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ORIGINAL PAPER

Batch and fed-batch growth of *Pichia pastoris* under increased air pressure

Marlene Lopes · Isabel Belo · Manuel Mota

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Abstract *Pichia pastoris* CBS 2612 behavior under air pressures of 1, 3 and 5 bar in culture media of glycerol (pure and crude) and methanol was studied. Generally, the increase in oxygen transfer rate due to the increase of total pressure improved cellular growth for all carbon sources and for batch and fed-batch processes with different feeding rate strategies. In batch cultures, 1.4-, 1.2-, and 1.5-fold improvement in biomass production was obtained with the increase of air pressure up to 5 bar, using methanol, pure glycerol, and crude glycerol, respectively. The increase of air pressure to 5 bar using exponential feeding rate led to 1.4-fold improvement in biomass yield per glycerol mass consumed, for crude and pure glycerol. The current low cost of crude glycerol from the biodiesel production together with the present results shows the possibility of improving cell mass production of *P. pastoris* using increased air pressure.

Keywords *Pichia pastoris* · Increased air pressure · Oxygen transfer rate (OTR) · Crude glycerol

List of symbols

C	Dissolved oxygen concentration in the liquid (mg O_2/L)
C^*	Solubility of oxygen in the liquid (mg O_2/L)
CDW	Cell dry weight
D	Dilution rate (h^{-1})
DO	Dissolved oxygen tension (%)
F	Feed rate (mL/min)
H_{O_2}	Henry constant for oxygen
k_La	Volumetric oxygen mass transfer coefficient (h^{-1} or s^{-1})
NAD	Nicotinamide adenine dinucleotide
OTR	Oxygen transfer rate (mg O_2/L h)
qO_2	Specific oxygen uptake rate (mg O_2/g h)
q_s	Maximum specific substrate consumption rate (g/g h)
p_{O_2}	Oxygen partial pressure (bar)
P	Absolute pressure (bar)
P_T	Total air pressure (bar)
t	Time (h)
V_0	Initial culture volume (mL)
y_{O_2}	Oxygen molar fraction in the gas
$Y_{x/O}$	Cell mass yield per oxygen mass consumed (g/g)
$Y_{x/s}$	Cell mass yield per carbon source mass consumed (g/g)
μ	Specific growth rate (h^{-1})
υ	Superficial gas velocity (m/s)

Subscripts

O	Oxygen
S	Substrate
T	Total
X	Biomass
0	Initial value

M. Lopes · I. Belo (✉) · M. Mota
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: ibelo@deb.uminho.pt

Introduction

Pichia pastoris has many biotechnological applications and, in particular, two aspects of the species have contributed to its application: (1) the strong preference of *P. pastoris* for respiratory growth, a key physiological trait that greatly facilitates its culturing at high cell densities relative to fermentative yeasts [1]; and (2) since *P. pastoris*

assimilates methanol, the expression system is linked with alcohol oxidase, which is abundantly produced in presence of methanol [2].

Glycerol is regularly used as the main initial carbon source in *P. pastoris* fermentations to increase cell concentration. As the main by-product of biodiesel production, crude glycerol can now be found in abundance and at prices lower than glucose, which makes possible to use crude glycerol as carbon source for bioprocesses with the methylotrophic *P. pastoris* [3]. The rapidly expanding market for biodiesel has decreased glycerol's cost and increased its availability, as typical biodiesel production processes generate around 10 % (wt) glycerol of the total amount of biodiesel produced.

Fed-batch is the dominating mode of operation in high cell density cultures of *P. pastoris* in processes where the high oxygen demand of these cultures makes its supply an important and difficult task. In unicellular organisms such as yeasts, oxygen, for carrying out any oxidative reaction within the cell, is generally incorporated through the intermediate state of the dissolved oxygen molecule. Thus, the organism responds to the liquid phase oxygen concentration or partial pressure in regulating its overall metabolic activities.

The oxygen transfer rate (OTR) from the gas phase into the broth is controlled by the oxygen solubility and the volumetric oxygen mass transfer coefficient ($k_L a$), and can be stated mathematically as:

$$\mathrm{OTR} = k_L a(C^* - C) \quad (1)$$

where C^* is the solubility of oxygen in the liquid, and C is the dissolved oxygen concentration in the liquid.

The oxygen solubility in the liquid medium can be raised by increasing the total air pressure in the cultivation system. The saturation concentration of oxygen from air in broth, C^*, is affected by the oxygen partial pressure and, consequently, by the total air pressure. The equilibrium relation between these two parameters is given by Henry's law:

$$p_{O_2} = H_{O_2} \times C^* \quad (2)$$

where

$$p_{O_2} = y_{O_2} \times P_T \quad (3)$$

and where p_{O_2} is the oxygen partial pressure, H_{O_2} the Henry constant, y_{O_2} the oxygen molar fraction in the gas, and P_T the total air pressure.

