eq. (1) (Yee and Blanch, 1992).

$$F(t) = \frac{\mu X_0 V_0 \exp(\mu t)}{S_F Y_{X/S}} \quad (1)$$

where X_0 is the initial cell concentration in the fed-batch phase (3 g L^{-1}) and V_0 is the culture volume when the medium feed started (130 mL). The other variables assumed the following values: $S_F = 60\text{ g L}^{-1}$, $Y_{X/S} = 0.5\text{ g g}^{-1}$ (grams of cell dry weight per gram of glucose) and $\mu = 0.1\text{ h}^{-1}$. The medium was pumped into the reactor using a high-pressure pump (Jasco 880-PU) with flow rates between 1 mL h^{-1} and 25 mL h^{-1} according with Eq. (1). The pump automatically performed the flow rate increment. In the pump programme, the exponential function was approximated by a sequence of several linear functions of increasing slopes.

A constant specific glucose uptake rate of $0.2\text{ g g}^{-1}\text{ h}^{-1}$ was expected for the feeding flow profile used, assuming the cells followed a oxidative metabolism. The same feed flow rates were used for all the experiments.

2.3. Analytical procedures

Cell concentration (g L^{-1} dry weight) was obtained by measuring the absorbance of the suspension at 620 nm using a previously determined correlation. Glucose concentration was measured by the 3,5-dinitrosalicylic acid method (Miller, 1959). Ethanol was quantified by gas chromatography (column CP-WAX 57 CB, Chrompack). Catalase and SOD activities were assayed in cell extracts by the method of Beers and Sizer (1952) and the method of McCord and Fridovich (1969), respectively. To distinguish mitochondrial SOD (MnSOD) from cytosolic SOD (CuZn-SOD) the same method was applied but in the presence of 0.6 M KCN to inhibit the cytosolic isoform. Activities were expressed as units of enzymatic activity per milligram of total soluble protein (U mg^{-1}), which was assayed by method of Bradford (1976). Cells were harvested by centrifugation ($5000 \times g$, 10 min, 4°C) for the cell extracts preparation. The cell pellets were washed once with 50 mM potassium phosphate buffer pH 7.8, containing 1 mM EDTA and centrifuged. Pellets were resuspended in the same buffer, frozen and stored at -20°C . Cell disruption was achieved by vigorously shaking the cellular suspension with 0.5 mm diameter glass beads. Cellular extracts were then obtained by centrifugation at $5000 \times g$ for 15 min at 4°C and overnight dialysis (14,000 cut-off membrane) of the supernatant against buffer at 4°C . Preliminary experiments showed there was no loss of enzymatic activities by overnight storage at 4°C .

2.4. Microscopic procedures

An optical microscope (Zeiss, Axioskop) was used for all microscopic procedures. Cell viability was assessed by the methylene blue staining technique (Jones, 1987).

Genealogical profile of the samples was assessed by the method of the bud scars analysis (Soares and Mota, 1996). The cells were stained with calcofluor and observed using incident ultraviolet light with appropriate filters (excitation filter, 365 nm, dichroic mirror, 395 nm; barrier filter, 420 nm). The number of bud scars per cell was scored for 300 cells per sample. The genealogical age of the cells was defined as the number of bud scars per cell. Cells were distributed in six

classes: cells without bud scars, cells having one, two, three, four and more than four bud scars.

Cell-size distribution of the samples were automatically evaluated by an image processing system composed of an optical microscope coupled with a black and white camera (Sony CCD AVC D5CE) and linked to a microcomputer by a frame grabber (DT3155, Data Translation, Inc.). The images were acquired and processed using Image-Pro Plus 3.0/Windows, Media Cybernetics. Traditional tools generally used for image processing were employed (Coelho et al., in press) contours enhancement, background subtraction, feature extraction and objects separation. Objects were labelled permitting individual properties to be extracted. The projected area of each cell was used as cell-size parameter. The cells were observed immediately after sampling with $400\times$ magnification and at least 300 cells per sample were used for the evaluation of cell-size distribution.

