Universidade do Minho
Escola de Engenharia

Marlene Alexandra da Silva Lopes

Characterization of non-conventional yeasts under hyperbaric conditions: cellular response to oxidative stress

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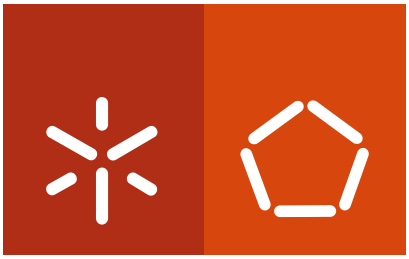

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Characterization of non-conventional yeasts under hyperbaric conditions: cellular response to oxidative stress

Doctoral Dissertation for PhD degree in
Chemical and Biological Engineering

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Characterization of non-conventional yeasts under hyperbaric conditions: cellular response to oxidative stress

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"In the beginning there was yeast, and it raised bread, brewed beer, and made wine. After many not days but centuries and even millennia later, it was named Saccharomyces cerevisiae. After more years and centuries there was another yeast, and it was named Schizosaccharomyces pombe, now there were two stars in the yeast heaven. In only a few more years there were other yeasts, and then more, and more. The era of the non-conventional yeasts had begun."

Spencer et al. (2002)

LIST OF PUBLICATIONS

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, this thesis is based on the following original articles:

- Lopes M., Mota M., Belo I. Advantages of microbial cultivation under increased air pressure. *Submitted for publication to Biotechnol Adv* [Chapter 2].

- Lopes M., Mota M., Belo I. Study of oxygen mass transfer rate in pressurized lab-scale stirred bioreactor. *Submitted for publication to Chem Eng Technol* [Chapter 3].

- Lopes M., Mota M., Belo I. Comparison of *Yarrowia lipolytica* and *Pichia pastoris* cellular response to different agents of oxidative stress. *Submitted for publication to Appl Biochem Biotechnol* [Chapter 4].

- Lopes M., Gomes N., Mota M., Belo I. (2009) *Yarrowia lipolytica* growth under increased air pressure: influence on enzyme production. *App Biochem Biotechnol* 159: 46-53 [Chapter 5].

- Lopes M., Mota M., Belo I. (2009) *Yarrowia lipolytica* adaptation to oxidative stress induced by increased air pressure. New Biotechnol 25(S1): S77-S78 [Chapter 5].

- Lopes M., Mota M., Belo I. (2011) Enhanced *Pichia pastoris* biomass under increased air pressure: batch and fed-batch strategies. Curr Opin Biotech 22S: S60 [Chapter 6].

- Lopes M., Belo I., Mota M. (2013) Batch and fed-batch growth of *Pichia pastoris* under increased air pressure. *Biop Biosys Eng* (in press) DOI: 10.1007/s00449-012-0871-5 [Chapter 6].

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SUMMARY

Providing an adequate oxygen supply is critical to the growth and maintenance of most aerobic microbial cultures used in biotechnological processes. Oxygen mass transfer from gas phase to the culture medium is often a major growth limiting factor because of oxygen´s low solubility in an aqueous solution. Thus, ensuring adequate oxygen supply to submerged cultures in bioreactors is not trivial.

The use of increased air pressure as a way of improving oxygen mass transfer from gas phase to liquid phase has been developed by some authors. However, the effect of reactor pressurization must be considered on cellular growth and metabolism. The increase of oxygen partial pressure could result in reactive oxygen species (ROS) formation and lead to an oxidative environment to the cells. As the effect of increased air and oxygen pressure is strongly dependent of the cell type, species and strains, due to their different abilities of cellular response to possible oxidative stress that can arise, this thesis is focused on the study the behavior of non-conventional yeasts under hyperbaric air. In spite of the well-known importance of *Yarrowia lipolytica* and *Pichia pastoris* in several biotechnological processes, few studies are available on the application of air pressure increase for the cultivation of these yeasts.

This work was started with the study of the oxygen mass transfer phenomenon from gas phase to the medium in a lab-scale pressurized bioreactor. The influence of operation parameters (aeration and stirring rates and increased air pressure up to 5 bar) on oxygen transfer rate (*OTR*) was analyzed. An empirical correlation for the prediction of the volumetric oxygen mass transfer coefficient (k_La) as a function of air pressure, power input and superficial gas velocity was attempted. The results demonstrated that the increased air pressure is valuable option for *OTR* enhancement in bioreactors, competing favorable with raising stirring and aeration rates, which can cause cell damage by shear stress.

Yeast cells exposed to adverse conditions employ a number of defense mechanisms to respond effectively to the stress effects of reactive oxygen species. The cellular response of *Y. lipolytica* W29 and *P. pastoris* CBS 2612 to the exposure to the ROS-inducing agents paraquat (1 mM), hydrogen peroxide (50 mM) and increased air pressure (1 bar and 5 bar) was analyzed. For both strains the cellular viability loss and lipid peroxidation was lower for the cells exposed to increased air pressure than for those exposed to chemical oxidants. Under superoxide stress (paraquat and air pressure), the SOD induction was the main observed mechanism, whereas the hydrogen peroxide was the most efficient inducer of catalase. The results suggested that *Y. lipolytica* have a more potent antioxidant system than *P. pastoris*.

Batch cultivations of *Y. lipolytica* W29 under air pressures up to 6 bar were performed to investigate whether increasing air pressure may lead to increasing biomass yields, without giving raise to oxidative stress. The levels of antioxidant enzymes induced were also monitored. Cell growth was strongly enhanced by the pressure raise, since 5- and 3.4-fold improvement in the biomass production and in specific growth rate, respectively, were observed under 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. Moreover, the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production by *Y. lipolytica* cells was investigated. The extracellular lipase activity increased 96% using a 5 bar air pressure instead of air at 1 bar pressure during the enzyme production phase. These results demonstrated that air pressure increase in bioreactors is an effective way for the enhancement of cell mass and enzyme productivity in bioprocesses involving *Y. lipolytica* cultures.

P. pastoris CBS 2612 behavior under air pressures of 1 bar, 3 bar and 5 bar in culture media of glycerol (pure and crude) and methanol was studied. Generally, an enhancement on cellular growth, for all carbon sources, was achieved with the raise of air pressure 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 up to 5 bar using exponential feeding rate led to a 1.4-fold improvement in biomass yield per glycerol mass consumed, for pure and crude glycerol. The results show the possibility of improving cell mass production of *P. pastoris* under moderate air pressure, using low cost carbon sources.

P. pastoris GS115/pPICZ/lacZ (Mut^+), expressing intracellular β-galactosidase, and *P. pastoris* KM71H/pPICZαA/frutalin (Mut^S), expressing extracellular frutalin, were used to investigate the effect of increased air pressure on yeast growth and heterologous protein expression. The increase of air pressure up to 5 bar had a small effect on biomass production, but led to a 9-fold improvement in β-galactosidase specific activity compared to 1 bar. The recombinant frutalin secretion was enhanced by the increased air pressure up to 5 bar and the protease specific activity reached was 2.4 times lower than that obtained at atmospheric pressure in baffled flasks.

RESUMO

Um dos pontos críticos de processos biotecnológicos consiste no fornecimento de oxigénio suficiente para o crescimento e manutenção das culturas microbianas aeróbias. A velocidade de transferência de oxigénio do gás para o meio de cultura é normalmente um fator limitante do crescimento, devido à baixa solubilidade do oxigénio em soluções aquosas. Assim, garantir o adequado aprovisionamento de oxigénio a culturas submersas num bioreator não é uma tarefa menosprezável.

São poucos os investigadores que têm recorrido ao aumento da pressão de ar como forma de melhorar a transferência de oxigénio da fase gasosa para a fase líquida. Neste caso, o efeito da pressurização do reator no crescimento e metabolismo celular deve ser tido em consideração. Além disso, o aumento da pressão parcial do oxigénio pode resultar na formação de espécies reativas de oxigénio e originar um ambiente oxidativo para as células. Uma vez que o efeito do aumento da pressão de ar e de oxigénio depende das espécies e estirpes, devido à diferente capacidade de resposta ao stresse oxidativo, esta tese foca-se no estudo da resposta celular de leveduras não-convencionais ao ar hiperbárico. Apesar da reconhecida importância das espécies *Yarrowia lipolytica* e *Pichia pastoris* em muitos processos biotecnológicos, são poucos os estudos sobre a aplicação do aumento da pressão de ar na cultura destas leveduras.

Este trabalho começou com o estudo da taxa de transferência de oxigénio da fase gasosa para o meio líquido num reator pressurizado, à escala laboratorial. Foi analisada a influência de parâmetros operacionais (taxa de arejamento e de agitação e aumento da pressão de ar até 5 bar) na taxa de transferência de oxigénio (*OTR*). Obteve-se uma correlação empírica do coeficiente volumétrico de transferência de oxigénio (k_La) em função da pressão de ar, da potência de agitação e da velocidade superficial do gás. Os resultados demonstraram que o aumento da pressão de ar é uma opção viável para o incremento de *OTR* nos bioreatores, em alternativa ao aumento da taxa de arejamento e de agitação, que podem causar stresse hidrodinâmico às células.

As células de levedura, quando expostas a condições adversas, desenvolvem um sistema de defesa contra os efeitos causados pelas espécies reativas de oxigénio. Assim, foi analisada a resposta celular das estirpes *Y. lipolytica* W29 e *P. pastoris* CBS 2612 à exposição aos agentes indutores de espécies reativas de oxigénio *paraquat* (1 mM), peróxido de hidrogénio (50 mM) e pressão total de ar (1 bar e 5 bar). Em ambas as estirpes, a perda de viabilidade e a peroxidação lipídica foram menores nas células expostas ao aumento da pressão de ar do que nas expostas aos oxidantes químicos. Em ambiente de stresse provocado pelo ião superóxido (*paraquat* e pressão de ar), o mecanismo de defesa mais observado foi a indução de SOD,

enquanto o peróxido de hidrogénio foi o maior indutor da catalase. Os resultados sugerem que a estirpe *Y. lipolytica* tem um sistema antioxidante mais eficaz que a estirpe *P. pastoris*.

Com o objectivo de investigar se o aumento da pressão de ar podia conduzir a um incremento no rendimento em biomassa, sem originar stresse oxidativo, foram realizados ensaios em modo *batch* de *Y. lipolytica* W29 a valores de pressão total de ar até 6 bar. Foi igualmente avaliada a capacidade da levedura em induzir a expressão de enzimas antioxidantes. O crescimento celular foi consideravelmente beneficiado com o aumento da pressão de ar, uma vez que a produção de biomassa e a taxa específica de crescimento aumentaram 5 e 3.4 vezes, respectivamente, no ensaio realizado a 6 bar. A atividade específica da enzima SOD obtida no ensaio a 6 bar foi 53.4 vezes maior do que a alcançada a 1 bar. Foi também analisada a influência de uma fase de pré-adaptação das células às condições hiperbáricas na produção de lipase por *Y. lipolytica*. A atividade da lipase extracelular aumentou 96% com a aplicação de uma pressão de ar igual a 5 bar, comparativamente ao ensaio realizado a 1 bar, durante a fase de produção da enzima. Estes resultados demonstraram que o aumento da pressão total de ar é uma forma eficaz de aumentar a produtividade em biomassa e em enzima SOD em bioprocessos que utilizem a levedura *Y. lipolytica*.

Foi estudado o comportamento da estirpe *P. pastoris* CBS 2612 em meios de glicerol (puro e bruto) e metanol, com valores de pressão iguais a 1 bar, 3 bar e 5 bar. De uma maneira geral, foi observado um incremento do crescimento celular com o aumento da pressão de ar, em todas as fontes de carbono e em processos em modo *batch* e *fed-batch* com 2 estratégias de alimentação diferentes. Nas culturas em modo *batch* usando metanol, glicerol puro e glicerol bruto, obtiveram-se aumentos de 1.4, 1.2 e 1.5 vezes, respectivamente, na produção de biomassa com a pressão de 5 bar. No processo *fed-batch* com alimentação exponencial, o rendimento em biomassa por massa de glicerol consumido (puro e bruto) aumentou 1.4 vezes com o uso de pressão de ar igual a 5 bar. Os resultados demonstram a possibilidade de aumentar a produção de biomassa de *P. pastoris* sob pressão de ar moderada, usando fontes de carbono de baixo custo.

A estirpe *P. pastoris* GS115/pPICZ/*lac*Z (Mut$^+$), que expressa β-galactosidase intracelular, e a estirpe KM71H/pPICZαA/frutalina (Mut^s), que expressa frutalina extracelular, foram usadas com o intuito de estudar o efeito do aumento da pressão de ar no crescimento destas estirpes e na expressão de proteínas heterólogas. O aumento da pressão total de ar até 5 bar não teve um efeito significativo no crescimento celular destas estirpes, mas conduziu a um incremento de 9 vezes na actividade específica da enzima β-galactosidase, comparativamente à obtida a 1 bar. A expressão de frutalina também aumentou a 5 bar e a actividade específica de protease obtida foi 2.4 vezes inferior à obtida nos ensaios em matraz (pressão atmosférica).