Published works have reported the use of increased air pressure as a way of improving the OTR that can be applied for cell cultivation with energy and capital cost efficiencies acceptable for industrial application [4]. In fact, the authors proved that the use of increased pressure can reduce the running costs when high OTRs are needed, since the air pressurization up to 5 bar can improve the energy efficiency of a STR bioreactor. Moreover, high pressure bioreactors and technology are intensively applied in chemical industry, thus it could be adapted to microbial cultures technology. Some results have demonstrated that increased air pressure could be successfully applied to the cultivation of yeast species such as *Yarrowia lipolytica* [5] and *Kluyveromyces marxianus* cultivation [6]. However, the effect of increased air and oxygen pressure is strongly dependent of the species and strains [7–9] due to different abilities of cellular response to possible oxidative stress that can arise. In spite of the well-known importance of *P. pastoris* as a cell factory, mainly for biopharmaceuticals production, few studies are available on the application of air pressure increase for the cultivation of this yeast, and only slight pressure increase was applied, elevating the air pressure from 1.2 to 1.9 bar [10].

In this study, we investigated whether increasing air pressures (fivefold above atmospheric pressure) may be applied as an alternative way of OTR improvement in *P. pastoris* cultures growing in methanol or glycerol (pure and crude) as carbon sources, in batch and fed-batch cultures.

Materials and methods

Oxygen transfer rate

OTR in bioreactors was estimated in blank assays using the sulfite oxidation method [11], as described by Lopes et al. [12].

Batch operation

Pichia pastoris CBS 2612 was grown in YP (10 g/L yeast extract and 20 g/L peptone) medium with 10 g/L of pure or crude (byproduct of biodiesel production from waste vegetable oils obtained at the CVR-Centre for Waste Valorization, University of Minho, Portugal) glycerol and methanol, prepared in a potassium phosphate buffer 100 mM, pH 6. The glycerol media were sterilized by autoclaving at 115 °C for 30 min and the methanol medium was sterilized by filtration through 0.2 μm filter.

The crude glycerol used had a dark brown color and pH 8.60, containing 58 and 25 % (mass) glycerol and methanol, respectively, and a total protein content of approximately 8.8 mg/L. The crude glycerol used in this work did not suffer any pre-treatment, but most of the suspended solids were separated by sedimentation.

Yeasts cells were pre-grown overnight in 250 mL Erlenmeyer flasks filled with 100 mL of YP, with each carbon source at 140 rpm and at 30 °C. Batch cultivations

were carried out using a 600 mL stainless steel stirred tank bioreactor (PARR 4563, Parr Instruments, USA), with 400 mL of each carbon source medium, at 30 °C and 400 rpm. Compressed air was continuously sparged into the culture at an aeration rate of 1 vvm. The values of air absolute pressure studied were 1, 3, and 5 bar. The operating pressure was set by the manipulation of the pressure of the inlet compressed air and the regulatory valve position in the exit gas line. The reactor was equipped with a pressure transducer (PARR 4842, PARR Instruments, USA) to monitor total internal pressure.

Batch cultures in a 2-L fermenter (BIOLAB, B. Braun, Germany) with 1.6 L working volume were also performed with each carbon source. The operating conditions were 30 °C, 400 rpm, and 1 vvm of aeration rate. This bioreactor is equipped with a polarographic oxygen probe (12/220 T-type, Metler Toledo, USA) and the respective meter (type 170) that allowed monitoring of dissolved oxygen tension during cell cultivation. The short interruption of aeration allowed the determination of the specific oxygen uptake (qO_2) rate at exponential phase for each carbon source.

Fed-batch operation

Yeasts cells were pre-grown overnight in 250 mL Erlenmeyer flasks filled with 100 mL of YP medium, with pure or crude glycerol at 140 rpm and 30 °C.

The fed-batch fermentation was carried out in the pressurized reactor (PARR) described above. The values of absolute air pressure studied were 1 and 5 bar. The operating conditions were 30 °C, 400 rpm, and 1 vvm of aeration.

A three-stage fermentation protocol was used in this part of the study: the first stage was a glycerol (pure or crude) batch fermentation; then, 24 h after inoculation, the process was switched to glycerol fed-batch with a glycerol feed (pure or crude glycerol 50 g/L, yeast extract 10 g/L and peptone 20 g/L) added to the bioreactor using two strategies: (1) a constant feeding flow rate (F) of 0.05 mL/min, where the dilution rate (D) ranged from 0.02 to 0.007 h^{-1}, or (2) an exponential feeding rate in order to keep dilution rate of 0.01 h^{-1}, with the feed flow rate varying from 0.02 to 0.06 mL/min, according with the equation:

$$F = DV_0 e^{Dt} \quad (4)$$

where F is the feed rate, D the dilution rate, V_0 the culture volume when the medium feed started and t is the time.

The medium was pumped into the reactor using a high-pressure pump (Jasco 880-PU). In the third stage, about 105 or 120 h of the fed-batch phase, the process was switched to batch mode during 24 h.

