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Hyperbaric bioreactors use with Yarrowia lipolytica cultures: cellular adaptation to hyperbaric conditions

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Abstract

Increase air pressure for aeration of microbial cell cultures can prevent oxygen limitation but cause oxidative stress and consequently affect cell physiology. A pressurized bioreactor was used for *Y. lipolytica* batch cultivation under increased air pressure up to 6 bar and no inhibition of cell growth was observed. Moreover, an increase of 6-fold led to a 3.4-fold increase in specific growth rate under atmospheric pressure. The antioxidant enzyme superoxide dismutase was induced by the air pressure raise, which indicates that the defensive mechanisms of the cells were effective and cells could cope with increased pressure. The extracellular lipase activity increased from 22.3 to 43.7 U/l using a 5-bar air pressure instead of air at 1 bar pressure.

1 Introduction

Y. lipolytica is a non-conventional yeast, non-toxic, that can grow to very high densities. It has a haploid genome and sexual life cycle, and therefore is amenable to both classical and molecular analysis (Juretzek et al., 2001; Barth and Gaillardin, 1996). It is also advantegeous in studies of the biodegradative pathways for a variety of hydrophobic compounds including alkanes, oils, and fatty acids and thus for its capacity to produce lipid-degrading enzymes, such as lipases.

The amount of oxygen available to *Y. lipolytica* seems to be an important parameter since that strain is strictly aerobic. Many efforts have been made to overcome the oxygen limitation in the culture medium and previous work demonstrated that hyperbaric air could be successfully applied to yeast cultivation, as a way of improving the oxygen transfer rate to aerobic cultures (Lopes et al., 2008; Aguedo et al., 2005; Belo et al., 2003).

In industrial bioreactors, levels and gradients of total and partial pressures are considerably higher than on the laboratory scale. Thus, cells in bioreactors are often exposed to O_2 partial pressures higher than 210 mbar (corresponding to air at 1 bar). In many cases, increased O_2 partial pressure (higher than approx. 1 bar) is toxic to aerobic cultures and inhibits microbial growth and product formation (Onken and Liefke, 1989). During the reduction of molecular oxygen to water through acceptance of four electrons, active oxygen species such as superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$) are generated. These active oxygen species may give rise to damage of enzymes, nucleic acids, or lipids (Izawa et al., 1995). To counter oxidative stress, cells constitutively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them. In addition, yeast cells have adaptive responses to elevated levels of oxidative stress, indicating that these cells sense increased levels of reactive oxygen species and transduce the signal into increased expression of defence activities (Storz and Imlay, 1999). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), constitute the primary

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defences of the cells because they are responsible to transform these reactive oxygen species into nonreactive ones (Moradas-Ferreira et al., 1996).

High pressure is typically viewed as a denaturing factor (Dong et al., 2007), however there is a little report about the effects of slight increased air pressure on the growth and metabolism of microbe cells. The aim of this work is to investigate whether increasing air pressures may lead to increasing biomass yields of *Yarrowia lipolytica* W29, without giving rise to oxidative stress. This paper also reports an investigation into the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production by *Y. lipolytica* cells.

2 Materials and Methods

Yarrowia lipolytica W29 (ATCC 20460) was grown in YPD medium. The medium for lipase production was YNB/olive oil as described in Lopes et al. (2008).

Yeast cells were pregrown in 250 ml Erlenmeyer flasks filled with 100 ml of the YPD medium at 140 rpm, 27 ºC of temperature and for 24 h. Batch cultivations were carried out using a 600 ml stainless steel stirred tank bioreactor (Parr 4563, Parr Instruments, USA), with 400 ml of YPD media, at 27 ºC and 400 rpm in order to asses the effect of pressure in cellular growth and on antioxidant enzymes induction. Compressed air was continuously sparged into the culture at a flow rate of 1 vvm. The values of air pressure studied were 1 bar to 6 bar. An experiment in an Erlenmeyer flask (500 ml) with 200 ml of YPD medium, under atmospheric pressure (1 bar) and an agitation rate of 140 rpm was used as a control. With the aim of investigate the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production by *Y. lipolytica* cells, experiments were conducted in the pressurized bioreactor in which the lipase production phase (YNB/olive oil medium, 400 rpm, at 27 ºC for 48 h, Lopes et al. 2008) was preceded by a 24 h growth in YPD medium at 1 bar or 5 bar of total air pressure.