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LIST OF ABBREVIATIONS

a	Specific interfacial area (m^{-1})
Arg	Arginine
BMG	Buffered minimal medium containing glycerol
BMGH	Buffered minimal medium containing glycerol and histidine
BMM	Buffered minimal medium containing methanol
BMMH	Buffered minimal medium containing methanol and histidine
c	Constant dependent on the impeller used
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 (g/L)
CuZnSOD	Cytosolic superoxide dismutase
D	Dilution rate (h^{-1})
D_i	Impeller diameter (m)
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen tension (%)
F	Feed rate (mL/min)
F_g	Volumetric gas flow rate (m^3/s)
g	Relative centrifuge force
GR	Gluthathione reductase
GSH	Gluthathione
GSSG	Oxidized glutathione
His	Histidine
HPLC	High performance liquid chromatography
H_{O_2}	Henry constant for oxygen
k_L	Liquid side mass transfer coefficient (m/s)
k_La	Volumetric oxygen mass transfer coefficient (h^{-1} or s^{-1})
K_T	Constant dependent on the impeller used
M	Molarity (mol/L)
mM	Molarity (mmol/L)
MDA	Malondialdehyde

MnSOD	Mitochondrial superoxide dismutase
Mut$^+$	Methanol utilization plus
Mut^S	Methanol utilization slow
N	Agitation rate (rps)
NADPH	Nicotinamide adenine dinucleotide phosphate
N_P	Power number
N_{Re}	Reynolds number
NAD	Nicotinamide adenine dinucleotide
OTR	Oxygen transfer rate (mg O_2/L h)
OUR	Oxygen uptake rate (mg O_2/L h)
P	Absolute pressure (bar)
PBS	Phosphate buffered saline
PQ	Paraquat
pAOX	Alcohol oxidase promoter
p_{CO_2}	Carbon dioxide partial pressure
pDNA	Plasmid DNA
p_{O_2}	Oxygen partial pressure (bar)
P_g	Power input to the aerated system (W)
P_g'	Power input to the non-aerated system (W)
P_x	Biomass productivity (g/L h)
qO_2	Specific oxygen uptake rate (mg O_2/g h)
q_s	Maximum specific substrate consumption rate (g/g h)
R&D	Research and development
RI	Refractive index
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
STR	Stirred-tank reactor
t	Time (h)
V	Working volume (m^3)
V_0	Initial culture volume (mL)
V_{sa}	Sample volume (mL)
vvm	Volume of air per volume of reactor per minute

YP	Yeast extract and peptone medium
YPD	Yeast extract, peptone and dextrose medium
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)

Subscripts

O	Oxygen
S	Substrate
sa	Sample
T	Total
X	Biomass
0	Initial value

Greek letters

α	Numerical constant
B	Numerical constant
γ	Numerical constant
μ	Specific growth rate (h^{-1})
ρ	Liquid density (kg/m^3)
δ	Numerical constant
υ	Liquid viscosity (kg/m s)
υ_s	Superficial gas velocity (m/s)

Notes:

In general, the International System of Units (SI) was used in this work. However, sometimes multiples and sub-multiples of the fundamental units (which are not SI) were used since their use is so common that is allowed by that system. From Chapter 3 to Chapter 8 was used “bar” as the pressure unit, since it was more appropriate for the range of pressure values used in this work.

Units not recognized by the SI were also used to express some variables, such as the volume percent (% v/v) and the mass percent (% w/v) to denote the composition of some solutions, the revolutions per minute (rpm) to indicate the agitation rates and the volume of air per volume of reactor per minute (vvm) to designate the aeration rates.

1 MOTIVATION AND OUTLINE

This chapter introduces the background information about the theme of the work, as well as its objectives.

The outline of the thesis is also presented.

1.1 CONTEXT AND MOTIVATION

Laboratory research of microbial processes is usually performed at atmospheric pressure, but in industrial bioreactors of several tens of meters high, pressure and consequently gas solubility is a function of the local position in the reactor, generally increasing by 1 bar for every 10 m increase in depth (Onken and Liefke, 1989). Due to the differences observed in the residence time distribution in large reactors, cells are distinctly exposed to high pressures (at the bottom) and to low pressures (on top). Many differences in process productivities found between lab-scale and plant-scale can be partially explained by these different environmental conditions in each scale system. This is particularly important when dissolved oxygen is a determinant factor in the process, as is the case in high cell density aerobic cultures. In such cultures, the cellular oxygen demand far exceeds the oxygen transfer capacity of conventional bioreactors such as stirred tanks, meaning that the dissolved oxygen becomes limiting for microbial growth. Many efforts have been made to overcome the oxygen limitation in the culture medium, being the most commonly used the increase in power input. Special aeration systems, e.g. aeration using oxygen enriched air and increased reactor pressure are techniques applied to increase oxygen availability (Belo et al., 2003; Knoll et al., 2005; Lopes et al., 2008). Also, the use of in situ production of oxygen (Sonnleitner and Hahnemann, 1994) or the use of a second liquid phase of oxygen-carriers compounds such as perfluorodecalin (Amaral et al., 2008) or n-hexadecan (Nielsen et al., 2003) in the culture medium can increase the availability of oxygen to the microorganisms.

Bioreactor pressurization has been proven to be an efficient way of oxygen mass transfer to aerobic cultures and could be successfully applied to yeast cultivation (Aguedo et al., 2005; Belo et al., 2003; Pinheiro et al., 2003). However, above certain limits the increased air pressure and the consequent increase in oxygen partial pressure may causes oxidative stress to the cells and have detrimental effects on cell physiology and metabolites production. It was proven that oxygen toxicity, besides total pressure, is the main cause of cell inhibition (Pinheiro et al., 2002).

Oxidative stress is caused by exposure to reactive oxygen species (ROS), especially superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$), which can damage proteins and modify bases and sugars in DNA (Esterbauer et al., 1991). To protect against the damage caused by oxidative stress, cells possess a number of biochemical systems, including enzymes (superoxide dismutase, catalase and glutathione reductase) as well as the non-enzymatic protective

molecules (glutathione and thioredoxin), most of which are expressed at low levels during normal growth. In response of elevated concentrations of ROS, the expression of many antioxidant defenses is induced.

Moderate pressure may have delicate effects on the metabolism and the gene expression of cells. It is then crucial to study the effects of the air pressure increase on cell growth and proteins production. Also, the antioxidant defense induced by the yeasts growing under increased air pressure is of great importance. The effects of oxidative stress related to hyperbaric air on microbial behavior have been studied for *E. coli* (Belo and Mota, 1998), *Thermus* sp. (Belo et al., 2000), *K. marxianus* (Pinheiro et al., 2003) and *S. cerevisiae* (Belo et al., 2005). However, the effect of increased air and oxygen pressure is strongly dependent of the species and strains due to their different cellular responses. The purpose of this thesis was to investigate whether total air pressure could be used within a range of values easily applicable in an industrial environment, in order to improve cell cultivation and consequently enzyme productivities in *Yarrowia lipolytica* W29, *Pichia pastoris* CBS 2612 and in the recombinant strains *Pichia pastoris* GS115 and KM71H. For this purpose, the following main topics were focused:

- Characterization of oxygen mass transfer rate (*OTR*) in pressurized lab-scale stirred bioreactor.
- Analysis of the cellular response of *Y. lipolytica* and *P. pastoris* to the exposure of ROS-inducing agents.
- Study of the effects of increased air pressure on *Y. lipolytica* batch growth, antioxidant defense induction and lipase production.
- Study of the effects of increased air pressure on *P. pastoris* growth in batch and fed-batch cultures.
- Evaluation of the effects of bioreactor pressurization on the expression of heterologous proteins by recombinant *P. pastoris* strains.

1.2 OUTLINE OF THE THESIS

The main goal of this thesis was to answer to the question "**how does two different non-conventional yeast species respond to air pressure?**". The thesis was structured in nine chapters:

- The context and motivation of this thesis and the research aims are presented in the current chapter (**Chapter 1**). The structure of the thesis is also outlined.

- **Chapter 2** concerns literature review, where a general overview of previous studies developed with microbial cultures under increased air pressure is presented.

The different sections of **Experimental Results** are presented from Chapter 3 to Chapter 7. In these chapters a brief *introduction*, *materials and methods*, *results and discussion* and *conclusions* for the chapter topics are given.

- In **Chapter 3** the results concerning the oxygen mass transfer rate in a pressurized bioreactor are reported. The oxygen volumetric mass transfer coefficient (k_La) as a function of the air pressure is also described.

- In **Chapter 4**, the strains *Y. lipolytica* W29 and *P. pastoris* CBS 2612 were exposed to the ROS-inducing agents paraquat, hydrogen peroxide and increased air pressure. The cellular response of the yeast strains to each agent was assessed by the analysis of antioxidant enzymes and GSH.

- Batch cultivation of *Y. lipolytica* W29 under increased air pressure from 1 bar to 6 bar is reported in **Chapter 5**. The effect on cellular growth and the ability of the strain to induce antioxidant enzymes such as SOD and catalase was evaluated. Moreover, the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production was also reported.

- **Chapter 6** presents the study of *P. pastoris* CBS 2612 behavior under total air pressure up to 5 bar in culture media of glycerol (pure and crude) and methanol, which was performed in batch and fed-batch cultures.

- Two recombinant *P. pastoris* strains (GS115/pPICZ/*lacZ* and KM71H/pPICZαA/frutalin), producing intracellular β-galactosidase and extracellular frutalin respectively, were used to investigate the effect of reactor pressurization on heterologous protein expression. The results concerning this study are presented in **Chapter 7**.

- **Chapter 8** presents the overall conclusions as well as suggestions for future work in this field of research.

- Finally, **Chapter 9** gathers all the references used in the elaboration of this work.

2 LITERATURE REVIEW

In a number of biological systems, life strategies may be significantly influenced by pressure. In industrial biotechnology microbial cultures are exposed to different local pressures inside the bioreactors. The increased pressure may have detrimental or beneficial effects on cellular growth and products formation, depending on the microbial species and strains.

In this Chapter, the focus will be on the effects of increased air pressure on various microbial cultures growing in hyperbaric bioreactors under moderate pressures. Revisiting general principles of pressure effects on biological systems, recent data illustrating the diversity of increased air pressure effects may have at different levels in microbial cultivation, with particular attention to effects on cellular growth, products formation and antioxidant defense mechanisms is present.

The information presented in this Chapter was submitted to *Biotechnol Adv:*

Lopes M., Mota M., Belo I. Advantages of microbial cultivation under increased air pressure (January 2013).

2.1 INTRODUCTION

All biological processes of life on Earth experience varying degrees of pressure. Aquatic organisms living in the deep-sea, as well as chondrocytic cells of articular cartilage are exposed to hydrostatic pressures that raise up to several hundred times that of atmospheric pressure. The effects of hydrostatic pressure on physiological or biochemical system basically result from the compression of the system and can be related to the changes in the protein structures, lipid bilayers of membranes and gene expression.

In industrial processes, microorganisms are required to have an efficient metabolism, with high productivities, in order to achieve an economical production process. During a biotechnological process based in microbial cultures differences in operational conditions take place, like pressure gradients (in general up to 0.2 or 0.3 MPa, maximum approx. 1 MPa), leading to changes in cell metabolism. In a typical industrial cell cultivation system, quite high cell densities are reached and oxygen is usually the major growth limiting factor. The use of pressure in bioreactors may be a way of improving oxygen transfer rate (*OTR*) of aerobic cultures avoiding oxygen limitation.

Some authors have demonstrated that increased air pressure could be applied to microbial cultivation, as a way of improving the *OTR* to aerobic cultures (Belo et al., 2000; Charoenrat et al., 2006; Knoll et al., 2005; Lopes et al., 2008; Pinheiro et al., 2003). However, the effect of increased air pressure is strongly dependent of species and strains due to the different cellular responses to oxidative stress. Above certain limits, increased air pressure and the consequent increase in oxygen partial pressure may have detrimental effects on microbial cell activity and on product formation. In hyperbaric bioreactors, cells are often exposed to O_2 partial pressures higher than 0.021 MPa (corresponding to air at 0.1 MPa), leading to the formation of reactive oxygen species (ROS). To counter oxidative stress, cells constitutively express enzymes that detoxify the ROS and repair the damages incurred.

It is important to recognize the impact of increased air pressure on cellular physiology and morphology, product formation and induction of antioxidant defenses. The purpose of this Chapter is to review the revelant available knowledge about the effects of increased air pressure on microbial cultures.

PRESSURE AND LIFE

The majority of the biosphere is aquatic, mainly oceanic, with an average depth of 3800 m. At this depth marine organisms whithstand pressures of 38 MPa, approximately 380-fold greater than atmospheric pressure (Abe, 2007). This means that the majority of the biosphere stays in high-pressure environments. In the marine environment, pressure is a natural parameter, which may play a role in adaptation processes. Aquatic environments exhibit a wide range of hydrostatic pressure from micro-pressures generated by a few centimeters of water column up to 110 MPa in oceanic depths. Consequently, pressure appears to be an important parameter for live on Earth. Taking into account that the maximum pressure at the center core of Earth is evaluated to be 400 GPa and that such a value is higher for the Giant Planets approximately 90% of the Universe is submitted to a pressure higher than 10 GPa (Jayaraman, 1984).

Three main factors can characterize the pressure effects: energy, densification effect and chemical reactivity. The energy developed by high pressure is quite low and, consequently, high pressure will only affect weak chemical bonds (Rivalain et al., 2010). Due to compressibility, the difference between final and initial volumes under high pressure is always negative. This factor induces different phenomena such as the formation of new structural forms (Knorr et al., 2006) and the modification of the equilibrium, for example the dissociation of water (Heremans et al., 1996). Due to the compressibility of the solutions and the improvement in the solubility, pressure enhances the chemical reactivity, inducing an increase of the kinetics (Schettino and Bini, 2007).

The discovery of piezophiles (or barophiles) microorganisms in the deep marine environments contributed to the development of the study of high pressure effects in the microorganisms adaptation and in the role of high pressure in the origin of life (Pradillon and Gail, 2007). Pressure effects are also of interest in the biomedical science field since they are responsible for a number of pathologies. In the human hip joint, pressures of 10–20 MPa have been recorded (Hall et al. 1993). Cells of articular cartilage are constantly influenced by mechanical stress when forces are transmitted across joints (Hodge et al. 1986). In addition to this interest in deep-sea life and biomedical science, high pressure treatment of food has been studied as a technique to pasteurize food without a heating process, and an increasing number of food products treated under high pressure have been commercialized (Hayashi, 2002; Knorr et al., 2006; Smelt et al., 2006).

High pressure represents an interesting form of stress and pressure effects on any physiological or biochemical system basically results from the compression of the system. High pressure treatment has been repeatedly reported to cause cellular death, cellular arrest and growth inhibition. Abe (2004) reported that hydrostatic pressure in the range of 15-25 MPa caused arrest of the cell cycle in G_1 phase in an exponentially growing culture of a *S. cerevisiae* tryptophan auxotroph. Fernandes (2005) observed that the *S. cerevisiae* viability during hydrostatic pressure treatment decreases with pressures above 100 MPa, while at 220 MPa all wild-type cells are killed. Iwahashi et al. (2005) found that a pressure of 30 MPa induced the increase on *S. cerevisiae* cell size and decrease cellular viability.

Lipid bilayers of biological membranes are one of the most pressure sensitive biological components. High pressure orders phospholipid bilayers, causing the fatty acyl chains to pack together more tightly, reducing membrane fluidity in 0.1 MPa-adapted organisms (Mentré and Hui Bon Hoa, 2001). Membranes of deep-sea organisms exhibit an increase of the unsaturated to saturated lipid ratio, allowing the maintenance of fluidity under pressure.

High hydrostatic pressure affects protein polymerization and also induces protein denaturation, thus interfering with enzyme activity (Silva et al., 2001). Balny et al. (2002) attributed these pressure-induced effects to the penetration of water into the protein structure, resulting in unfolding. However, high pressure can maintain some parts of the molecule unchanged due to the fact that only weak bonds are affected, contrary to the heat and chemical denaturation (Rivalain et al., 2010).