Analytical procedures

Culture samples were collected (every 2 h in batch operation and twice per day in fed-batch mode) for analysis of cell concentration (optical density at 600 nm and converted to dry cell weight per liter), pH, and carbon source consumption. A blank assay at 600 nm without cells was performed and showed that the influence of crude glycerol color was insignificant due to its dilution. Glycerol and methanol were quantified by HPLC with a Metacarb 67H column (Varian, Palo Alto, CA) and a RI detector (Knauer K-2300, Germany). The eluent was H_2SO_4 0.005 mol/L at 0.5 mL/min, and the column temperature was 60 °C, maintained with a column thermostat (Chrompack, Brasil).

Total protein of crude glycerol was obtained by Bradford's method [13].

Results and discussion

Air pressure effect on batch cultures

Glycerol and methanol were used as carbon sources for *P. pastoris* growth. These substrates were chosen because: (1) glycerol is traditionally used as the main initial carbon source in *P. pastoris* fermentations to increase the cell concentration, and the low price of crude glycerol offers new opportunities to this substrate; and (2) methanol, another low-cost carbon source, is an inducer of the foreign gene expression and a substrate with high oxygen demand.

First, batch cultures in BIOLAB bioreactor coupled with an oxygen probe were performed to assess the oxygen needs of the cells in each carbon source. Typical batch growth and substrate curves profiles for the experiments at atmospheric pressure in BIOLAB bioreactor are shown in Fig. 1.

At atmospheric pressure in a BIOLAB bioreactor, no significant differences were found for cellular growth in pure and crude glycerol and higher final cell mass concentration was found in glycerol than in methanol. In this last substrate, the cells presented longer lag phase than in the other carbon sources.

All carbon sources used in this study, with exception of methanol, were completely consumed in about 24 h. The highest biomass yield was obtained with glycerol (0.79 and 0.72 mass of cells per mass of substrate, respectively with crude and pure glycerol). The lowest value was obtained with methanol (0.29 mass of cells per mass of substrate).

Each culture of *P. pastoris*, growing on three carbon sources, had different oxygen demands (Fig. 2). The literature reports the high oxygen demand of methanol metabolism and presumes that the oxygen limitation generally has a detrimental effect on the expression of foreign

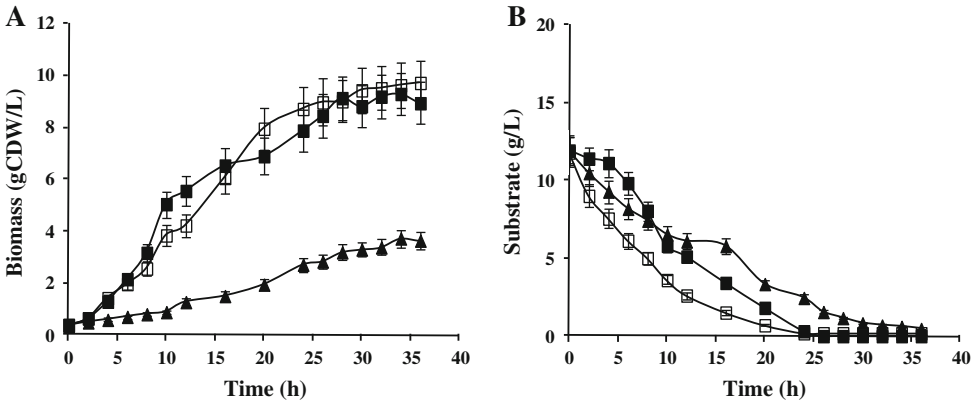

Fig. 1 Batch growth of *P. pastoris* (**a**) and substrate consumption (**b**) in 2 L-Biolab bioreactor with methanol (*filled triangle*), pure glycerol (*filled square*) and crude glycerol medium (*open square*)

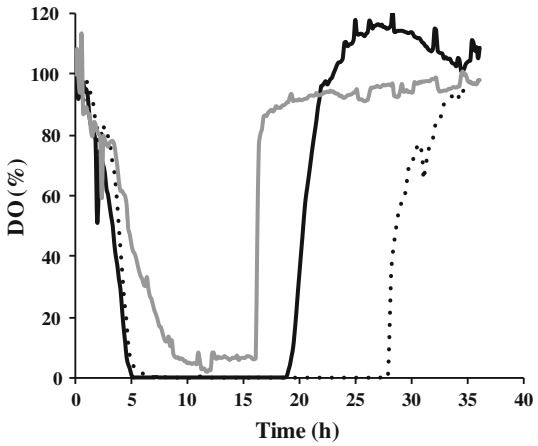

Fig. 2 Time course of dissolved oxygen concentration at methanol (*gray line*), pure glycerol (*black line*) and crude glycerol (*dotted line*) medium