Culture samples were collected for analysis of cell concentration (optical density at 600 nm and cell number and converted to g cell dry/l), total soluble protein, glucose consumption and enzymatic assays. Total soluble protein was obtained by Bradford's method. Glucose was determined using the 3,5-dinitrosalycilic acid (DNS) method. Extracellular lipase was measured in the samples supernatant using *p*-nitrophenyl-butirate (pNPB) in sodium acetate buffer 50 mM at pH 5.6 as a substrate, at 37 ºC for 15 min. One unit of activity was defined as the amount of enzyme that produces 1 µmol of *p*-nitrophenol per minute under assay conditions. Protease in cell-free samples was quantified as described in Lopes et al. (2008). The antioxidant enzymes were measured after dialysis of the cell extracts, which were obtained as described in Pinheiro et al. (2000). Catalase was assayed using the method described by Beers and Sizer (1952) and superoxide dismutase (SOD) was quantified by the method of Marklund and Marklund (1974).

3 Results and Discussion

Air effects on cell growth

Typical batch growth curves and glucose consumption profiles for the experiments under increased air pressure and atmospheric pressure are shown in Figure 1.The application of 6 bar stimulated cell growth compared to the atmospheric conditions.

Pressure increase had a clear positive effect on this yeast metabolism, since the biomass production increased and reached its maximal value for an air pressure of 6 bar. An increase of the cell dry weight at 6 bar of 3.5- and 5-fold was obtained compared with the experiments under atmospheric pressure in the control and in the bioreactor, respectively.

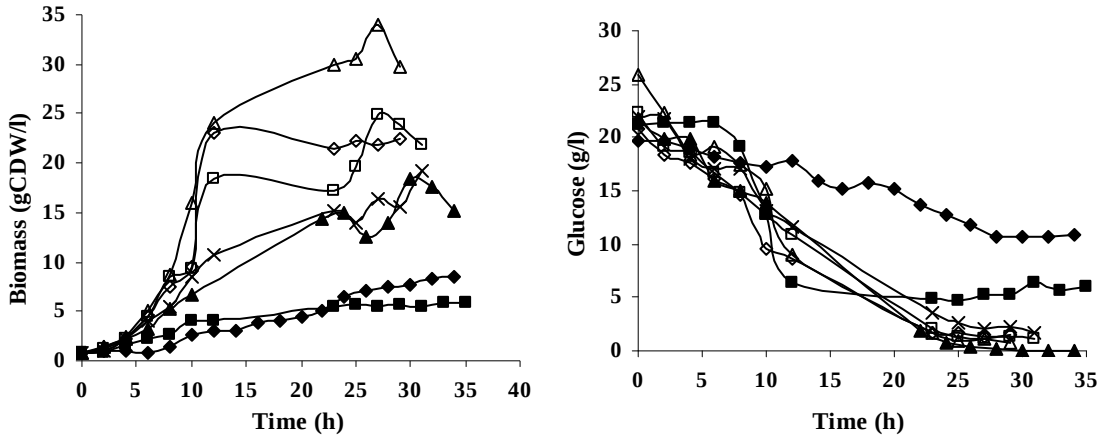

Fig. 1 Batch growth and glucose consumption of *Yarrowia lipolytica* at atmospheric pressure (♦) and in hyperbaric reactor under pressures of 1 bar (■), 2 bar (▲), 3 bar (×), 4 bar (□), 5 bar (◊) and 6 bar (Δ).

The results described above are in accordance with the previous work of Lopes et al. (2008), in which no cellular activity inhibition by pressure was detected in batch cultures of *Y. lipolytica* in culture media without glucose. Also, Belo et al. (2005) observed for semi-continuous cultures of *Saccharomyces cerevisiae* that an increase of pressure up to 10 bar had no metabolic impact in the cells. Aguedo et al. (2005) reported that the application of 5 bar air pressure stimulated the cell growth of *Y. lipolytica* W29 comparatively to atmospheric growth conditions.

In experiments under atmospheric pressure and 1 bar of air pressure, glucose was not totally consumed. The raise of air pressure up to 6 bar led to a completely consumption of glucose.

The use of pressure might be exploited to improve the biomass productivity of *Y. lipolytica* W29 (Table 1). An increase of air pressure up to 6 bar led to a 4.1-fold improvement in biomass productivity comparatively to atmospheric pressure. This result is in accordance with previous work of Pinheiro et al. (2000), in which the productivity in biomass of *K. marxianus* CBS 7894 was increased with the increase in air pressure up to 6 bar. Also, Charoenrat et al. (2006) observed that the cell mass productivity of *Pichia pastoris* can be improved by application of increased air pressure. However, for *S. cerevisiae*, Pinheiro et al. (1997) reported that an increase of 6-bar air pressure led to a decrease on biomass productivity for a batch mode of operation. This shows that microorganisms react differently to the air pressure rise, depending also on other culture conditions.

Table 1 Changes in biomass yield, specific growth rate and productivity with air pressure in batch experiments.