Pressure treatment caused induction of genes in the subcategory of “stress response”, including genes involved in energy metabolism, such as *PAU* genes, oxidative stress, such as *GRX*1 and *CCT*1, and heat shock response, such as *HSP*12, *HSP*150, *SSE*2 and *HSP*104 (Iwahashi et al., 2003; Jamieson, 1998; Rachidi et al., 2000). Iwahashi et al. (2005) found that a pressure of 30 MPa induced the production of certain heat-shock proteins and activated genes controlling membrane structure. Fernandes et al. (2004) reported that high hydrostatic pressure treatments shown to induce a decrease in mRNA levels of genes involved in cell-cycle progression.

2.2 PRESSURE IN BIOTECHNOLOGY

The effects of high pressures of several hundred MPa are not of relevance to aerobic processes, where in industrial bioreactors pressures of the order of up to 10 bar can only be reached locally. In fact, due to the differences observed in the residence time distribution in large reactors, cells are distinctly exposed to high pressures (at the bottom) and to low pressures (on top). In each local position, the total pressure is the sum of operational pressure (pressure at the reactor top) and the hydrostatic pressure exerted by the liquid height above this point. As a consequence, equilibrium solubilities of gaseous compounds will also be a function of the local position inside the reactor. Thus, in an industrial bioreactor containing a liquid medium up to a level of 10 m and with a pressure at the top of 0.15 MPa, total pressure at the bottom will amount to 0.25 MPa; this means, that gas solubility, e.g. for oxygen or carbon dioxide at the bottom, will be nearly 70% higher than at the top of the bioreactor. A few examples of industrial large bioreactors applied in aerobic processes are given in Table 2.1.

Table 2.1 Examples of industrial large bioreactors (adapted from Onken and Liefke, 1989).

Reactor	Company	Height (m)	Process
BIOHOCH	HOECHST	30	Aerobic effluent treatment
Tower Biology Reactor	Bayer	30	Aerobic effluent treatment
Pressure Cycle Fermenter	ICI	60	SCP from methanol
Deep-Shaft Reactor	ICI	100	Aerobic effluent treatment

The effects of these pressure values have to be taken into account on the scale-up, since many differences in process productivities found between R&D phase (lab-scale) and production phase (industrial scale) can be partially explained by these different environmental conditions.

Since the total and partial pressures are especially important in the kinetics of aerobic processes, the laboratoy simulation of local changes of environmental conditions on microbial cultures in industrial bioreactors is of great importance. Investigations of the effects of the increased pressure on microbial cultures can be performed with air, varying total pressure inside the bioreactor. Since

several years, stirred bioreactors for higher pressures are intensively applied in chemical industry and the adaptation of such technology to microbial cultures could be easily performed.

Published works have reported the use of increased air pressure in several microbial cultures and the effects on microorganism behavior are strongly dependent of the species and strains (Coelho et al., 2004; Onken and Liefke, 1989; Pinheiro et al., 2000).

2.3 EFFECTS OF INCREASED AIR PRESSURE

Providing an adequate oxygen supply is critical to the growth and maintenance of most aerobic microbial cultures used for biotechnological processes. Oxygen mass transfer from air to the growth medium is often a major growth limiting factor because of oxygen's low solubility in an aqueous solution (Bliem and Katinger, 1988). Thus, it is important to ensure an adequate oxygen supply to a submerged culture.

The use of increased air pressure to improve the oxygen mass transfer from the gas phase to the liquid has been developed by some authors (Belo et al., 2000; Knoll et al., 2007; Lopes et al., 2008; Pinheiro et al., 2003). In this case, the effect of increased air pressure on cellular growth and morphology must be considered. Moreover, the increase of oxygen partial pressure could result in reactive oxygen species formation and lead to an oxidative environment to the cells.

2.3.1 Oxygen mass transfer dynamics

Availability of oxygen strongly affects the process performance of aerobic bioprocesses. These bioprocesses are mostly carried out in aqueous media where the solubility of oxygen is low owing to the presence of ionic salts and nutrients, and the rate of oxygen utilization by the microorganisms is high (Gogate and Pandit, 1999; Gupta et al., 2003). Hence, oxygen mass transfer between phases is an important and rate limiting step in bioprocesses.

There are several methods to enhance oxygen mass transfer rate to a culture: increasing stirrer speed and/or air sparging rate or enriching air inlet with pure oxygen (Pan et al., 1987). Other non-

conventional methods to enhance the oxygen supply include in situ generation of molecular oxygen with hydrogen peroxide and catalase (Ibrahim and Schlegel, 1980; Schlegel, 1977), co-immobilization or mixed culture with oxygen-producing photosynthetic algae (Adlercreutz and Mattiasson, 1982; Khang et al., 1988), and the introduction of an immiscible phase of perfluorocarbons (Amaral et al., 2006) with high oxygen solubility. However, these approaches are limited by one or more problems: chemical compatibility, toxicity, increased cost of downstream processing to remove added chemicals, competition for common nutrients, and complications in bioreactor design and operation (Yang and Wang, 1992).

The mass balance for the dissolved oxygen in the well-mixed liquid phase can be established as:

$$\frac{dO}{dt} = OTR - OUR - DO \tag{2.1}$$

where O is the dissolved oxygen concentration in the medium, t is the time, OTR is the oxygen mass transfer rate from the gas phase into the broth, OUR is oxygen uptake rate by the microorganisms and D is the dilution rate.

OUR can be expressed by the product of the specific oxygen consumption rate of the microorganism (q_{O_2}) and the biomass concentration (X):

$$OUR = q_{O_2} \cdot X \tag{2.2}$$

OTR is controlled by the oxygen solubility and the volumetric oxygen mass transfer coefficient ($k_L a$), and can be stated mathematically as:

$$OTR = k_L a \, (C^* - C) \tag{2.3}$$

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:

$$p_{O_2} = H_{O_2} \cdot C^* \tag{2.4}$$

where

$$p_{O_2} = y_{O_2} \cdot P_T \tag{2.5}$$

and where

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

Published works have reported the use of increased air pressure to improve the oxygen transfer rate for cell cultivation. Yang and Wang (1992) reported that the bioreactor headspace pressurization increased 2.7 times the *OTR* in the bulk medium for operating pressures up to 0.27 MPa. Belo et al. (2000) reported a 6-fold improvement in *OTR* when the total air pressure increases from 0.1 MPa to 5.6 MPa. Pinheiro et al. (2003) demonstrated that *OTR* is clearly enhanced by air pressure raise from 0.12 MPa to 0.6 MPa, obtaining values of 316 mg O_2/(L h) and 1099 mg O_2/(L h), respectively. Knoll et al. (2005) showed that the increased air pressure up to 1.1 MPa could be a way of improving *OTR* of microbial cultures with energy cost efficiencies acceptable for industrial application. Also, Knoll et al. (2007), during the *E. coli* fed-batch, achieved approximately 2-fold improvement in *OTR* increasing stepwise the air pressure from 0.1 MPa to 1.1 MPa. Lopes et al. (2008) observed an increase of oxygen mass transfer from 1248 mg O_2/(L h) to 2924 mg O_2/(L h) shifting the total air pressure from 0.2 MPa to 0.8 MPa.

Cultivation under pressurized conditions also presents positive economic advantages in *OTR* improvement compared to oxygen enriched air strategies. For the same value of *OTR*, the power input for oxygen enriched air cultivation was higher than the one applied under pressurized conditions, mainly due to the higher stirring speed that must be used. Lara et al. (2011) found that the *OTR* reached in the pressurized cultures up to 0.8 MPa was around 20% higher than the corresponding value of the oxygen enriched air cultivation for the same type of *E. coli* pCMV-S culture conditions.

2.3.2 Microbial growth and morphology

Besides cell age, size and shape of microbial cells depend on several factors, such as growth phase, medium composition, operating conditions, among others. Growth rate, mutation and environmental conditions affect yeast size and shape distribution but, in general, the influence of spatial variations in large-scale bioreactors is not considered. As a consequence, analysis of the pressure effects on cell physiology and morphology must be considered.

Though several works demonstrate that hyperbaric air can be successfully applied to microbial cultivation, as a way of improving the oxygen transfer rate to aerobic cultures (Belo et al. 2003; Lopes et al., 2008; Pinheiro et al. 2003), above certain limits the increased air pressure and the consequent increase in oxygen partial pressure may have detrimental effects on microbial cell activity.

For the range of pressure up to 1.5 MPa few investigations have been published regarding possible effects of pressure on microbial growth (Table 2.2). Results from these papers demonstrate that the effect of air pressure on microbial cultures is dependent of the microbial strain.

Table 2.2 Effects of increased air pressure on microbial growth.

Microorganism	P (MPa)	Effect	Reference
Streptomyces rimosus	Up to 0.8	Complete inhibition of cell growth	Liefke et al. (1990)
S. aureofaciens	Up to 0.8	Retardation of cell growth	Liefke et al. (1990)
Pseudomonas fluorescens	Up to 0.8	Complete inhibition of cell growth	Onken (1990)
E. coli TB1	Up to 0.4	No effects on cell mass production	Belo and Mota (1998)
Thermus sp. RQ-1	Up to 0.56	Improvement of cell productivity	Belo et al. (2000)
Kluyveromyces marxianus	Up to 0.6	Slight retardation of cell growth	Pinheiro et al. (2003)
S. cerevisiae	Up to 0.6	52% increase in cell growth rate	Coelho et al. (2004)
Pseudomonas putida CA-3	Up to 1.1	Detrimental effects on cell growth	Knoll et al. (2005)
S. cerevisiae	Up to 0.6	Increase of cell mass production	Belo et al. (2005)
S. cerevisiae	0.6 – 1.5	Inhibition of cell mass production	Belo et al. (2005)
Pichia pastoris	Up to 0.19	12% increase of cell mass production	Charoenrat et al. (2006)
E. coli K-12	6	Cellular growth stopped after 9 h	Matsui et al. (2006)
S. cerevisiae	Up to 1.5	Inhibition of cell mass production	Dong et al. (2007)
Adeninivorans G1211	Up to 0.5	No effects on cell mass production	Knoll et al. (2007)
C. glutamicum DM1730	Up to 1.0	No effects on cell mass production	Knoll et al. (2007)
Y. lipolytica	Up to 0.8	No effects on cell mass production	Lopes et al. (2008)
E. coli VH33	Up to 1.1	No effects on cell mass production	Knabben et al. (2010)
E. coli pCMV-S	Up to 0.8	No effects on cell mass production	Lara et al. (2011)

2.3.2.1 Bacteria

For *E. coli*, one of the most popular hosts, it is well known that oxygen availability affects cellular yield (Ko et al., 1993). This is particularly important because almost all of the recombinant proteins expressed in this microorganism remain inside the cell, in spite of all the efforts made in heterologous protein secretion research (Karim et al., 1993). Therefore, one of the goals of cultivation conditions optimization is to reach high cell densities and it is crucial to ensure an adequate oxygen supply to the media. Belo and Mota (1998) observed that, in batch cultures, pressure up to 0.4 MPa led to a slightly decrease on cell growth of *E. coli* TB1/pUC13. However, for fed-batch experiments, the *E. coli* TB1 cells grew better in the pressurized bioreactor than in the fermenter at atmospheric pressure and high stirring rate. Knabben et al. (2010) reported that genetically engineered *E. coli* strain VH33 in batch fermentations under pressurized conditions up to 1.1 MPa, fully aerobic conditions could be achieved, acetate accumulation could be prevented and a high cell-density culture was reached. Gregory and Fridovich (1973) observed that *E. coli* cells keep their reproduction ability at oxygen partial pressure up to 4.6 MPa. Lara et al. (2011) reported that the pressurization of *E. coli* pCMV-S cultivations up to 0.8 MPa lead to a similar biomass concentration than that obtained with the oxygen enriched air strategy. Matsui et al. (2006) cultured non-recombinant *E. coli* K-12 cells with pressurized air up to 6 MPa at a constant flow rate of 1 vvm and 3 vvm. The authors observed that the cellular growth stopped at 9 h and 10 h at 1 vvm and 3 vvm, respectively. However, their results indicate that the cellular growth was stopped by the inhibitory effect of increased p_{CO_2}, and the inhibition could be overcome by increasing the gas flow rate to release the dissolved CO_2 into gas phase.

In experiments with increased air pressure the growth of wild types of *S. rimosus* and *S. aureofaciens* were quite different. While the metabolic activities of *S. rimosus* were completely inhibited under 0.8 MPa of air pressure, the growth of *S. aureofaciens* was only retarded, prolonging the lag phase about threefold (Liefke et al., 1990).

Onken (1990) reported that in batch cultivations of *P. fluorescens*, bacterial growth was completely inhibited with air at 0.8 MPa total pressure. The same effect was observed with aeration by pure oxygen at 0.115 MPa. Carbon dioxide partial pressure did not show inhibitory effects.

Thermophilic microorganisms are important sources of thermostable enzymes and, despite their adaptation to low gas concentrations in natural habitat due to high temperatures, significantly high values of specific oxygen up-take rate have been reported for some aerobic strains (Cometta et al., 1982). Thus, the industrial cultivation of thermophiles in high cell density systems can present oxygen limitations. Belo et al. (2000) concluded that the raise of air pressure up to 0.56 MPa led to a 2.2-fold improvement in cell productivity on *Thermus* sp. RQ-1 batch cultivation and a reduction of liquid loss (Figure 2.1).

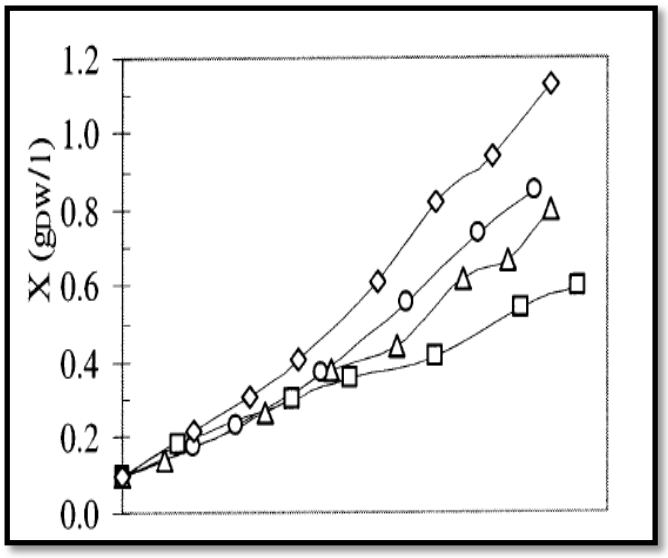

Figure 2.1 Time course of cell in batch cultivations of *Thermus* sp. RQ-1 in the pressurized reactor. The operating conditions used were a stirring rate of 200 rpm, an aeration rate of 0.3 L/min (at standard conditions) in 300 mL total volume and different air pressures: 0.10 MPa (□), 0.30 MPa (Δ), 0.43 MPa (○) and 0.56 MPa (◊) (adapted from Belo et al., 2000).