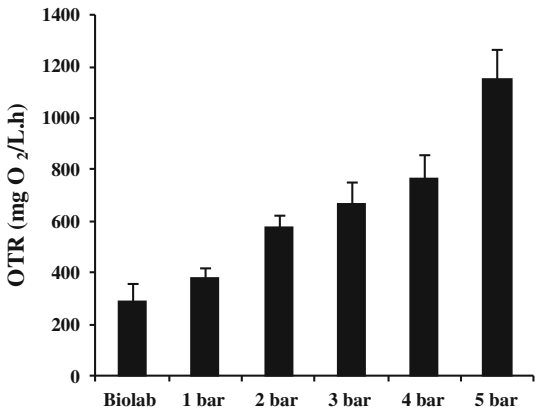

Fig. 3 Effect of air pressure on the maximum oxygen transfer rate (OTR) in BIOLAB reactor (atmospheric pressure) and in pressurized reactor (increased air pressure up to 5 bar)

genes [14]. In this study, the oxygen demand of the cultures were determined during the first hours of growth, and specific oxygen uptake rate (qO_2) values of 53 ± 4 mg O_2/(g h), 70 ± 6 mg O_2/(g h), and 163 ± 15 mg O_2/(g h) were observed for methanol, pure glycerol, and crude glycerol medium, respectively. Chen et al. [15] observed a qO_2 value of 57 mg O_2/(g h) for recombinant *P. pastoris* in fed-batch with methanol. Solà et al. [16] in *P. pastoris* chemostat cultures with a 60 % glycerol/40 % methanol mixture as carbon source, found a qO_2 value of 125 mg O_2/(g h). To our knowledge, this is the first report on qO_2 on crude glycerol. Using the ratio of the specific cellular growth and the qO_2 values, the cell mass yield per oxygen mass consumed ($Y_{x/O}$) can be obtained. Accordingly, the yields of dry cell mass per oxygen mass of 1.5, 1.7, and 0.8 g/g were obtained for methanol, pure glycerol, and crude glycerol, respectively. These results show that cultures of *P. pastoris* have high oxygen demand needs, particularly in crude glycerol, probably due to the metabolisation of other components present in this biodiesel subproduct. In fact, in crude glycerol, oxygen depletion from the medium was observed for a longer period of time (Fig. 2) than in the other carbon sources which indicate the need of improving OTR in bioreactors for *P. pastoris* growth in this low-cost carbon source. In the BIOLAB bioreactor (atmospheric pressure), the OTR value was 288 mg O_2/(L h), which is insufficient for the oxygen demand of the culture growing in crude glycerol. In fact, a 4 g/L cell culture, with the qO_2 found in crude glycerol, will need a OTR higher than 656 mg O_2/(L h).

For a specific culture medium and bioreactor, the OTR enhancement can be performed by increasing the stirring rate, airflow rate, and oxygen solubility in the medium. The oxygen solubility in the medium can be enhanced by air pressure increase, according with Henry's law, as an alternative to the use of O_2 enriched air. The OTR, in PARR bioreactor, increased from 384 mg O_2/(L h) at 1 bar to 672 mg O_2/(L h) at 3 bar, and to 1,152 mg O_2/(L h) at 5 bar (Fig. 3). The increase of OTR by total air pressure raise has been reported and applied in microbial cultures by some researchers [6, 12, 17].

For the hyperbaric bioreactor used, the variation of k_La with pressure fits well with the following function:

$$k_L a = 372 \times P^{0.81} \times v^{0.33} \quad (5)$$

where $k_L a$ was determined by the ratio between OTR and oxygen solubility at each pressure value, P is the absolute pressure and υ is the superficial gas velocity.

According with Eq. (5), the increase of pressure slightly decreases $k_L a$, which is due to the decrease of air flow rate inside the reactor at increased air pressure. Since $k_L a$ decreases with pressure, the observed increase in OTR with pressure was smaller than the theoretical one (Eq. 1).

Batch cultures under increased air pressure up to 5 bar were performed in order to prevent oxygen limitation observed during the exponential growth phase.

Typical batch biomass profiles for the experiments under increased air pressure, for the carbon sources tested, are shown in Fig. 4.

At 1 bar of total air pressure, the cells grew better in glycerol (pure and crude), reaching higher final cell mass concentration than in methanol. In this last substrate, the cells presented longer lag phase than in the other carbon sources, as occurred in the BIOLAB reactor operating at atmospheric pressure.

Regardless of the carbon source, the rise of total air pressure from 1 to 5 bar led to an increase in the final cell dry weight. Compared to 1 bar, a 1.4-, 1.2-, and 1.5-fold improvement in biomass production was obtained with the increase of air pressure up to 5 bar, for the trials with methanol, pure glycerol, and crude glycerol, respectively. That was due to the improvement of OTR from the air to the liquid phase, thus allowing the unlimited cellular growth. Similarly, Knabben et al. [18] used increased pressure pilot-plant bioreactors to minimize overflow metabolism in *E. coli* fed-batch cultures.

All carbon sources used in this study were completely consumed. Typical substrate consumption curves profiles for the experiments under increased air pressure are shown in Fig. 5.