3. Results

An initial glucose feed below the critical glucose flux for this strain ($0.36 \text{ g g}^{-1} \text{ h}^{-1}$) was used in all of the fed-batch experiments at different values of total pressure of air and pure oxygen. Thus, it was expected to observe pure oxidative metabolism in the absence of oxygen limitation. Aeration of the culture with air at atmospheric pressure (0.1 MPa) was insufficient to prevent oxygen limitation (Fig. 1) and a significant switch of the metabolism to the fermentative pathway was observed, leading to a low global cell mass yield and a significant ethanol production (Table 1). The specific growth rate was quite lower than the pre-

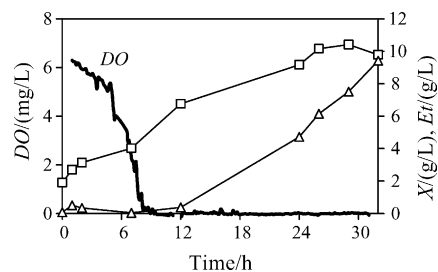


Fig. 1. Time course of dissolved oxygen concentration (DO), cell mass concentration (X , \square) and ethanol concentration (Et, \triangle) in the fed-batch experiment at 0.1 MPa.

Table 1

Pressure effects on the global cell mass yield, $Y_{X/S}$, ethanol mass yield, $Y_{E/S}$, specific cell growth rate, μ , and maximal cell concentration, X_{\max} in the fed-batch cultivations of *Saccharomyces cerevisiae* with air and pure oxygen

Pressure (MPa)	$Y_{X/S}$ (%)	$Y_{E/S}$ (%)	μ (h^{-1}) ^a	X_{\max} (g L^{-1})
0.1 air	24.3	25.4	0.04–0.08	9.8
0.6 air	35.6	13.6	0.09–0.13	15.4
1.0 air	37.1	13.2	0.08–0.12	13.7
1.5 air	8.3	14.0	0.06–0	1.7
0.13 O ₂	35.9	14.4	0.08–0.11	14.4
0.32 O ₂	1.8	8.3	0.05–0	4.6

Data are means of two independent experiments.

^a Experimental values of μ determined from the beginning to the end of the fed-batch experiment.

dicted value (0.1 h^{-1}) for the feeding profile used and the final cell mass concentration was 50% lower than the maximum attainable value. Consequently, the experimentally determined specific glucose uptake rate ($0.3\text{--}0.4 \text{ g g}^{-1} \text{ h}^{-1}$) deviated from the expected value and exceeded the critical value.

A 1.5-fold increase in the cell mass yield and a 1.8-fold reduction of ethanol yield were obtained by increasing total air pressure to 0.6 MPa or by the use of pure oxygen at 0.13 MPa. Under these conditions, the specific cell growth rate increased through time closely to the expected value and the specific substrate uptake rates varied from $0.2 \text{ g g}^{-1} \text{ h}^{-1}$ to $0.3 \text{ g g}^{-1} \text{ h}^{-1}$. No significant further improvement of cell mass production was found for hyperbaric air at 1.0 MPa, which corresponds to 0.21 MPa of oxygen partial pressure. Nevertheless, the results demonstrated that hyperbaric pressure of 1.0 MPa, does not inhibit cellular respiration of the yeast strain. Moreover, the absence of possible deleterious effects of pressure on the physiological state of the cells is also confirmed by the high numbers of the viable cells observed for the cultures under hyperbaric pressure of 1.0 MPa (Fig. 2).

Fed-batch experiments were performed at higher values of hyperbaric pressure of air (1.5 MPa) and pure oxygen (0.32 MPa), but a drastic inhibition of cell growth was observed. Glucose uptake was also repressed since a glucose build-up in the medium occurred, contrarily to the other pressure conditions where glucose concentration was kept at a residual value smaller than 0.2 g L^{-1} . Also, a large reduction in cell viability with the exposure time to increased

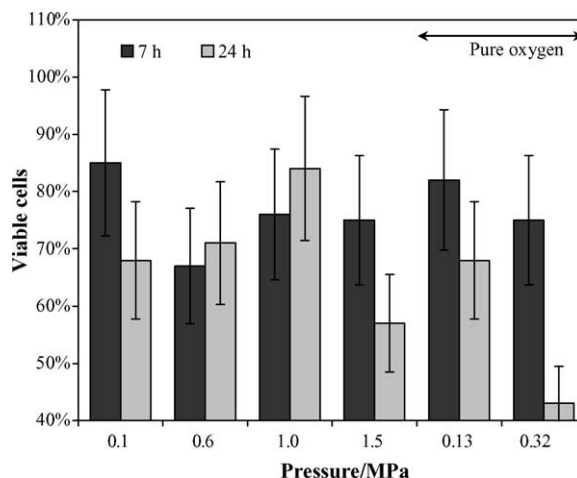


Fig. 2. Effect of air pressure (from 0.1 MPa to 1.5 MPa) and oxygen pressure (at 0.13 MPa and 0.32 MPa) on cell viability assessed by the methylene blue staining method, in cell samples collected at 7 h and 24 h of fed-batch growth. Data are mean \pm confidence interval at 95% level.

oxygen and air pressure (Fig. 2) was obtained. Due to the experimental errors associated to the cell viability determination method no statically significant differences (at a 95% confidence level) were found on the percentage of viable cells at 7 h exposure to the different atmospheres and the most significant change was obtained for the culture under 0.32 MPa of oxygen for 24 h.