Puhar et al. (1983) related the effects of high partial pressures of O_2 and CO_2 on *Methylomonas clara* continuous cultures growing on methanol. Above a p_{O_2} of 0.07 MPa and a p_{CO_2} of 0.033 MPa the cell mass yield decreased with increasing $p_{O_2}$and p_{CO_2}. No cells washout was observed even when pure oxygen was used, but the cell mass yield was very low.

2.3.2.2 Yeasts

Over-provision of O_2 in the pre-fermentation aeration stage of brewing may have an adverse effect on yeast cells. Pinheiro et al. (1997) observed a 2.2-fold reduction in specific growth rate of *S. cerevisiae* in batch cultures at 0.6 MPa compared to the experiment under 0.12 MPa. Also, a 0.8 MPa pure O_2 pressure leads to nearly complete inhibition on yeast growth. However, Belo et al. (2003) reported that the gradual pressurization of the bioreactor up to 1.5 MPa increased specific cell growth rate and biomass yield of *S. cerevisiae* compared to the experiment at atmospheric pressure. The results obtained for the experiment with air at 1.5 MPa constant pressure demonstrated that this high value of air pressure imposed to the cells, without previous periods of adaptation, dramatically inhibited cellular activity. Campelo and Belo (2004) reported that the rising of air pressure from 0.1 MPa to 0.6 MPa stimulated cell growth but had no effect on leavening ability or viability of the cells.

The effect of pressure on *S. cerevisiae* cell activity strongly depends on the nature of the gas used for pressurization. While nitrogen and air to a maximum of 0.6 MPa of pressure were innocuous to yeast, oxygen and carbon dioxide pressure caused cell inactivation, with impact in cellular reproduction, as was shown by the reduction of budding cells percentage in the overall cell population. Moreover, a decrease in the average cell size was found for cells exposed for 7.5 h to 0.6 MPa CO_2 (Coelho et al., 2004). However, Belo et al. (2005) reported that no differences were found between the genealogical age of *S. cerevisiae* cell population under environments of air at 0.1 MPa, 0.6 MPa and 1 MPa of the cultures samples collected after 24 h of growth. The majority of the cells were young with no bud scars (daughter cells), or with only one bud scare. However, changes in the genealogical age profile were obtained for the final cultures exposed to 1.5 MPa of air pressure. 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. The raise of air pressure from 0.1 MPa to 1.5 MPa led to a decrease of the cell area (Figure 2.2), which can be attributed to the cell compression, since cells were not growing and there was an increase on the percentage of older cells that under normal conditions are bigger in size.

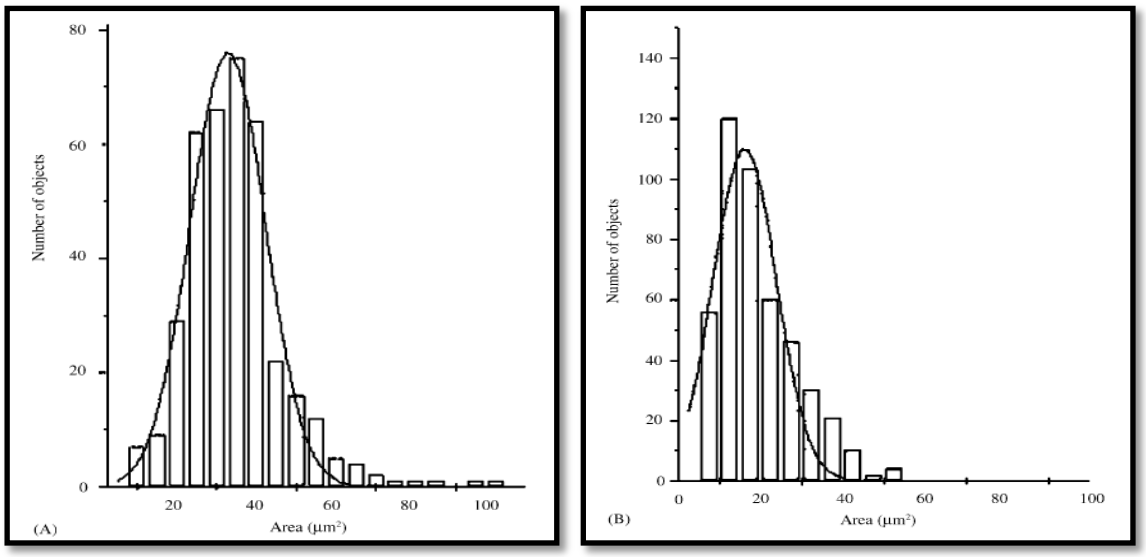

Figure 2.2 Histograms and Gaussian fits for the projected area of the final cells exposed to 0.1 MPa (A) and 1.5 MPa (B) of air pressure (adapted from Belo et al., 2005).

Coelho et al. (2007) concluded that the cell separation step (when the bud size is about 30% – 50% of the whole budding cell size) may be considered the limiting step in cell duplication. The influence of environmental conditions, specially the oxygen partial pressure, on the constant rate related to the beginning of the START event was related to the oxygen availability, giving a decrease in bud separation time and lower G_1 phase within the pressure raise. Under anaerobic conditions, no significant differences were verified, demonstrating that the nature of the gas is crucial for the yeast cell cycle development and not the total pressure itself. Dong et al. (2007) observed that the *S. cerevisiae* growth at higher pressure of 0.5 MPa, 1 MPa and 1.5 MPa were much slower than that under atmospheric pressure, resulting in a lesser biomass. Under atmospheric pressure, *S. cerevisiae* cells were round and smooth, but when cultured at 0.5 MPa, the cells became more flat and wrinkles showed up on the cell membrane. With the pressure increase up to 1.5 MPa, more disrupted cell membrane structure and cell deaths were observed (Figure 2.3).

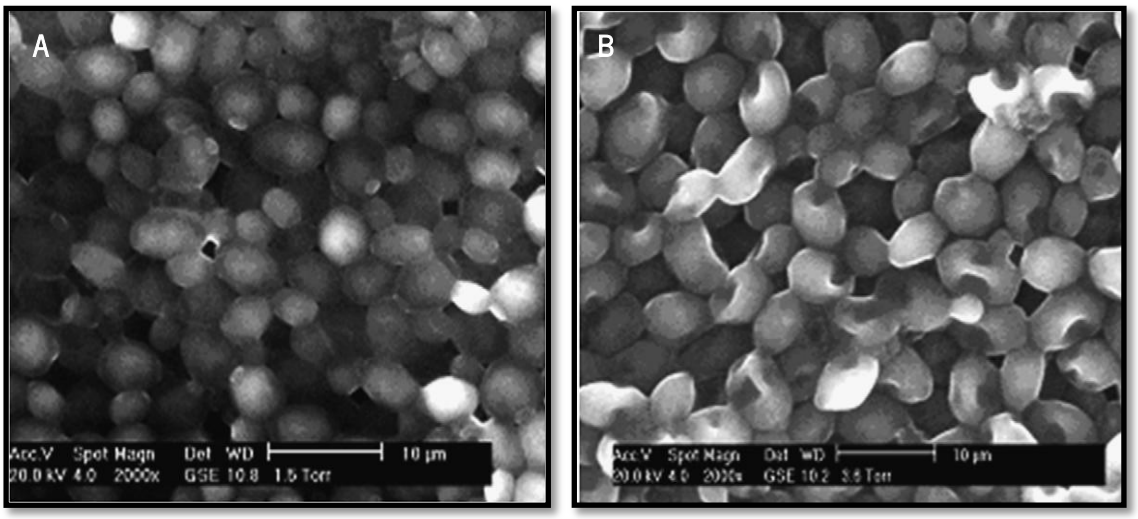

Figure 2.3 Scanning electron microscope photographs of yeast cells under different pressure: (A) 0.1 MPa and (B) 1.5 MPa (adapted from Dong et al., 2007).

In typical *Kluyveromyces* cultivation, quite high cell densities are reached and oxygen is usually the major growth limiting factor (Onken and Liefke, 1989). The use of increased air pressure up to 0.6 MPa has positive effect on the growth behavior of both *Kluyveromyces marxianus* strains, ATCC10022 ("Kluyver-negative") and CBS 7894 ("Kluyver-positive") and air pressure may be a way of eliminating oxygen limitation, leading to high biomass productivities. For the "Kluyver-negative" strain, with a lactose concentration of 20 g/L and an air pressure of 0.6 MPa, a 3-fold improvement in biomass yield was achieved compared with a micro-aerated culture. In turn, as pressure increased, ethanol production decreased 71%. With cultures of "Kluyver-positive" strain a 4-fold improvement in biomass productivity was observed in experiment under 0.6 MPa comparatively to the micro-aerated culture (Pinheiro et al., 2000).

The methylotrophic yeast *Pichia pastoris* is a common host for the production of recombinant proteins. Fermentation process usually occurs at very high cell density cultures, on the one hand and the use of the reduced energy source methanol demands high oxygen transfer rates. Charoenrat et al. (2006) investigated the effect of increased air pressure from 0.12 MPa to 0.19 MPa on *P. pastoris* Y-11430 cultivation and concluded that a 12% higher final cell mass was obtained under 0.19 MPa of air pressure. However, a 1.3-fold decrease on biomass yield was achieved in experiment with 0.19 MPa of air pressure comparatively to the trial under 0.12 MPa.

The raise of air pressure up to 0.8 MPa did not improve the cellular growth (Lopes et al., 2008). The cell exposure to increased air pressure did not induce hyphae formation. Cells remained oval under pressures up to 0.8 MPa with elongation factors below 2.0 for the majority of cells (> 85% of cells). A cell size decrease was found for the 0.8 MPa culture, since a decrease of the percentage of cells with a projected area higher than 100 μm^2 was obtained at 0.8 MPa (7%) compared with the 25% of cells with this size obtained at 0.4 MPa and at atmospheric pressure.

2.3.3 Products formation

The oxygen demand in high cell density cultivation exceeds by far the maximum oxygen transfer capacity of conventional bioreactors such as stirred tanks or bubble columns. Thus, the dissolved oxygen concentration in the culture medium limits the microbial growth and activity. In some processes, the reduction of growth and production rates was observed, thus affecting the productivity. There are also processes where oxygen limitation has a much stronger impact. For example, if the microorganisms are able to use anaerobic metabolism, the metabolic pathway can be shifted to an unfavorable outcome (Doelle et al., 1982; Futatsugi et al., 1993). This can result in the formation of by-products, thereby declining the product yield.

E. coli is capable of producing acetic acid from glucose, especially during periods of oxygen starvation. Yang and Wang (1992) observed that the incipient acetic acid production closely coincided with DO limitation, and clearly more acetic acid was produced at low pressures (0.106 and 0.136 MPa). Moreover, pressurization (up to 0.27 MPa) facilitated cells utilization of acetic acid as a secondary substrate after the exhaustion of the glucose. Knabben et al. (2010) also reported that by combining the genetically engineered *E. coli* strain VH33 with batch fermentations under pressurized conditions, acetate accumulation can be prevented without using additional control schemes or expensive supplementary equipment. Belo and Mota (1998) found that no differences on cytochrome b5 yield were found between experiments conducted at atmospheric pressure and 0.2 MPa. However, the raise of air pressure up to 0.4 MPa had a negative effect on the protein production. For *E. coli* TB1/pUC13 cells, a 4-fold increase in the cyt. b5 final productivity was achieved by an air pressure increase to 0.48 MPa as compared with an increase in the stirring rate to 500 rpm.

The plasmid DNA (pDNA) is produced by aerobic cultivation of *E. coli*, and to achieve high productivities, the typical strategy is to obtain high cell densities. However, the amount of obtainable biomass is often limited by the maximum oxygen mass transfer of the bioreactor. Lara et al. (2011) reported that the amount of pDNA vaccine produced with pressurization of *E. coli* pCMV-S culture up to 0.8 MPa was practically the same than the one obtained with oxygen enriched air strategy. Also, the final product yield and global productivities were similar for both strategies.

In the wild strain of *S. aureofaciens* the raise of air pressure up to 0.8 MPa increases the specific product formation and total yield, whereas in *S. rimosus* the specific product formation was negatively affected (Liefke et al., 1990).

β-galactosidase is used as an industrial enzyme in the dairy industry as it allows for the modification or the use of products containing lactose (Dickson and Martin, 1980). According to some authors (Barberis and Gentina, 1998; Garcia-Garbay et al., 1987) the expression of this enzyme is associated with the oxygen transfer rate in bioreactors, so it is important to establish well-defined and optimized conditions to culture medium oxygenation for yeast growth and β-galactosidase production. Pinheiro et al. (2003) concluded that it is possible to use air pressure up to 0.6 MPa, as an optimization parameter of this enzyme production in high-density cell cultures where oxygen is a limiting factor. The maximum specific activity was attained for the highest air pressure studied (0.6 MPa) and the highest rate of activity loss was observed at 0.12 MPa air pressure (Figure 2.4).

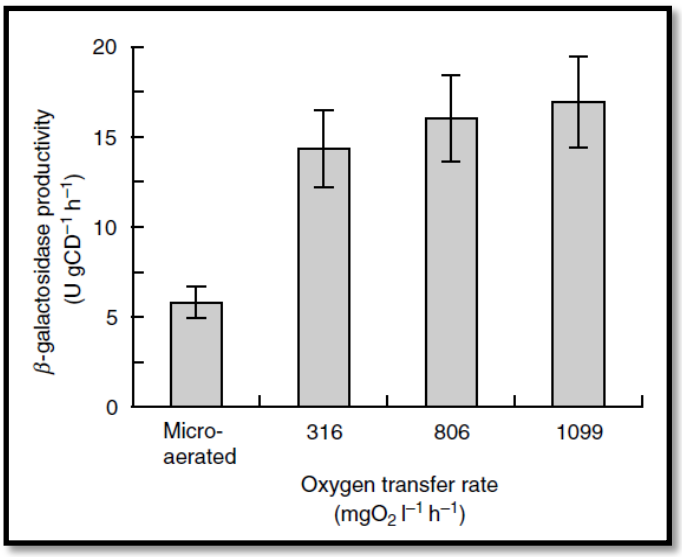

Figure 2.4 Effect of *OTR* on the specific β-galactosidase specific productivity in cultures of *Kluyveromyces marxianus* during batch growth. The sequential values of *OTR* corresponded to atmospheric pressure, 0.12 MPa, 0.4 MPa and 0.6 MPa (adapted from Pinheiro et al., 2003).