The increase of total air pressure led to an earlier consumption of carbon sources. Among the substrates studied, the highest biomass yield was obtained with glycerol (crude and pure), followed by methanol (Table 1). The increase of total air pressure to 5 bar caused a 1.6- and 1.4-fold improvement in biomass yield for crude glycerol and methanol, respectively. However, in the pure glycerol medium, no significant effect on yield was obtained by the increase of total air pressure. The biomass yield obtained with crude glycerol in experiments under 1 bar was similar to that achieved with pure glycerol. Surprisingly, a 1.3-fold improvement in biomass yield with crude glycerol was attained at 5 bar, compared to the yield obtained with pure glycerol at 5 bar. It is reasonable to speculate that the increase of total air pressure resulted in complete

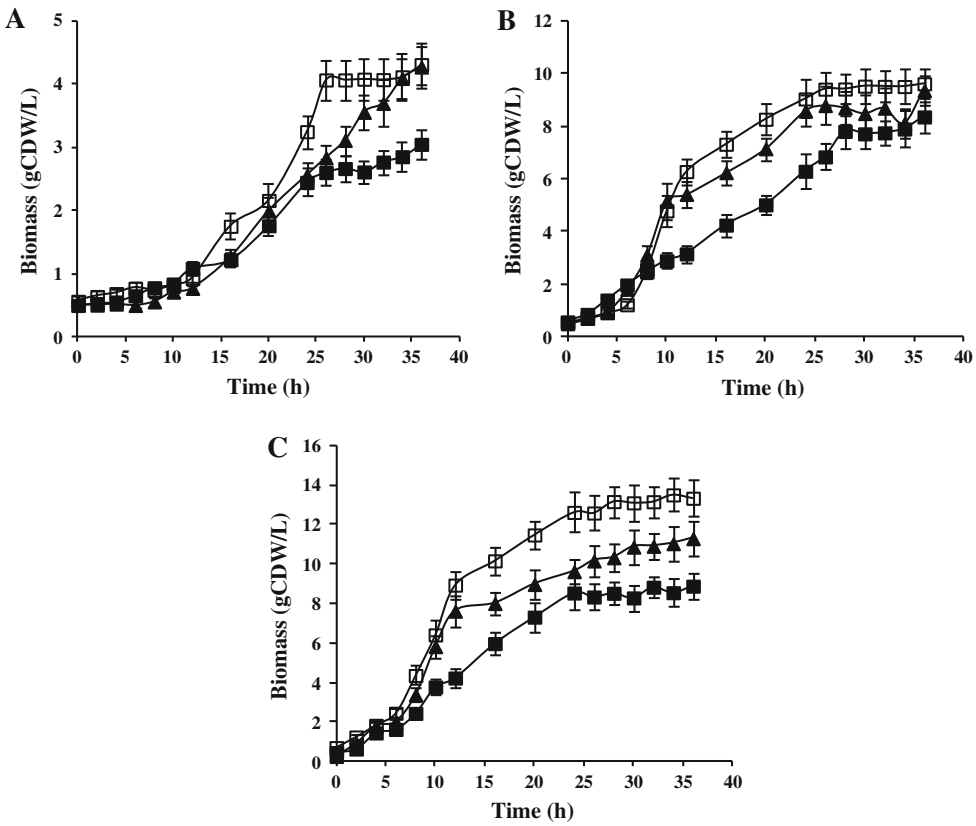

Fig. 4 Batch growth of *P. pastoris* in hyperbaric reactor under pressures of 1 bar (*filled square*), 3 bar (*filled triangle*) and 5 bar (*open square*), in **a** methanol, **b** pure glycerol and **c** crude glycerol medium