The stronger effects of 0.32 MPa of pure O₂ pressure on cell activity and viability than the effects of 1.5 MPa of air pressure indicates that is not the total pressure to a maximum of 1.5 MPa that causes cell inhibition, but the toxicity of oxygen higher than 0.21 MPa.

Fig. 3 presents the data of the activities of the antioxidant enzymes, catalase and SOD, mitochondrial (MnSOD) and cytosolic (CuZnSOD), measured at the end of the cell cultivation under hyperbaric conditions.

Catalase and MnSOD were induced by hyperbaric air to a maximum of 1.0 MPa and 0.13 MPa of pure O₂ pressure, but no statistically significant changes (at a 95% confidence level) were observed for CuZnSOD activity. The respiratory activity and cell growth rate also increased with pressure rise, which implies that mitochondrial activity also increased. Mitochondria are the major sources of ROS, which explains the MnSOD induction. When cell growth was retarded by pressure an induction of CuZnSOD was observed, in opposition

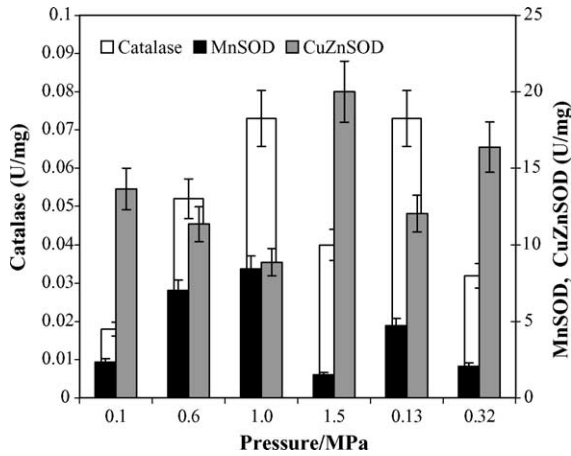


Fig. 3. Effect of air pressure (from 0.1 MPa to 1.5 MPa) and oxygen pressure (at 0.13 MPa and 0.32 MPa) on catalase and superoxide dismutase (mitochondrial and cytosolic isoforms) activities, in the final fed-batch cell cultures (24 h of growth). Data are mean \pm confidence interval at 95% level.

to the MnSOD and catalase. The response of CuZnSOD also indicates that ROS could be generated in the cytosol, due to oxygen over provision by other mechanisms not associated to respiration.

The effects of hyperbaric pressure on yeast reproduction was analysed by genealogical age determination. The genealogical age profiles of the cultures samples collected at 24 h of growth under hyperbaric pressure are presented in Fig. 4. No differences were found between the genealogical age of cell populations under environments of air at 0.1 MPa, 0.6 MPa, 1.0 MPa and pure oxygen at 0.13 MPa. The majority of the cells are young with no bud scars (daughter cells), or with only one bud scar. This behaviour is in accordance with the cell growth rate at a near constant value (Soares and Mota, 1996). Changes in the genealogical age profile were obtained for the final cultures exposed to 1.5 MPa of air pressure and 0.32 MPa of O₂ pressure. The differences in the genealogical age distribution of these cell populations and the cell populations grown at lower values of pressure were statically significant ($P < 0.01$, χ^2 test). An increase in the fraction of cells with more than four bud scars was observed, which indicates that the old cells are more resistant to pressure than young cells. Once again no synergic effects of total pressure and oxygen concentration were recorded for increased pressure on genealogical age distribution. Thus, oxy-