Charoenrat et al. (2006) concluded that the recombinant Thai Rosewood β-galactosidase yield reached the highest value with the pressure process (0.19 MPa), comparatively to reference process (0.12 MPa of air pressure). A 1.4-fold improvement on β-galactosidase activity was reached by increasing the total air pressure from 0.12 to 0.19 MPa.

The air pressure raise had different effects on protease and lipase secretion by the *Y. lipolytica* W29, which indicates that pressure can be an important factor of enzymes expression regulation and can be used as a control parameter for lipase production optimization. Lopes et al. (2008) observed that in the assay under 0.8 MPa a delay in the peak of lipase activity was observed, which indicates that the increase of pressure induces a phase of cellular adaptation and retards the enzyme expression; however, after this phase, the cells were able to produce more lipase. Results also show that 0.8 MPa of air pressure retards the production of protease, with a strong inhibiting effect on enzyme production, what could explain the increase in lipase productivity obtained for this pressure.

γ-Decalactone (4-hydroxydecanoate) is an aroma compound of industrial interest that can be produced biotechnologically by the strictly aerobic yeast *Y. lipolytica*. Cultures that were grown

under moderate pressure, i.e., under increased O_2 solubility, lead to a decrease on γ-decalactone production. However, by applying 0.5 MPa during growth and biotransformation yielded increased concentrations of other compounds such as dec-2-en-4-olide and dec-3-en-4-olide (Aguedo et al., 2005).

A survey of published data on effects of increased air pressure on aerobic cultures with product formation is given in Table 2.3. From these findings it is evident that elevated total air pressure may have distinct effects, depending on microorganism strain and biotechnological process.

Table 2.3 Influence of total air pressure on product formation.

Microorganism	Product	P (MPa)	Effect	Reference
E. coli	Cytochrome b5	Up to 0.4	Decrease on protein production	Belo et al. (1998)
E. coli	Acetate	1.1	Acetate accumulation can be prevented	Knabben et al. (2010)
E. coli	pDNA vaccine	Up to 0.8	No changes on pDNA topology and production	Lara et al. (2011)
S. aureofaciens	Antibiotics	Up to 0.8	Increase on specific product formation	Liefke et al. (1990)
S. rimosus	Antibiotics	Up to 0.8	Decrease on specific product formation	Liefke et al. (1990)
K. marxianus	β-galactosidase	Up to 0.6	Increase on enzyme specific activity	Pinheiro et al. (2003)
P. pastoris	β-galactosidase	Up to 0.19	Increase on enzyme specific activity	Charoenrat et al. (2006)
Y. lipolytica	γ-decalactone	Up to 0.5	Decrease on lactone production	Aguedo et al. (2005)
Y. lipolytica	Lipase	Up to 0.8	Increase on enzyme specific activity	Lopes et al. (2008)
Y. lipolytica	Protease	Up to 0.8	Decrease on enzyme specific activity	Lopes et al. (2008)

2.3.4 Antioxidant response

In industrial bioreactors, levels and gradients of total and partial pressures are considerably higher than in the laboratory scale. Thus, cells in bioreactors are often exposed to O_2 partial pressures higher than 0.21 MPa (corresponding to air at 0.1 MPa). In many cases, increased O_2 partial pressure (higher than approximately 0.1 MPa) 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, reactive oxygen species are generated.

Oxidative stress is caused by exposure to ROS, especially superoxide anions, hydrogen peroxide, and hydroxyl radicals, which can damage proteins by causing modifications of amino acid side chains, formation of crosslinks between proteins, and fragmentation of the polypeptide backbone. In addition, ROS can modify bases and sugars in DNA, leading to DNA chain breaks and causing lipid peroxidation in cell membranes. To protect against the damage caused by oxidative stress, cells possess a number of biochemical systems, including enzymes (superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase) as well as the non-enzymatic protective molecules (glutathione and thioredoxin), most of which are expressed at low levels during normal growth. In response to elevated concentrations of ROS, the expression of many antioxidant defenses is induced.

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH) acts as a radical scavenger with the redox-active sulphydryl group reacting with oxidants to produce reduced glutathione (GSSG) (Marchler et al., 1993). Thioredoxin is a small sulphydryl-rich protein which can be used as a reductant for thioredoxin peroxidase and for ribonucleotide reductase (Muller, 1996).

Superoxide dismutase (SOD) is a metalloenzyme that detoxify superoxide radicals by conversion to hydrogen peroxide and oxygen.

Catalases are ubiquitous enzymes, which protect aerobic organisms from the toxic effects of H_2O_2 by catalyzing the conversion of H_2O_2 to molecular O_2 and H_2O (Angelova et al., 2005).

The enzyme glutathione reductase is primarily responsible for the reduction of oxidized glutathione and maintenance of the GSH/GSSG ratio in cells (Grant et al., 1996) and glutathione peroxidase catalyzes the reduction of hydroperoxides, using GSH as a reductant (Galiazzo et al., 1987).

For *E. coli* it was reported that cells overproducing SOD were more sensitive to generators of superoxide anions (Schellhorn and Hassan, 1988). Belo and Mota (1998) observed that the antioxidant enzyme SOD was slightly induced at the end of fed-batch culture under 0.48 MPa of air pressure. Induction of SOD by pressurized oxygen was also reported by Taniguchi et al. (1992) for *Streptococcus lactis* cells. However, it was reported that catalase is not very important in defending the *E. coli* cells against oxidative stress, but a raise in oxygen tension particularly induced the manganese form of SOD (Gregory and Fridovich, 1973).

During the cultivation of *Thermus* sp. RQ-1 in the pressurized reactor, an induction of the antioxidant enzymes SOD and catalase were observed, mainly at the beginning of the exponential growth phase. A pressure raise of 0.4 MPa led to a 4-fold increase of SOD activity. Despite the observed induction of catalase by pressure, the activity values of this enzyme were very low for *Thermus* sp. RQ-1 cells. This probably indicates that there are other peroxidases more important to this strain for the elimination of H_2O_2 (Belo et al., 2000).

During fed-batch of *S. cerevisiae*, Belo et al. (2005) observed that catalase and MnSOD were induced by hyperbaric air to a maximum of 1.0 MPa and 0.13 MPa of pure O_2 pressure, but no statistically significant changes were observed for CuZnSOD activity. Similar results have been obtained by Pinheiro et al. (1997). The authors reported that above 0.3 MPa activities of mitochondrial superoxide dismutase and glutathione reductase increased with air pressure, but cytosolic superoxide dismutase and catalase increased activity only in pure oxygen. Dong et al. (2007) reported that incubation of *S. cerevisiae* cells under air pressure of 0.5, 1.0 and 1.5 MPa for 2 h caused obvious increase in the contents of GSH. At 1.0 MPa, the intracellular concentration of GSH reached the maximum value and it showed a 27% increases comparatively to the obtained under atmospheric pressure. Also, the use of air pressure up to 1.0 MPa stimulated *S. cerevisiae* cells to improve the trehalose synthase activity and increased intracellular concentrations of trehalose (Figure 2.5). Lee and Hassan (1987) studied the influence of increased oxygen partial pressure on chemostat cultures and observed that the exposure of the cells to 100% O_2 induced superoxide dismutase and repressed formation of catalase.

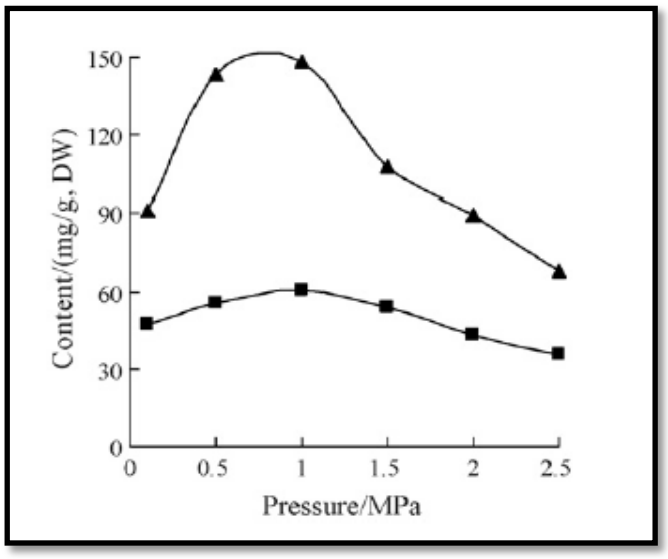

Figure 2.5 Changes of trehalose and GSH contents with pressure: (▲) trehalose; (■) GSH (adapted from Dong et al., 2007).

The ability of the strain *K. marxianus* to respond to the increase of ROS formation because of hyperoxygenation was demonstrated by Pinheiro et al. (2003). The total SOD activity had a slight increase from 36.6 to 65.1 U/mg protein, after 24 h of cell exposure to 0.12 and 0.6 MPa air pressure respectively. Also Pinheiro et al. (2000) observed that superoxide dismutase, catalase and glutathione reductase were at high activity levels for an air pressure of 0.4 MPa, suggesting that *K. marxianus* could tolerate the increased in oxygen partial pressure. The authors also reported that when MnSOD was at a low activity level, catalase and glutathione reductase were at high activity levels. On the contrary, when CuZnSOD was induced at a high level, the other enzymatic activities decreased.

2.4 CONCLUSIONS

Pressure has a wide range of effects on biological systems, and as an environmental parameter, may influence the cellular physiology and intracellular structures.

In biotechnology, the increased air pressure has been applied in microbial cultures, as a way of *OTR* improvement. Depending on microbial strain the increased air pressure can cause several effects including inhibition of growth, decrease in product formation and lower cell yield. In some

cases, however, increased pressure may improve process efficiency, i.e. enhancement of biomass and product yields, particularly in strictly aerobic strains.

The ability of the microorganisms to respond to the increase of ROS formation by induction of antioxidant defense, because of hyperoxygenation, was demonstrated herein. Thus, the bioreactor pressurization may also be used as a way of inducing high activity levels of antioxidant enzymes, which might have potential applications on dairy and pharmaceutical industry.

3 OXYGEN MASS TRANSFER RATE IN PRESSURIZED LAB-SCALE STIRRED BIOREACTOR

Oxygen mass transfer from air to the liquid phase in bioreactors with aerobic cultures has long been a serious impairment to the productivity of various bioprocesses. Increase of oxygen mass transfer rate (*OTR*) can be the key to overcome oxygen limitation. In this work, the influence of increased air pressure up to 5 bar on *OTR* was measured and correlated. A 7.1-fold *OTR* improvement was obtained by the total air pressure increase from 1 bar to 5 bar. The oxygen volumetric mass transfer coefficient (k_La) was described by a function of the air pressure in a stirred lab-scale pressurized bioreactor. The correlation obtained showed that k_La slightly decreased with the raising in air pressure, following a power function.

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Lopes M., Mota M., Belo I. Study of oxygen mass transfer rate in pressurized lab-scale stirred bioreactor (January 2013).

3.1 INTRODUCTION

In aerobic bioprocesses, oxygen is a key substrate; due to its low solubility in aqueous solutions, it is important to ensure an adequate delivery of oxygen from a gas stream to the culture broths. The oxygen mass transfer rate (*OTR*) and volumetric oxygen mass transfer coefficient (k_La) must be known, and if possible predicted to achieve an optimum design operation and scale-up of bioreactors. *OTR* and k_La are influenced by a high number of parameters (physical properties of gas and liquid, operating conditions, geometrical parameters of the bioreactor) and also by the presence of biomass, that is, the consumption of oxygen by the cells (Garcia-Ochoa and Gomez, 2009; Suresh et al., 2009). Both parameters can be related and stated mathematically as:

$$OTR = k_L a\,(C^* - C) \tag{3.1}$$

where C^* is the solubility of oxygen in the liquid, C is the dissolved oxygen concentration in the liquid and k_La is made up of the mass transfer coefficient (k_L) and the interfacial area (a). In this equation the term $(C^* - C)$ is considered to be the driving force which causes oxygen to transfer from the gas phase to the liquid phase (Sinclair, 1984).

Several empirical correlations have been proposed to estimate the k_La in mechanical agitated bioreactors (STR), being the most well-known the following function:

$$k_L a = \alpha \left(\frac{P_g}{V}\right)^{\delta} (v_s)^{\gamma} \tag{3.2}$$

where P_g represents the power input to the aerated bioreactor, V is the bioreactor working volume and v_s is the superficial gas velocity. The parameters α, δ and γ are dimensionless constants.

To calculate the power input to the aerated system (P_g), the Reynolds number (N_{Re}) is determined by equation 3.3 and power number (N_p) by equation 3.4.

$$N_{Re} = \frac{D_i^2 \times N \times \rho}{v} \tag{3.3}$$

$$N_p = \frac{P_g}{\rho \times N^3 \times D_i^5} \tag{3.4}$$

where ρ represents the liquid density, N the agitation rate, υ the liquid viscosity and D the impeller diameter.

According to Cheremisinoff and Gupta (1983), if the flow regime inside the system is turbulent ($19070 < N_{Re} < 38141$), N_p is not a function of N_{Re} when the vessel is fully baffled. Consequently, P_g without aeration (P_g') can be determined by equation 3.5.

$$P_g' = K_T \times D_i^5 \times N^3 \times \rho \tag{3.5}$$

where K_T is a constant dependent on the impeller used.

Finally, to determine P_g in an aerated system, equation 3.6 can be used.

$$P_g = c \times \left(\frac{P_g' \times N \times D_i^3}{F_g^{0.56}} \right)^{0.45} \tag{3.6}$$

where c is a constant dependent on the impeller and F_g is the volumetric gas flow rate.

In order to overcome the oxygen limitation in aerobic microbial cultures, selection of adequate, normally high, *OTR* values is crucial. Special aeration systems, e.g. aeration using oxygen enriched air and increased reactor pressure are techniques applied to increase oxygen availability (Belo et al., 2003; Knoll et al., 2005; Maier et al., 2004). Also, the use of *in situ* production of oxygen (Sonnleitner and Hahnemann, 1994) or the use of a second liquid phase of various organic compounds such as perfluorodecalin (Amaral et al., 2008) or n-hexadecan (Nielsen et al., 2003) in the culture medium can increase the availability of oxygen to the microorganisms.