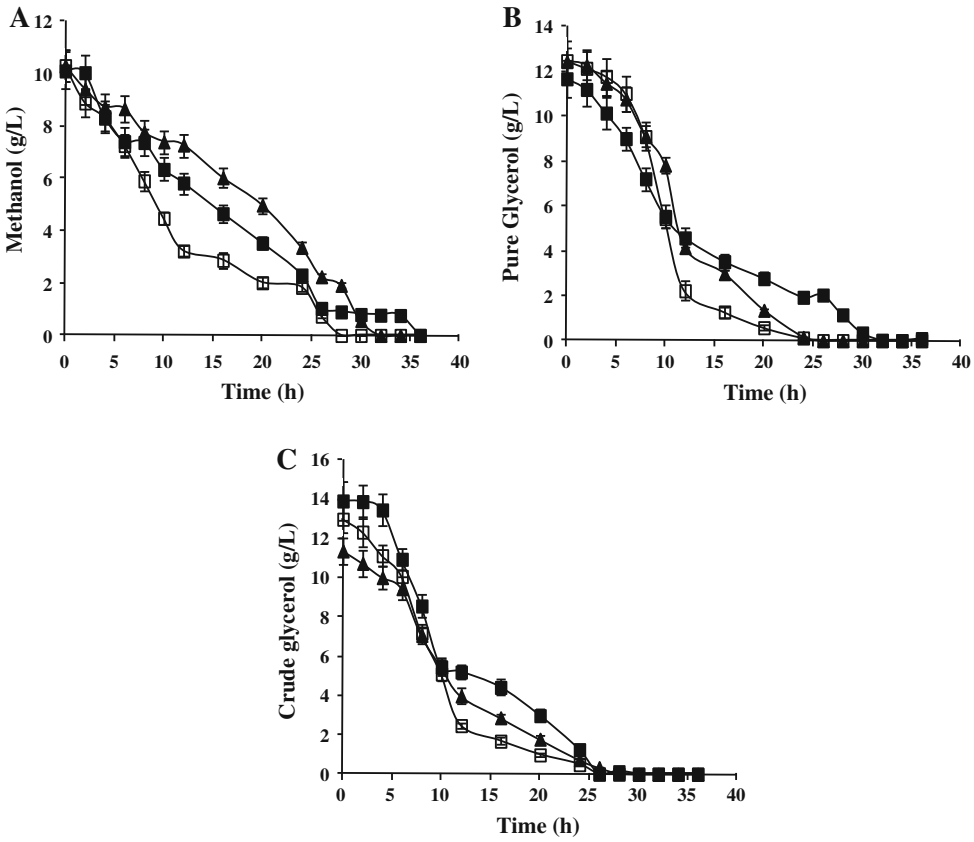

Fig. 5 Methanol (**a**), pure glycerol (**b**) and crude glycerol (**c**) consumption of *P. pastoris* in hyperbaric reactor under pressures of 1 bar (*filled square*), 3 bar (*filled triangle*) and 5 bar (*open square*)

consumption of all glycerol and by-products present in crude glycerol. This may be due to the presence of fatty acids, vitamins A, E and K [19, 20], and trace elements [21] in the vegetable oils diffusing the glycerol phase during the biodiesel formation reactions, thus enriching the glycerol-based production medium, and pressure increase improves its utilization by the yeast. These compounds have positive effects on the yeast physiology and metabolism such as improved membrane integrity [22] and increase in intracellular NAD level [23]. Moreover, the yeast *P. pastoris* has the ability to use fatty acids as sole carbon and energy source [24]. This additional carbon source present on crude glycerol could explain the higher biomass yield obtained with this medium, since methanol is mostly evaporated during sterilization. The values of biomass yields of *P. pastoris* growing on glycerol range from 0.32 mass of cells per mass of substrate [25] and 0.51 mass of cells per mass of substrate [26] and even 0.86 mass of cells per mass of substrate [27], depending of the strain and the experimental conditions. The relatively high cell mass yields at 5 bar with crude glycerol, when compared to the medium with pure glycerol, point out the remarkable influence of the additional nutrients present in crude glycerol. Çelik et al. [3] also reported an improvement in biomass yield of *P. pastoris* E17 from 0.44 mass of cells per mass of substrate to 0.57 mass of cells per mass of substrate when the growth medium was switched from pure to crude glycerol. On the other hand, the cell mass yield obtained at 5 bar with crude glycerol (0.97 mass of cells per mass of glycerol) indicates that the carbon source is mostly used for biomass formation, instead of energy formation and maintenance. The low maintenance demand of *P. pastoris* is a requirement for the very high cell density that is achieved with this organism. Jahic et al. [28] observed that *P. pastoris* SMD 1168 had a maintenance demand of 0.013 g/(g h) for growth on pure glycerol (low value compared to *E. coli*, with 0.04 g/(g h) for growth on glucose [29]).

The specific cellular growth rate of *P. pastoris* was slightly enhanced by the increase of total air pressure for all carbon sources used (Table 1). The most significant difference was found for pure glycerol. At 5 bar, the specific cellular growth rate was 1.5-fold higher than at 1 bar, but no significant improvement in the growth rate was observed in experiments with methanol medium. According with the values of qO_2 obtained for this substrate, the increase in OTR by the values of pressure used overcame the oxygen demand of the culture.

The specific substrate consumption rate for all carbon sources was calculated by the ratio between the maximum specific growth rate and the biomass yield. The effect of increased air pressure in this parameter depends on the

Table 1 Changes in biomass yield, maximum specific growth rate and maximum specific substrate consumption rate in batch experiments under increased air pressure

	1 bar	3 bar	5 bar
$Y_{x/s}$ (mass of cell per mass of substrate)			
Pure glycerol	0.67 ± 0.06	0.71 ± 0.09	0.73 ± 0.08
Crude glycerol	0.60 ± 0.06	0.97 ± 0.09	0.97 ± 0.11
Methanol	0.25 ± 0.02	0.27 ± 0.03	0.36 ± 0.04
μ (h^{-1})			
Pure glycerol	0.15 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
Crude glycerol	0.18 ± 0.02	0.21 ± 0.01	0.20 ± 0.01
Methanol	0.07 ± 0.002	0.08 ± 0.01	0.08 ± 0.01
q_s (mass of substrate per mass of cell per hour)			
Pure glycerol	0.22 ± 0.04	0.31 ± 0.05	0.32 ± 0.06
Crude glycerol	0.30 ± 0.06	0.22 ± 0.04	0.21 ± 0.04
Methanol	0.28 ± 0.05	0.30 ± 0.06	0.22 ± 0.04

Values are average ± standard deviation of three experiment replicates

carbon source used. Similarly to the observed effect on the specific cellular rate, for pure glycerol, the specific consumption rate at 5 bar was 1.5-fold higher than at 1 bar. However, for the other substrates, it slightly decreased with pressure.