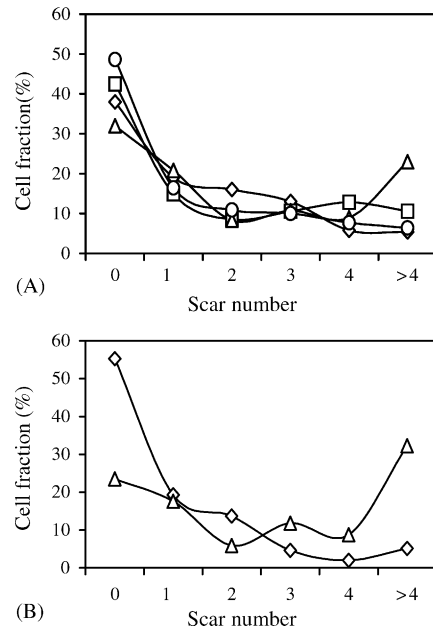


Fig. 4. Effect of air pressure (A) (\diamond) 0.1 MPa, (\circ) 0.6 MPa, (\square) 1.0 MPa and (Δ) 1.5 MPa and oxygen pressure (B) (\diamond) 0.13 MPa and (Δ) 0.32 MPa on the genealogical age distribution of the final fed-batch cell cultures (24 h of growth).

gen toxicity is confirmed as the true cause of cell inhibition by hyperbaric air.

The cell-size distribution of yeast cells exposed to hyperbaric pressures of air was analysed by a computer-aided image analysis technique (Coelho et al., in press). Typical Gaussian distributions were obtained for all the cell population analysed. Fig. 5 illustrates two examples and Table 2 presents the Gaussian parameters of the final cell cultures under hyperbaric air (Fig. 5).

While the decrease in the mean cell area obtained for cells under 0.6 MPa and 1.0 MPa of air pressure can be correlated with the faster cell growth, the decrease of the cell area obtained at 1.5 MPa of air pressure means

Table 2

Air pressure effects on the mean projected area of the cells at 24 h of growth

Air pressure (MPa)	Projected area ^a
0.1	35 \pm 2
0.6	27 \pm 1
1.0	25 \pm 1
1.5	20 \pm 1

^a Mean \pm standard deviation ($n > 300$).

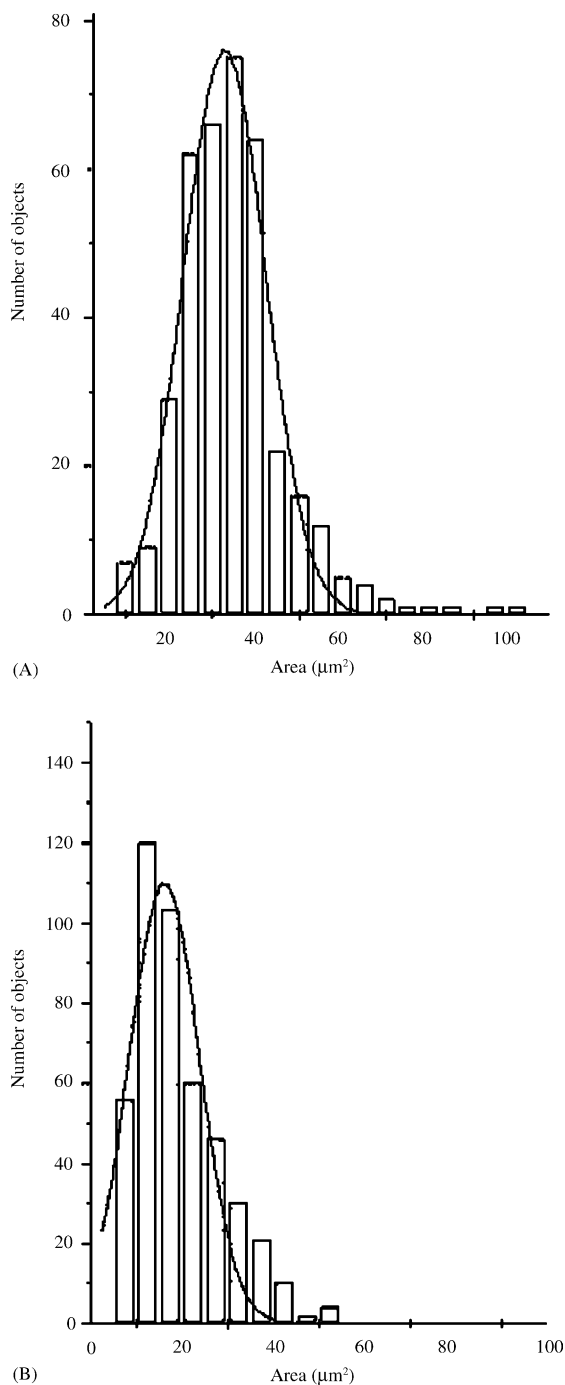


Fig. 5. Histograms and Gaussian fit for the projected area of the final cells exposed to 0.1 MPa (A) and 1.5 MPa (B) of air pressure.

a cell compression because cells were not growing and there was an increase on the percentage of older cells that under normal conditions are bigger in size.