A number of methods have been developed to determine the oxygen transfer rate in bioreactors. The techniques vary according to the accuracy required and have advantages and disadvantages depending on the availability of the necessary analytical instruments and material and labor costs (Novak and Klekner, 1988). In the absence of microbial cells the *OTR* can be estimated by the oxygen absorption rate of a sodium sulfite solution (Cooper et al., 1944). This technique is based on the reaction of sodium sulfite, a reducing agent, with the dissolved oxygen to produce sodium sulfate, in the presence of a catalyst (usually a divalent cation of Cu^{2+} or Co^{2+}); the reaction can be expressed as:

$$Na_2SO_3 + \frac{1}{2}O_2 \xrightarrow{Cu^{2+}/Co^{2+}} Na_2SO_4 \quad (3.7)$$

The reaction rate is much faster than the oxygen transfer rate and there is a concentration range of sodium sulfite (from 0.04 M to 1 M) for which the oxygen concentration can be assumed as zero. According to the stoichiometry (equation 3.7), the *OTR* is half of the variation in time of the molar concentration of aqueous sulfite. Therefore, the oxidation rate is controlled by the rate of mass transfer, and measures the overall rate. Thus, knowing *OTR* and oxygen solubility, the volumetric oxygen mass transfer can be determined by the equation 3.8:

$$OTR = k_L a\, C^* \quad (3.8)$$

Some authors have demonstrated the applicability of pressurized bioreactors in microbial cultures, with enhancements in biomass and product yields (Knoll et al., 2007; Pinheiro et al., 2003). Since these improvements could be related with enhancement in oxygen mass transfer due to the increase of oxygen solubility with pressure, it seems important to describe *OTR* and k_La in such bioreactors. Thus, experimental values of *OTR* were obtained in a laboratory-scale pressurized bioreactor, by varying the air pressure, the aeration and the stirring rates. Based on equation 3.2, data fitting to an empirical correlation for the prediction of the k_La as a function of air pressure, power input of the aerated bioreactor and superficial gas velocity was attempted.

3.2 MATERIALS AND METHODS

A 600 mL stainless stirred tank bioreactor (PARR 4563, Parr Instruments, USA) with 400 mL of operating volume was used (Figure 3.1). The bioreactor vessel is a cylinder of 0.063 m diameter and with a ratio of 3 between height and diameter. The bioreactor is equipped with an impeller with two turbines of four pitched blades (0.035 m of diameter), a temperature probe and a sparger tube for aeration. The gas flow rate was measured with calibrated mass flow controller (Alicat scientific, Model MC-5SLPM-D).The parameters studied were stirring rate (200 rpm, 400 rpm and 600 rpm), aeration rate (0.5 vvm, 1 vvm and 2 vvm, measured under standard temperature and pressure conditions) and total air pressure inside the bioreactor (from 1 bar to 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 the total internal pressure.

Figure 3.1 Stainless stirred bioreactor (PARR 4563, Parr Instruments, USA).

3.2.1 Oxygen transfer rate (*OTR*)

OTR in bioreactors operating under different conditions was estimated in blank assays using the sulfite oxidation method (Cooper et al., 1944) at 30 °C. A sodium sulfite solution (0.2 M) is oxidized to sodium sulfate in the presence of a catalyst ($CuCl_2$ 0.001 M). At regular times samples of known volumes were collected and mixed with an excess of iodine solution (0.05 M). The amount of iodine that not reacted with sulfite ion was determined by measuring absorbance at 595 nm and converted to molar concentration using a previous calibration. The amount of residual sulfite can be estimated by:

$$[SO_3^{2-}] = \frac{0.05V_I - (V_I + V_s)\,[I_2]}{V_a} \tag{3.9}$$

where V_s is the sample volume, V_I is the iodine solution volume and $[I_2]$ is the molar concentration of iodine solution.

As this method measures the rate of O_2 absorption by a Na_2SO_3 solution, it enables to predict the effect of pressure increase on the oxygen mass transfer capacity of the system.

3.2.2 *k*L*a* modelling

A correction of equation 3.2 was made, in order to predict the effect of absolute pressure, *P*, in k_La:

$$k_L a = \alpha \left(\frac{P_g}{V}\right)^{\delta} (v_s)^{\gamma}\ P^{\beta} \tag{3.10}$$

k_La values were obtained dividing the experimental *OTR* data by the oxygen solubility at 30 °C.

P_g and v_s in equation 3.10 were calculated with the help of the equations shown in the introduction, converting aeration rate to real F_g. Air flow rate inside the reactor was corrected from the measured at standard conditions to the values of temperature and pressure of the assay using the ideal state gas equation.

In order to predict the effect of air pressure in the oxygen solubility, it was used the Krichevsky-Kasarnovsky equation (Prausnitz et al., 1986). The result proved that the Henry´s law was still valid for the air pressure range used in this work (up to 5 bar).

The dimensionless parameters α, δ, β and γ were estimated by minimizing the sum of least squares of the difference between the experimental and modeled value of k_La, using the Solver tool of Microsoft Excel 2010 software.

3.3 RESULTS AND DISCUSSION

3.3.1 *OTR* measurement

To evaluate the effect of total air pressure on *OTR* values, several experiments were carried out, changing stirring and aeration rates under increased air pressure up to 5 bar (Figure 3.2). From the analysis of each factor separately it was observed that the increment of aeration and stirring rates and total air pressure inside the bioreactor led to an enhancement in *OTR* value. At higher gas flow rates, gas holdup in the bioreactor increases, leading to higher surface area of bubbles, which in turn increases the k_La values. It may be said that the change in gas flow rate affects the fractional gas holdup, and hence, affects *a* and consequently k_La values (Belo et al., 2011). Thus, according to the equation 3.8, *OTR* value also increases. *OTR* improvement was more pronounced with the increment of the stirring rate or the raise of air pressure than with the increase of aeration rate. At 1 bar of air pressure, a 4.3-fold improvement was achieved with the increase of air flow rate up to 2 vvm. In turn, varying the stirring rate from 200 rpm to 600 rpm, at 0.5 vvm of aeration rate, the *OTR* value augments 10 times. This lower improvement in *OTR* with increment of aeration rate as compared to the agitation rate was similar for all values of pressure. For all the conditions studied the increase in agitation proved to be more efficient in the *OTR* enhancement than the increase in aeration. This behavior is in agreement with the results of Amaral et al. (2008), Chen et al. (1999), Gomes et al. (2007), Gómez-Díaz and Navaza (2003) and Juárez and Orejas (2001) that showed that k_La depends more strongly on agitation than on aeration rates for STR under atmospheric pressure conditions.

Impeller speed is the major factor that affects k_La values of a stirred-tank bioreactor as it determines the overall power dissipation for any specific impeller design. This effect was attributed to the rapid breakage of the gas bubbles into smaller sizes with an increase in the impeller speed, and thus, enhancement in the gas–liquid interfacial area for mass transfer (Suresh et al., 2009). Raising the stirring rate from 200 rpm to 600 rpm led to an improvement in *OTR* value more significant at 1 bar of air pressure and 0.5 vvm of aeration. A 10-fold improvement in *OTR* was observed by changing the stirring rate from 200 rpm to 600 rpm, at 1 bar and 0.5 vvm. Comparatively, only 4- and 3-fold improvement were found at 1 bar and 2 vvm and with 5 bar and 2 vvm, respectively. It seems that the stirring rate effect is more significant at low k_La values.

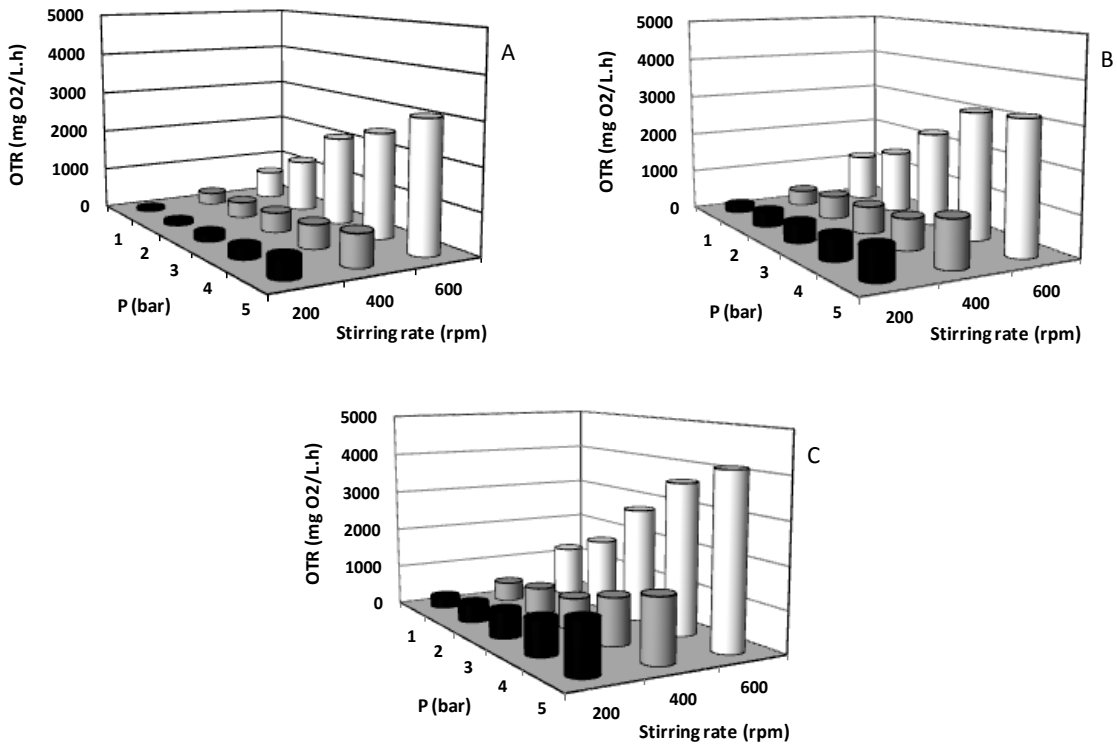

Figure 3.2 Experimental *OTR* values at various experimental conditions and aeration rate of (A) 0.5 vvm, (B) 1 vvm and (C) 2 vvm.

Independently of the stirring and aeration rates tested, the increased air pressure from 1 bar to 5 bar led to an improvement in *OTR* values. This enhancement was even more pronounced at lower stirring and aeration rates. A 7.1-fold improvement in *OTR* value at 0.5 vvm and 200 rpm was achieved when air pressure varied from 1 bar to 5 bar, whereas at 2 vvm and 600 rpm the *OTR* at 5 bar was 4 times higher than at 1 bar. In the experiment conducted at 2 vvm of aeration rate, the same improvement in oxygen mass transfer (4.3-fold) was achieved with the raise of air pressure from 1 bar to 5 bar and with the increase of stirring rate from 200 rpm to 600 rpm. This result proves that the increased air pressure is an alternative to stirring rate increase which is particularly important for high cell density cultures and when the cells are sensitive to shear stress that limits the increase of stirring. Belo and Mota (1998) observed that the *E. coli* TB1 cells showed to be more sensitive to high shear stress caused by stirring than to air total pressure up to 4 bar.

The oxygen transfer rate raise promoted by the increased air pressure inside the bioreactor is based on the fact that the equilibrium oxygen solubility in the nutrient broth increases linearly with

the total air pressure according to Henry's law. Although oxygen-enriched air can achieve the same result, it is costly and requires special handling (Yang and Wang, 1992).

Other authors have reported the enhancement in *OTR* values due to the increased air pressure inside the bioreactor, using different bioreactors and conditions. Yang and Wang (1992) found a 2.5-fold increase in *OTR* by the use of air pressure from 1.06 bar to 2.72 bar. Knoll et al. (2005) observed that the oxygen transfer capacity, the energy efficiency, and the cost efficiency of oxygen transfer can be greatly enhanced by employing elevated reactor pressures up to 11 bar.

3.3.2 k_La modelling

The determination of k_La in bioreactors is essential to establish aeration efficiency and to quantify the effects of the operating variables on the provision of oxygen. In order to predict bioreactor performance when using models that account for the effect of the increased air pressure, an empirical correlation (equation 3.10) for the k_La in a pressurized bioreactor was proposed. Using the experimental data obtained in experiments with increased air pressure up to 5 bar, ranging aeration rate from 0.5 vvm to 2 vvm and stirring rates from 200 rpm to 600 rpm, the values of α, δ, β and γ coefficients from equation 3.10 were estimated as shown on equation 3.11:

$$k_La = 535\left(\frac{P_g}{V}\right)^{0.70}(v_s)^{0.48}\ P^{-0.13} \qquad (3.11)$$

From equation 3.11 it can be observed that the measured k_La increases according to the specific power input, $\left(\frac{P_g}{V}\right)$, to the power of 0.70, to the superficial gas velocity, v_s, to the power of 0.48 and decreases with the air pressure, *P*, to the power of (- 0.13). The values of coefficients show that the k_La dependence was higher on specific power input than superficial gas velocity, once the coefficient of v_s was lower than the coefficient of $\left(\frac{P_g}{V}\right)$. The raise of total air pressure had a small negative effect on k_La, as demonstrated by the coefficient of *P*. This means that the increase of air pressure slightly decreases the volumetric mass transfer coefficient. Belo et al. (2000) also reported that raising oxygen solubility through the increase in total air pressure enhanced *OTR* in the pressurized bioreactor and decreased k_La, this effect was attributed by the use of constant gas

flow rate (at standard conditions) that in fact led to a decrease of true gas flow rate inside de bioreactor with pressure. The global effect of pressure in k_La is a balance between the positive effect of the air bubble compression, thus the increase of interfacial specific area for mass transfer, and the negative effect on gas hold-up decrease. Knoll et al. (2005) found similar values for coefficients of $\left(\frac{Pg}{V}\right)$ and v_s, respectively 0.74 and 0.42. However, the authors did not take into account the air pressure in k_La mathematical correlation.

Baldwin et al. (2000) reported that k_La appears to increase with superficial gas velocity at low gas sparging rates and decrease at higher values. Yang and Wang (1992) observed that the bioreactor pressurization up to 2.72 bar had little effect on k_La. Maier et al. (2001) have shown that the k_La values in a stirred tank reactor remain constant irrespective of the reactor pressure if the superficial gas velocity is kept constant.