Pressure (bar)	$Y_{x/s}$ (g biomass/g glucose) (%)	μ (h^{-1})	P (g biomass/l·h)
Control	53.3	0.09	0.25
1	34.7	0.18	0.17
2	70.2	0.19	0.45
3	96.7	0.23	0.62
4	104.1	0.26	0.71
5	117.9	0.28	0.77
6	121.1	0.31	1.02

The specific growth rate of *Y. lipolytica* was clearly enhanced by the increase of air pressure. An increase of 6-bar led to a 3.4- and 1.7-fold increase in specific growth rate under atmospheric pressure and 1 bar, respectively. Due to the high oxygen mass transfer rate, the cells have more oxygen in the medium giving higher growth rates, and less time is necessary to obtain maximum cell concentration. Belo et al. (2005) reported that an increase of air pressure up to 6 bar led to a 1.8-fold improvement in the specific growth rate of *S. cerevisiae*.

Also, Pinheiro et al. (2000) observed that an increase of 4-bar led to a high increase in specific growth rate for *K. marxianus*.

Biomass yield was also enhanced by air pressure rise. With 6 bar air pressure biomass yield was 121.1 % whereas at 1 bar it was 34.7 %. At values of air pressure up to 2 bar glucose was not totally consumed, probably due to oxygen limitation. An increase of the yield at 6 bar of 56 % and 71 % was obtained compared with the experiments under atmospheric pressure and 1 bar, respectively.

It is clear from these results that pressure had no inhibitory effects on the growth of this yeast strain. An increase of air pressure up to 6 bar might successfully be applied to the improvement of the biomass production of *Y. lipolytica* W29.

Yeast cells were observed by optical microscopy and was noted that the cells displayed a typical oval form in all assays up to 6 bar (data not shown). The results demonstrated that cell exposure to increased air pressure did not induce hyphae formation.

Pressure effect on antioxidant enzyme activities

To examine the effects of oxygen toxicity on yeast cells, with the increase in oxygen partial pressure, the changes of cellular antioxidant enzyme activities under different air pressures were determined. Figure 2 presents the data of the catalase and SOD specific activities, measured at the end of the cell cultivation under hyperbaric conditions.

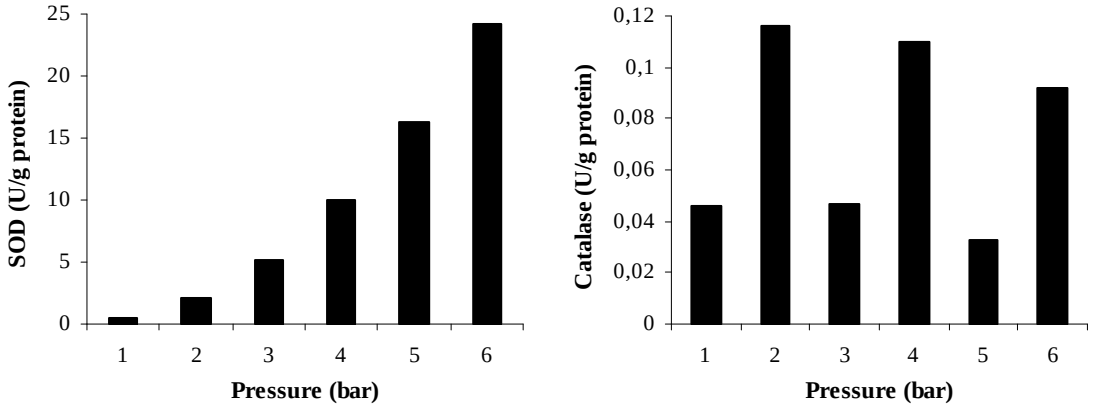

Fig. 2 Effect of air pressure on superoxide dismutase and catalase specific activities, in the final batch cell cultures (approximately 30 h of growth).

Superoxide dismutase specific activity was induced by hyperbaric air to a maximum of 6 bar. An increase of the SOD specific activity at 6 bar of 53.4-fold was obtained compared with the experiments under 1 bar. This shows the ability of *Y. lipolytica* cells to respond to the increase of reactive oxygen species formation because of hyperoxygenation. The SOD induction showed the cell sensitivity to high oxygen concentrations. However, as no cell growth inhibition was observed under pressurized conditions, it is quite safe to state that the cells of the strain used can cope with air pressure values up to 6 bar.

The influence of total air pressure on the catalase activity is not clear, thus it seems that this enzyme plays a minor role in the defensive mechanisms against the oxidative stress, for *Y. lipolytica*.

These results demonstrate that the raise of air pressure could be also applied to SOD production, once it is most induced.

Pressure effect on lipase production and pre-adaptation

In order to investigate the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production by *Y. lipolytica* cells under increased pressure, assays

were conducted in the pressurized bioreactor in which cells were pregrown on the bioreactor at normal and increased pressure following by a lipase production phase at normal and increased pressure.