4. Discussion

Yeasts are subjected to many types of stress throughout cultivation processes (e.g. pressure and oxidative stress). Knowledge on the impact of these stress factors and their lethal limits is crucial to establish and control operating conditions.

In aerobic yeast cultivation, total pressure is an important stress factor, largely because it changes the partial pressure of dissolved gases, and in particular dissolved oxygen concentration can cause oxidative stress to the cells.

In this work, a limit for the oxygen partial pressure of 0.21 MPa (i.e. 1.0 MPa of air) was found, above which cell inhibition was observed. Lower limits for the oxygen partial pressure at which cell growth was inhibited, has been report (Lee and Hassan, 1987; Abel et al., 1994), although other methods were used for yeast cultivation.

For the fed-batch operation used in this work, it was possible to improve cell mass production by a 6- and 10-fold increase of air pressure, but there was still some ethanol production. Previous work showed that at identical experimental operation conditions, theoretical cell mass yield could be attained if air pressure is gradually increased (up to 1.0 MPa) through operation time (Belo et al., 2003).

The oxygen toxicity caused respiratory and fermentative activity inhibition, as well as cell viability reduction. Oxidative stress emerges as a consequence of the limits of the anti-oxidant enzymes induction. Different effects could also explain enzyme behaviour. SOD activity is also affected by other factors, such as growth rate and phase. Westerbeek-Marres et al. (1988) reported a reduction of the MnSOD activity of yeast cells and an increase of CuZnSOD activity in the transition from the exponential to stationary phase of cell growth, which is in accordance with the results reported herein. On the other hand, according to Westerbeek-Marres et al. (1988) CuZnSOD is induced by catabolic repression, thus the increase in the activity of this enzyme could also result from glucose accumulation in the culture medium, since substrate uptake was inhibited by

pressure. The differences in the response to hyperbaric pressure of the anti-oxidant enzymes may explain the limits to cell protection against oxidative stress, with the consequent oxygen toxicity observed above certain limits of pressure. In particular, the imbalance of the catalase and cytosolic SOD activities can lead to insufficient scavenging of ROS inside cell. However, other peroxidases could demonstrate different behaviour to catalase and contribute to ROS elimination (Izawa et al., 1995). Moreover, other authors have reported catalase repression by pure oxygen at 0.1 MPa (Lee and Hassan, 1987).

Oxidative stress was also responsible for cell aging and cell size decrease. The genealogical age profile determination in addition to cell-size determination was a useful tool to conclude that oxidative stress caused cell compression. When pressure did not affect cell growth, distributions of the genealogical age of cells were similar to the ones reported to yeast cells at the exponential phase of growth (Soares and Mota, 1996).

Besides cell age, the size and shape of yeast cells depend on several factors, such as phase of growth, medium composition, operating conditions, among others. Hydrostatic pressure above values of 10^2 MPa of magnitude is a well-known environmental factor that alters yeast morphology (Shimada et al., 1993). Perrier-Cornet et al. (1995) reported a compression of yeast cells by 250 MPa of hydrostatic pressure. It was claimed that the reason for this compression was the cell mass transfer from the inside of the cell to the exterior medium, due to the change of membrane properties by pressure. This explanation applies to the results reported here since it is well known that oxidative stress can damage cells membranes by lipid peroxidation, leading to changes in membrane permeability and fluidity. This effect is supported by the decrease in viable cells in final cultures at 1.5 MPa air and 0.32 MPa oxygen. Moreover, the method used for the cell viability determination, the methylene blue staining method, depends on the membrane permeability, giving low viabilities as the membrane permeability increases. The damage of cell membrane could also be attributed to the abrupt decompression of dissolved gases during sample collection. However, this phenomenon does not affect membrane permeability of *S. cerevisiae*, for gas pressures as high as 5 MPa (Bavouzet et al., 1994).

Nevertheless, little information is still available about the effects of pressure, in a moderated range

of values, on the shape and size of yeast cells. The work reported here may contribute to the improvement of that knowledge and demonstrated the usefulness of hyperbaric bioreactors to perform oxidative stress studies.

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