Although the pH was not controlled during batch cultures, the buffered medium was effective in maintaining the pH value between 5.5 and 6 in glycerol (crude and pure) and methanol media.

These results demonstrate that pressure had no inhibitory effects on the batch growth of the *Pichia pastoris* strain CBS 2612. Thus, an increase of air pressure up to 5 bar may successfully be applied to the improvement of biomass production. Charoenrat et al. [10] also showed that the cell mass productivity of *P. pastoris* cultures can be improved by the OTR enhancement through increased air pressure from 1.2 to 1.9 bar. However, the results reported here demonstrate that for the methylotrophic yeast *P. pastoris* CBS 2612, values of total air pressure up to 5 bar can be applied.

Although the cell productivity of *P. pastoris* processes can be improved by increasing the OTR by application of moderate air pressure, the impact of pressure applied in protein expression and its activity could conduct to the same or to different results. Charoenrat et al. [10] reported that the total activity of β-glucosidase of *P. pastoris* was enhanced by increasing air pressure to 1.9 bar. Lopes et al. [5] showed that air pressure rise up to 6 bar can be imposed to the *Y. lipolytica* culture as a mean of enzyme production improvement such as lipases and SOD. Pinheiro et al. [6] also demonstrated that the specific β-galactosidase production by *K. marxianus* increased three times using a 6 bar air pressure instead of air at atmospheric pressure. However, Belo et al. [30] reported that the increase of air pressure from 2 to 4 bar showed a negative effect on cytochrome b5 heterologous expression by *E. coli* TB1 cells.

Air pressure effect on fed-batch cultures

As the results above demonstrated, the increase of air pressure up to 5 bar could be successfully applied for *P. pastoris* batch growth, improving the final cell mass productivity. However, because the mode of operation can influence the effect of moderate pressure on final cell productivity, fed-batch operation at increased air pressure was performed in order to study the cellular behavior and compare it to batch cultures. Pure and crude glycerols were used as carbon sources, and two strategies were applied: (a) constant feeding rate, and (b) exponential feeding rate, as described in the "Materials and methods".

The rise of air pressure up to 5 bar led to an increase in final cell mass for both carbon sources and feeding strategies (Figs. 6, 7). The application of 5 bar pressure resulted in a complete glycerol consumption, avoiding its accumulation in the medium, as occurred at 1 bar.

For the constant feeding rate strategy, a 1.6- and 2.2-fold improvement in cell dry weight was obtained at 5 bar compared to 1 bar, for pure and crude glycerol, respectively. The fed-batch growth with pure glycerol resulted in higher biomass concentration compared to crude glycerol. A 1.9- and 1.4-fold improvement of final cell mass concentration at 1 and 5 bar was attained with this carbon source, compared to the other one.

With the exponential feeding rate strategy, when air pressure varied from 1 to 5 bar, the biomass concentration increased 2.4- and 2-fold for pure and crude glycerol, respectively. Similarly to constant feeding rate, with this strategy, the pure glycerol medium led to a higher final biomass.

Among the feed strategies studied, the highest biomass yield was obtained with exponential feeding rate for pure glycerol and with constant feeding rate for crude glycerol (Table 2). With exponential feeding rate, the increase of air pressure to 5 bar caused 1.34- and 1.43-fold improvement in biomass yield per crude and pure glycerol, respectively. For the constant feeding rate, a 1.2- and 1.63-fold improvement in biomass yield was obtained at 5 bar compared to 1 bar, for crude and pure glycerol, respectively.

Jahic et al. [28] found a yield of 0.7 mass of cells per mass of substrate when *P. pastoris* cells were grown on glycerol medium. The results reported here proved that the increase of total air pressure up to 5 bar led to an improvement of cell yields obtained by other researchers.

The differences on biomass yield between the two fed-batch strategies were more pronounced at 1 bar. Probably,