The increase of total air pressure influences enzymatic activity, as demonstrated by previous work of Lopes et al. (2008). The authors observed that an increase of OTR by raising air pressure up to 8 bar resulted in an increase of the lipase production by *Y. lipolytica* cells grown at atmospheric pressure. In this work, an increase of the lipase activity and lipase productivity at 5 bar of 1.8-fold and 3.7-fold, respectively, was obtained compared with the experiments under 1 bar, independently of the pressure conditions used in the inocullum preparation (Fig. 3A).

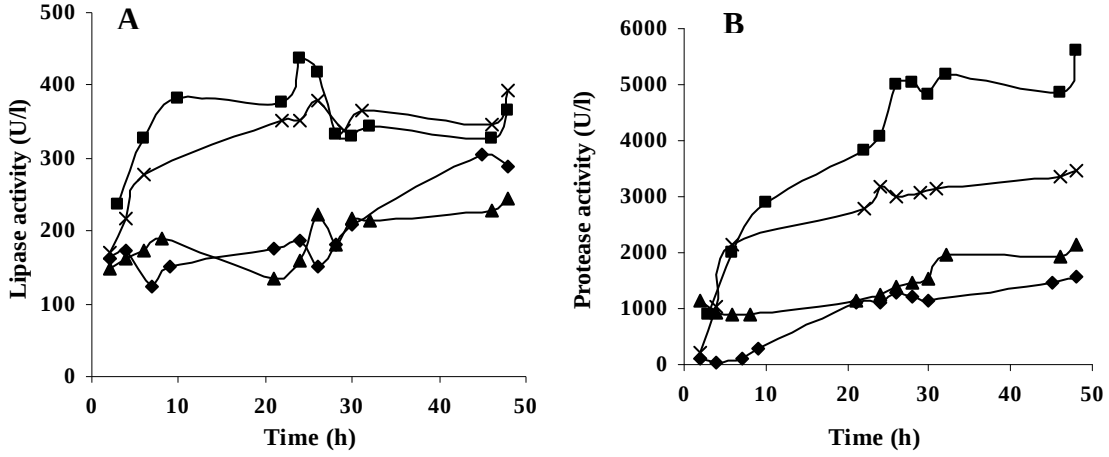

Fig. 3 Extracellular lipase (A) and protease (B) activities profiles by *Yarrowia lipolytica* in the pressurized reactor at different air pressures of growth and production, respectively: 1 bar and 1 bar (♦), 1 bar and 5 bar (■), 5 bar and 1 bar (▲) and 5 bar and 5 bar (×).

For the range of air pressure values applied, the pre-adaptation phase of cells to hyperbaric conditions did not improve the lipase production. The lipase production at 5 bar with cells pregrown at the same air pressure was similar to the obtained with grown cells under atmospheric pressure. Therefore, it can be concluded that the *Y. lipolytica* cells can quickly respond and adapt to hyperbaric conditions and no need of long phases of hyperbaric stress adaptation is needed.

Besides lipase production, the production of other enzymes, such as proteases, by *Y. lipolytica* strains has been reported (Puthli et al., 2006). Figure 3B shows the results of monitoring protease secretion along time, using azocasein as substrate. During the first hours of culture the protease activity was low, increasing gradually until the end of the cultivation time, suggesting that the decrease of the medium pH (data not shown) favours the production of an acid protease by yeast.

The highest value of protease production was found for the 5 bar assays, whereas in the experiments carried out under 1 bar its concentration in the medium was lower. The presence of protease in culture medium can influence the production kinetics of lipases since the prolonged time of fermentation can lead to the loss of product due to its decomposition. However, in this work, highest value of protease production was reached at the same air pressure (5 bar) that the maximum lipase productivity was obtained. Probably, lipase activity might be improved even more if protease secretion into the medium is inhibited by a chemical agent.

4 Conclusions

For the experimental conditions used in this work, air pressure rise up to 6 bar proved to be applicable to the batch cultivation of *Yarrowia lipolytica* W29. It has been demonstrated that the use of air pressure has positive effects on the growth behaviour of yeast and that air

pressure may be a way of increase the specific growth rate, leading to high biomass productivity. For *Y. lipolytica*, an increase of air pressure up to 6 bar led to a 4.1-fold improvement in biomass productivity comparatively to atmospheric pressure.
To protect against the damage caused by oxidative stress, cells possess a number of antioxidant enzymes and repair activities, most of which are expressed al low levels during normal growth. In spite of the SOD induction, air pressure rise did not inflict oxidative stress to the cells. Yarrowia lipolytica W29 adapt rapidly to hyperbaric conditions, thus this conditions can be imposed to cultures of this strain was a way of preventing oxygen limitation to cell growth and as a mean of several enzymes production improvement such as lipases, proteases and SOD.

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