In Figure 3.3, predicted *versus* experimental k_La values are shown with a deviation of 4% of the unitary slope, e.g., $k_La_{\text{predicted}} = 0.96\ k_La_{\text{experimental}}$ which indicates a good approximation between real k_La values and the values calculated by the correlation, despite the dispersion of the values (R^2 = 0.911) particularly for low k_La values, as was obtained in the experiments conducted at 200 rpm of stirring rate.

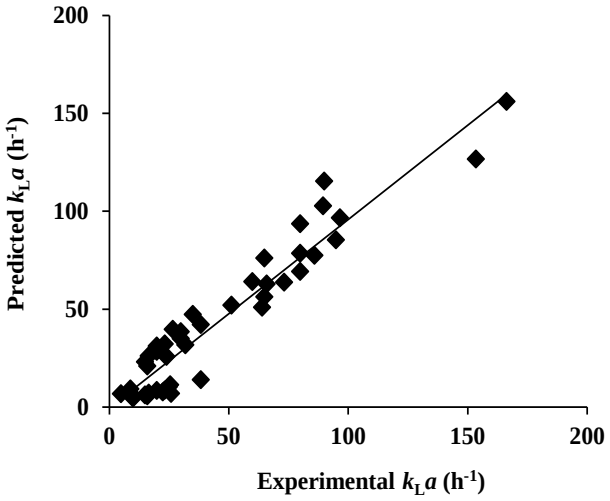

Figure 3.3 Correlation between the experimental and predicted k_La values using equation 3.11 with estimated parameters for increased air pressure up to 5 bar, stirring rates from 200 rpm to 600 rpm and aeration rates from 0.5 vvm to 2 vvm.

3.4 CONCLUSIONS

Mass transfer between gas and liquid phases in stirred-tank reactors is a very important process in the chemical and biochemical industry. Thus, the optimization of the bioreactor performance in what concerns the oxygen mass transfer requirement is a crucial task in industrial bioprocesses. In this work, the effects of increased air pressure on the oxygen transfer rate were investigated. The use of increased air pressure up to 5 bar proved to be a successful means to improve *OTR*. This means that pressure can be applied as an alternative to avoid the shear stress caused by the increased stirring rates that might be harmful to cells.An empirical correlation to predict k_La value as a function of pressure, power input and superficial gas velocity was established. It was demonstrated that the k_La increase was higher with the raise of specific power input than with the superficial gas velocity and that the increase total air pressure had a small negative effect on k_La. The correlation for k_La prediction, herein proposed, could be very useful for further work on the development of strategies for the optimization and scale-up of the processes where oxygen transfer is a limiting factor and air pressure increase could be used to prevent it.

4 COMPARISON OF *YARROWIA LIPOLYTICA* AND *PICHIA PASTORIS* CELLULAR RESPONSE TO AGENTS OF OXIDATIVE STRESS

Yeast cells exposed to adverse conditions employ a number of defense mechanisms in order to respond effectively to the stress effects of reactive oxygen species. In this work, the cellular response of *Yarrowia lipolytica* and *Pichia pastoris* to the exposure to the ROS-inducing agents paraquat (PQ), hydrogen peroxide (H_2O_2) and increased air pressure was analyzed. Yeast cells at exponential phase were exposed for 3 h to 1 mM paraquat, to 50 mM H_2O_2, or to increased air pressure of 3 bar or 5 bar. For both strains the cellular viability loss and lipid peroxidation was lower for the cells exposed to increased air pressure than for those exposed to chemical oxidants. The glutathione induction occurred only in *Y. lipolytica* strain and reached the highest level as a response to PQ exposure. In general, antioxidant enzymes where more expressed in *Y. lipolytica* than in *P. pastoris*. The enzyme superoxide dismutase was induced in both strains under all the oxidant conditions but was depend of the cellular growth phase, being undetectable in non-growing cells, whereas glutathione reductase was more induced in those conditions. Hydrogen peroxide was the most efficient inducer of catalase. Both yeast cultures underwent no cellular growth inhibition with increased air pressure, indicating that these yeast species were able to adapt to the oxidative stressful environment.

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Lopes M., Mota M., Belo I. Comparison of *Yarrowia lipolytica* and *Pichia pastoris* cellular response to different agents of oxidative stress (November 2012).

4.1 INTRODUCTION

All aerobic organisms use molecular oxygen for respiration and oxidation of nutrients to obtain energy efficiently. During the reduction of molecular oxygen to water through acceptance of four electrons, aerobic microorganisms have to face the toxic effects of oxygen, once active oxygen species such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) are generated. These reactive oxygen species (ROS) are highly noxious to all biological molecules, including DNA, proteins and lipids (Esterbauer et al., 1991).

The raise of total air pressure leads to an increase of oxygen partial pressure, which generates ROS (Giller and Sigler, 1995). Pro-oxidants compounds in the culture medium, such as paraquat and hydrogen peroxide have also the capacity to generate intracellular ROS.

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) is a quaternary nitrogen herbicide and in the presence of a sufficient supply of reducing equivalents, repeated cycles of herbicide reduction and re-oxidation can occur, producing large amounts of reactive oxygen species, oxidative stress, and lipid peroxidation (Joaquim et al., 2001).

The addition of exogenous hydrogen peroxide to microbial cultures may result in oxidative stress. The adaptation to this radical requires protein synthesis and the expression of at least 21 proteins increased following H_2O_2 adaptation (Davies et al., 1995).

The inadequate mixing and mass transfer that cells might face in an industrial submerged culture process may expose yeast cells to variations in dissolved oxygen, including transient exposure to regions of high dissolved oxygen (DO), leading to oxidative stress. Despite the industrial significance, few studies were performed to simulate the impact on microbial cells of oxidative stress caused by the exposure to increased oxygen partial pressure and high dissolved oxygen concentration (Belo et al., 2005; Pinheiro et al., 2003).

Cells possess several defensive enzymatic (such as superoxide dismutase, catalase and glutathione reductase) and non-enzymatic (such as glutathione) mechanisms to protect their cellular constituents and maintain cellular redox state.

Y. lipolytica, a non-conventional yeast, is most used in studies of the biodegradative pathways for a variety of hydrophobic compounds (Coelho et al., 2010) and the methylotrophic *P. pastoris* can

grow to high cell density and has the potential for high level expression of recombinant proteins (Wei et al., 2008). To learn more about the response of these yeast strains to oxidative stress, the effect of different ROS-generating agents on cell viability and on the induction of antioxidant enzymes were studied. The content of MDA and GSH and the induction of antioxidant enzymes (SOD, catalase, GR) in response to H_2O_2, PQ and increased air pressure were investigated. To our knowledge this is the first study concerning the interaction between these yeast strains and the pro-oxidant agents.

4.2 MATERIALS AND METHODS

4.2.1 Strains and media

Y. lipolytica W29 (ATCC 20460) and *P. pastoris* CBS 2612 were grown in YPD (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) medium. The yeast strains were maintained in YPD agar plates and stored at 4 °C to a maximum of 1 month.

4.2.2 Oxidants treatment

Yeasts cells were pre-cultured in 250 mL Erlenmeyer flasks filled with 100 mL of YPD medium till the beginning of exponential phase. Cells were then harvested by centrifugation, washed with PBS buffer and ressuspended in 400 mL of PBS buffer or YPD medium. In the experiments with chemical oxidants, PQ and H_2O_2 were added at non-lethal final concentration of 1 mM and 50 mM, respectively. The exposure to increased air pressure was performed in a 600 mL pressurized bioreactor (PARR 4563, Parr Instruments, USA) under 3 bar or 5 bar of air pressure at 400 rpm and 1 vvm of aeration.

4.2.3 Batch growth

Yeasts cells were pre-grown overnight in 250 mL Erlenmeyer flasks with 100 mL of YPD at 140 rpm and at 27 °C (*Y. lipolytica*) or 30 °C (*P. pastoris*). Batch cultivations were carried out in the

pressurized bioreactor, with 400 mL of each culture, at 27 °C or 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 bar, 3 bar, and 5 bar.

4.2.4 Analytical methods

Yeasts samples were collected after 3 h of exposure to oxidants for analysis of cell viability, MDA and GSH content and antioxidant enzymes activity. Cell viability was estimated by the Methylene Blue staining method (Jones, 1987). Antioxidant enzymes were measured after cell disruption and dialysis of cell extracts. Cells were disrupted by mechanical treatment with 0.5-mm glass beads during 6 min of vortex mixing (1 min bursts with 1 min cooling intervals). Whole cells and debris were removed by centrifugation at 5000 *g* for 15 min at 4°C. The clear supernatant was dialyzed overnight (Pinheiro et al., 2000). Catalase was assayed using the method described by Beers and Sizer (1952), SOD was quantified by the method of Marklund and Marklund (1974) and glutathione reductase was analyzed according to the procedure described by Smith et al. (1988). MDA was measured by the method of TBARS (thiobarbituric acid reactive species) as described by Espindola et al. (2003). GSH was quantified in the neutralized extracts using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) reagent according to the procedure described by Jamnik and Raspor (2003).

Samples from the batch cultures in the pressurized bioreactor were collected for analysis of cell concentration (optical density at 600 nm and converted to dry cell weight per liter) and glucose consumption. Glucose was 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 M at 0.5 mL/min and the column (Chrompack, Brasil) temperature was 60 °C.

4.3 RESULTS AND DISCUSSION

4.3.1 Cell viability

The effect of three different oxidative stress inductors, PQ, H_2O_2 and hyperbaric air, on yeast cell viability was tested. The experiments were conducted with cells at the exponential phase since

these cells are metabolically more active than stationary phase cells and were likely to be more susceptible to the possible lethality of aerobic stressors (Hassan and Fridovich, 1978).

The presence of a carbon source allowed the cells to grow during the oxidant exposure, and led to more active and viable cells. In the experiments with YPD medium there was no significant difference on the cellular viability between strains, independently of the stressors (Table 4.1). However, the exposure to oxidants agents in PBS buffer demonstrated that the *P. pastoris* cells were more susceptible than the *Y. lipolytica*. Hassan and Fridovich (1978) also observed that the *E. coli* cells were strikingly less sensitive to PQ in complex medium than in minimal medium.

Table 4.1 Cell viability (%), defined as the ratio of final and initial viable cells number, of two yeasts strains in exponential phase of growth, ressuspended in PBS buffer and YPD medium, exposed to different oxidants for 3 h. Values are average ± standard deviation of three independent experiments.

	PBS buffer				YPD medium			
	H_2O_2	PQ	3 bar	6 bar	H_2O_2	PQ	3 bar	6 bar
Y. lipolytica W29	87±11	90±10	95±12	95±13	92±15	94±13	97±14	96±15
P. pastoris CBS 2612	58±8	64±10	78±12	77±12	90±14	93±15	97±14	96±14

When cells of *Y. lipolytica* were exposed to H_2O_2 and PQ in PBS buffer respectively 87% and 90% of the cells survived, which shows that the strain is quite resistant to these oxidant agents. The exposure to hyperbaric air leads to a minor decrease in cell viability, indicating that the oxidative stress imposed by the partial oxygen pressure is less deleterious than the other stressors. The *P. pastoris* cells were more susceptible to the oxidative stress than the other strain, mainly in PBS buffer. When *Pichia* cells were subjected to the PQ and H_2O_2 treatment about 36% and 42% were killed, respectively. In turn, there was 78% and 77% of viable cells in treatments under 3 bar and 6 bar of air, respectively. Pinheiro et al. (2002) reported that *K. marxianus* cells respond better to the PQ than to the H_2O_2 exposure during 24 h of growth. Also, cell viability was higher for the cells growing under increase air pressure up to 6 bar than for those cells exposed to chemical oxidants.

Data suggest that different organisms respond differently to ROS and that they use different defense mechanisms against those substances.

4.3.2 Antioxidant markers

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA), a natural by-product of lipid peroxidation.

Regardless of the yeast strain, the exposure to oxidant agents leads to a MDA production. This production was more pronounced with the PQ treatment (Figure 4.1).

The MDA content in cells ressuspended in PBS buffer and exposed to PQ was 3- and 10-fold higher than in the experiments under 5 bar for *Y. lipolytica* and *P. pastoris* cells, respectively. The exposure of cells in YPD medium to H_2O_2 led to a 6- and 2-fold enhancement in MDA production compared to 5 bar of air pressure for *Y. lipolytica* and *P. pastoris* cells, respectively. Interestingly, it seems that lipid peroxidation with hydrogen peroxide is minimized, being *P. pastoris* less sensitive to the stress caused by H_2O_2. Thus, it is reasonable to conclude that the stress caused by an increase of partial oxygen pressure did not lead to membrane destructive processes.

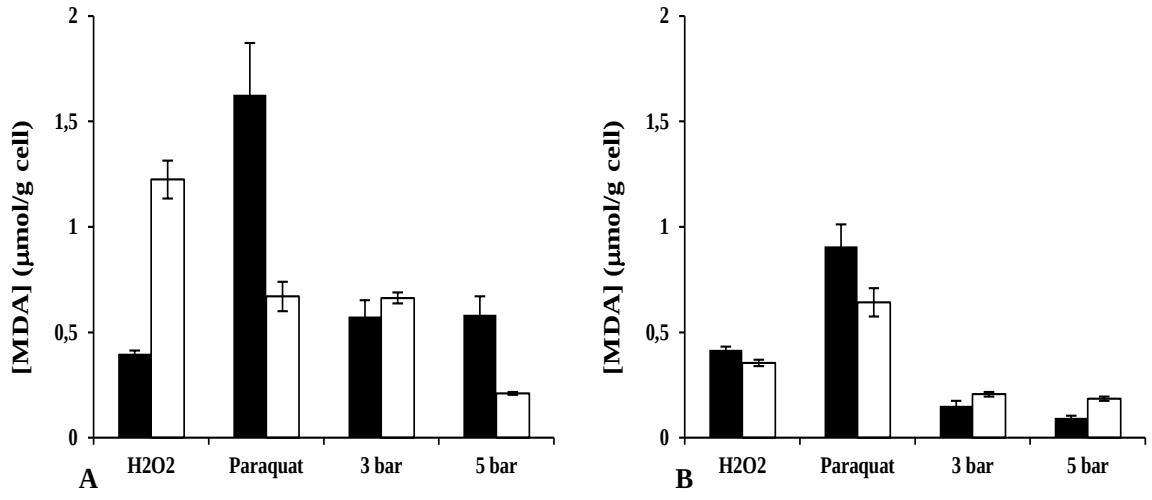

Figure 4.1 MDA content of (A) *Y. lipolytica* W29 and (B) *P. pastoris* CBS 2612 cells in exponential phase of growth, ressuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average ± standard deviation of three independent experiments.