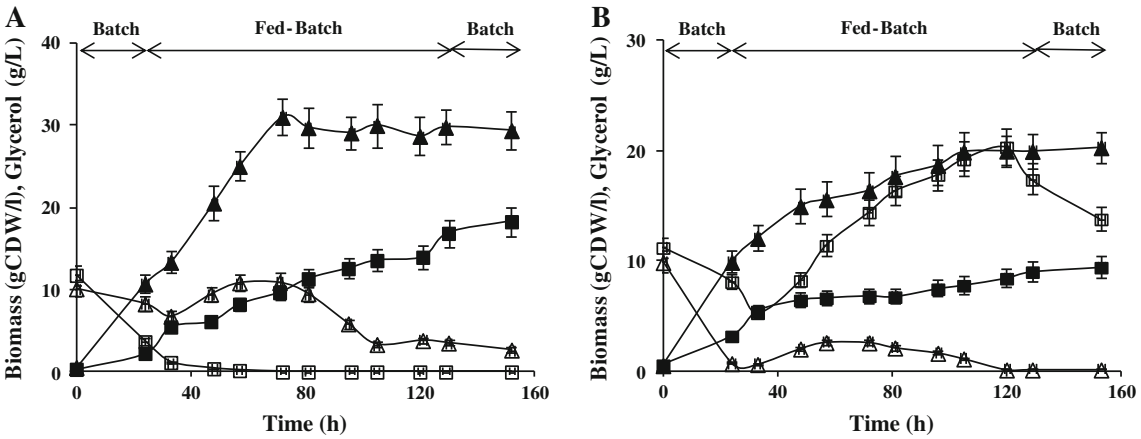

Fig. 6 Fed-batch growth of *P. pastoris* (*filled square* 1 bar; *filled triangle* 5 bar) and glycerol concentration (*open square* 1 bar; *open triangle* 5 bar) in **a** pure glycerol and **b** crude glycerol with constant feeding rate strategy. The glycerol concentration in the medium feed was 50 g/L

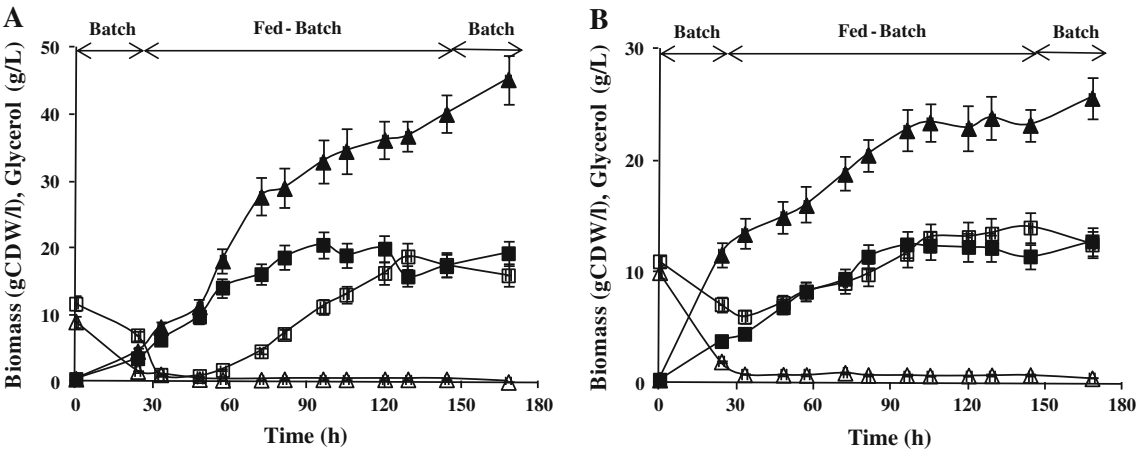

Fig. 7 Fed-batch growth of *P. pastoris* (*filled square* 1 bar; *filled triangle* 5 bar) and glycerol concentration (*open square* 1 bar; *open triangle* 5 bar) in **a** pure glycerol and **b** crude glycerol with exponential feeding rate strategy. The glycerol concentration in the medium feed was 50 g/L

Table 2 Changes in biomass yield (mass of cells per mass of substrate) with air pressure in fed-batch experiments for constant and exponential feeding rate strategies

	Constant feeding rate		Exponential feeding rate	
	1 bar	5 bar	1 bar	5 bar
Pure glycerol	0.57 ± 0.07	0.93 ± 0.11	0.74 ± 0.09	1.06 ± 0.14
Crude glycerol	0.55 ± 0.06	0.66 ± 0.07	0.41 ± 0.05	0.55 ± 0.05

Values are average ± standard deviation of three experiment replicates

at this pressure, the effects of dilution and substrate feeding flow rates had more influence than at 5 bar, where the increase of oxygen transfer capacity assumes an important role on yeast metabolism.

The final cell biomass obtained in fed-batch cultures was higher for pure glycerol. Also, the biomass yields obtained in fed-batch cultures with crude glycerol were lower than those obtained in batch cultures. Although it has been shown that crude glycerol from the biodiesel industry can support the batch and fed-batch growth of *P. pastoris*, the higher glycerol and by-products concentration in fed-batch mode could explain the results. In general, the composition of crude glycerol varies from plant to plant; it contains methanol and various elements such as calcium, potassium, phosphorus, magnesium, sulfur, and sodium. Crude glycerol also contains soaps, which are formed from a side reaction of biodiesel production, and it has been reported in a wide range from 23 to 25 % [31]. The complex interaction between the cell membrane and these surfactant type compounds can cause this biomass yield reduction in fed-batch process comparatively to batch cultures. Also, the presence of ions of sodium, calcium, and potassium could interfere with the ionic balance and affect the yeast metabolism.

Conclusions

For the experimental conditions used in this work, an air pressure rise of up to 5 bar proved to be applicable to the batch and fed-batch cultivation of *P. pastoris*. The use of air pressure had positive effects on the growth behavior of this yeast, whatever the carbon source used, even when crude glycerol was used as substrate. This significant increase in cell mass productivity using moderate pressure, combined with the availability and low cost of crude glycerol from biodiesel production, offers an opportunity for cheaper biotechnological processes using glycerol as substrate.

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