It seems that there was no direct relation between the medium composition and the induction of lipid peroxidation. Although the *Y. lipolytica* strain exposed to PQ showed a 2.4-fold increase in MDA content when the cells were in PBS buffer compared to YPD medium, the addition of the H_2O_2 led to a 3-fold enhancement of MDA in PBS buffer.

The involvement of oxidative mechanisms to mediate the damaging effects of oxidant agents tested has been first shown by the analysis of GSH. According to our results, only the *Y. lipolytica* strain has shown the capacity to induce GSH production in the experimental conditions (Figure 4.2). It is likely that *P. pastoris* possesses other defense mechanisms more important than GSH in antioxidant response to stressors. Also, this low GSH production in *Pichia* might be responsible for the lower cell viability in PBS medium observed for this yeast as compared to *Y. lipolytica*.

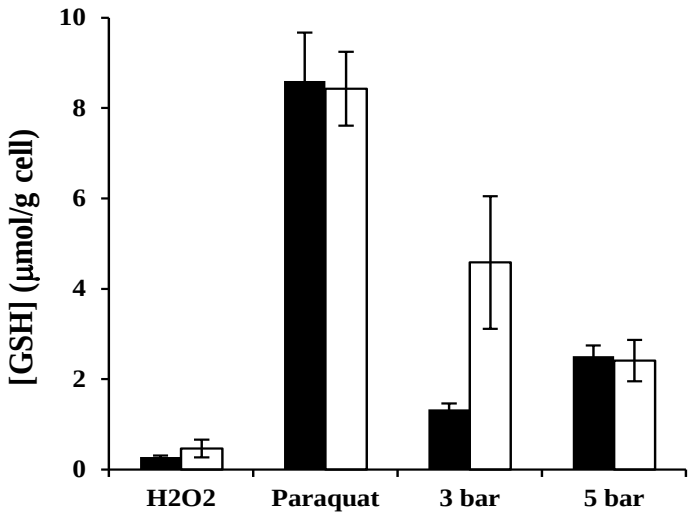

Figure 4.2 GSH content of *Y. lipolytica* W29 cells in exponential phase of growth, ressuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average ± standard deviation of three independent experiments.

The exposure of *Y. lipolytica* cells to PQ led to a 30-fold enhancement on GSH induction comparatively to the experiments conducted with hydrogen peroxide. Also, GSH was more induced with air pressure than with H_2O_2. The cells exposed to hydrogen peroxide showed a minor GSH induction. The GSH content in experiments with H_2O_2 was 30- and 9-fold smaller than the value obtained with PQ and 5 bar of air pressure. Among the oxidative stress agents, PQ is a thiol-oxidizing agent resulting in fast oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). The comparison of GSH in cells treated with PQ and increased air pressure indicates that the thiol oxidation should be small up to 5 bar of air pressure.

Glutathione is an essential reductant during normal metabolic processes in yeasts. Grant et al. (1996) demonstrated that a *S. cerevisiae* strain which lacks a functional copy of the *GSH 1* gene is hypersensitive to the peroxides and the oxidative stress conditions induced by the H_2O_2. Izawa et al. (1995) demonstrated that intracellular GSH played an important role in the stress response to H_2O_2 in *S. cerevisiae* using glutathione depleting agents and a glutathione-deficient mutant. This could explain the major decrease of yeast cells viability exposed to H_2O_2 comparatively to the others stressors agents, as reported above.

One of the principal antioxidant enzymes is superoxide dismutase that is involved in the dismutation of superoxide anions to dioxygen and hydrogen peroxide. In the experiments with yeast cells ressuspended in PBS buffer, both strains were unable to induce the SOD enzyme. This result may suggest that only in the presence of a respiratory carbon source the yeast strains used on this work have the capacity to induce this enzyme. The general inactivation of SOD observed after oxidant agents treatment in PBS buffer could also be the result of oxidative damage of this enzyme, because it is known to be inactivated by various peroxides. This probably reflects the decreased ability of the cells to adapt efficiently to the oxidative stress. Whereas SOD is considered to be an essential antioxidant enzyme, at the same time it can have pro-oxidant effects in vivo (Pigeolet et al., 1990), and thus SOD inactivation can also be the result of a cellular defense mechanism. Hassan and Fridovich (1978) found that *E. coli* cells responded to PQ by increasing their content of SOD in the presence of yeast extract, but not in its absence, leading to the assumption that yeast extract might have exerted its effect by eliciting the SOD biosynthesis.

Although there were differences in the other defensive mechanisms between the two yeasts tested (e.g. GSH and catalase), SOD induction was quite similar for both strains, with each oxidant treatment (Figure 4.3). This may be due to the fact that this enzyme is one of the primarily induced antioxidant mechanism involved in stress defense.

The experiments under air pressure up to 5 bar produced a clear increase in SOD activity compared to the exposure to H_2O_2 (69% higher for *Y. lipolytica* and 65% higher for *P. pastoris*) and PQ (57% higher for *Y. lipolytica* and 65% higher for *P. pastoris*). This higher SOD activity in the experiments under 3 bar and 5 bar of air pressure may explain the higher cell viability of the both strains. The importance of yeasts superoxide dismutase enzyme on triggering ROS generation by oxygen was also proved by Lushchak et al. (2005), who observed that *S. cerevisiae* strains carrying mutations in *SOD 1* and *SOD 2* genes were hypersensitive to oxygen, causing slow aerobic growth.

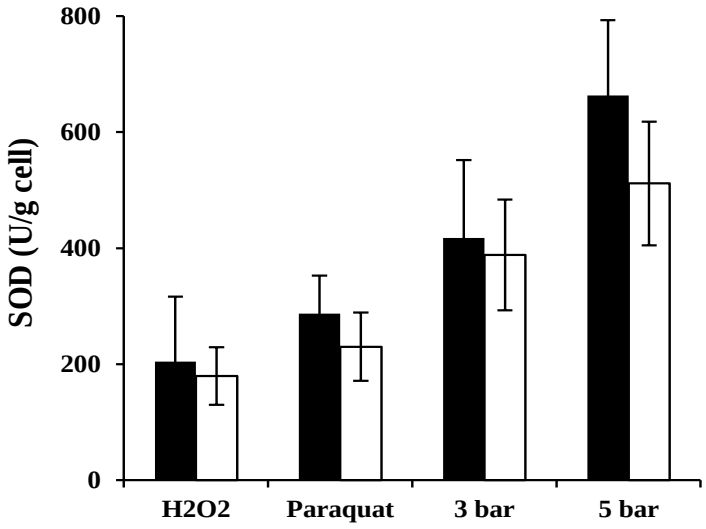

Figure 4.3 Superoxide dismutase specific activity of *Y. lipolytica* W29 (black bars) and *P. pastoris* CBS 2612 (white bars) cells in exponential phase of growth ressuspended in YPD medium and exposed to different oxidants. Values are average ± standard deviation of three independent experiments.

The lower SOD activity in yeast cells incubated with 50 mM H_2O_2 suggests that this enzyme does not participate in the cellular acclimatization to drastic oxidative H_2O_2-induced challenges. Abbeg et al. (2010) observed only a slight induction of SOD with the addition of H_2O_2 to the various *Candida* strains medium and Biryukova et al. (2006) found a slight induction of SOD with the pretreatment of *Y. lipolytica* cells with 0.3 mM H_2O_2.

The activation of catalase, observed following each oxidant stressors treatment, is one of the most common cellular responses to redox alterations, because this enzyme is easily induced by a wide range of stimuli often related to the energy status of the cell (Braconi et al., 2008).

In general, the oxidant treatments with cells ressuspended in PBS buffer showed a higher catalase activity compared to those with YPD medium (Figure 4.4). A 1.7-, 2.9-, 5- and 3.8-fold enhancement in enzyme activity was obtained when *Y. lipolytica* cells ressuspended in PBS buffer were exposed to H_2O_2, PQ, 3 bar and 5 bar of air pressure, respectively, compared to the activities with YPD medium. The glucose concentration can exert a negative pressure on the catalase activity of *Y. lipolytica* W29. The differences found in the experiments with *P. pastoris* were not so significant. On the other hand, as the yeast cells ressuspended in PBS buffer were not capable to

induce SOD, it can be concluded that both SOD and catalase operate in concert to protect cells from oxidative stress, acting in different ways.

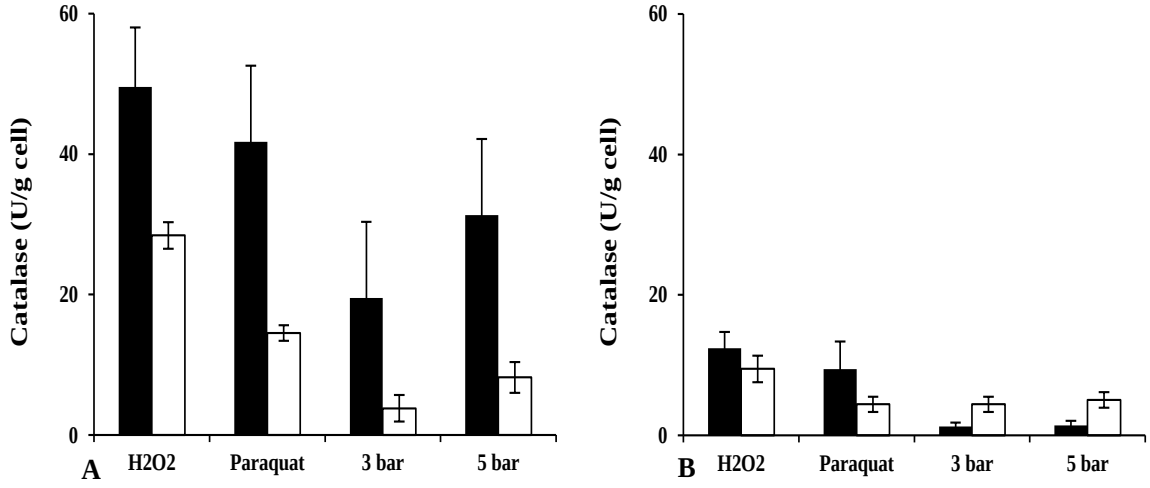

Figure 4.4 Catalase specific activity of (A) *Y. lipolytica* W29 and (B) *P. pastoris* CBS 2612 cells in exponential phase of growth, ressuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average ± standard deviation of three independent experiments.

When comparing the two yeasts, it was observed that *Yarrowia* showed a higher capacity to induce catalase after exposure to oxidant agents. A 4-, 16- and 23-fold enhancement was obtained in *Y. lipolytica* with H_2O_2 and PQ, 3 bar and 5 bar of air pressure, respectively, compared to the activities obtained with *P. pastoris*.

Particular attention should be paid to H_2O_2, the stress treatment that leads to a higher catalase synthesis in both yeast strains. The exposure of *Y. lipolytica* cells to H_2O_2 led to a 1.2-, 2.5- and 1.6-fold increase in catalase activity as compared to PQ, 3 bar and 5 bar of air pressure treatments. The enzyme activity of *P. pastoris* cells treated with H_2O_2 was 1.3, 9.9 and 8.9 times higher than that obtained in exposures to PQ, 3 bar and 5 bar of air pressure, respectively. Other authors have demonstrated that the treatment with H_2O_2 increased levels of catalase activity in *P. pastoris* (Smith et al., 1988), *C. albicans* (González-Párraga et al., 2003), various *Candida* strains (Abbeg et al.,

2010), *A. niger* (Kreiner et al., 2002), *Y. lipolytica* (Biryukova et al., 2006) and *S. cerevisiae* (Bayliak et al., 2006).

The enzyme glutathione reductase is involved in the glutathione recycling system. This enzyme enables the cell to sustain adequate levels of cellular GSH, once it is primarily responsible for the reduction of oxidized glutathione (GSSG) to reduced glutathione at the expense of NADPH.

Both yeast strains were able to induce glutathione reductase (Figure 4.5). However, the GR activity was considerably smaller than the SOD and catalase activities, suggesting that glutathione reductase plays a minor role on antioxidant defense against the agents tested. On the other hand, as the process of redox-cycling depletes intracellular NADPH (cofactor for glutathione reductase), the enzyme activity was likely to be affected by oxidative stress. It seems that there is a relation between the medium composition and the glutathione reductase induction, once the cells ressuspended in PBS buffer showed higher activity whatever the oxidant agent tested and yeast strain.

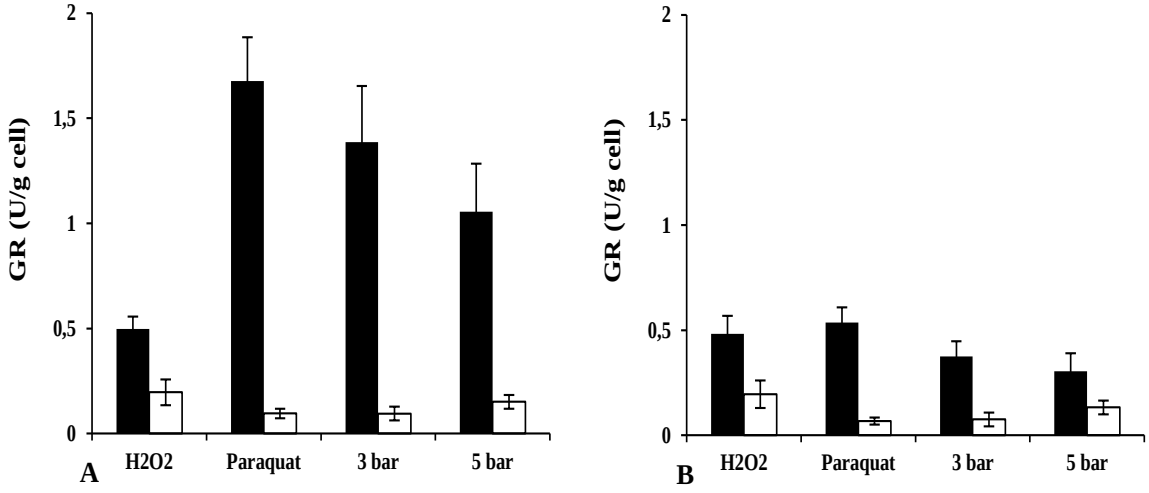

Figure 4.5 Glutathione reductase specific activity of (A) *Y. lipolytica* W29 and (B) *P. pastoris* CBS 2612 cells in exponential phase of growth, ressuspended in PBS buffer (black bars) and YPD medium (white bars) and exposed to different oxidants. Values are average ± standard deviation of three independent experiments.

In all the experiments with *P. pastoris* cells there was no significant difference in GR enzyme activity with any of the pro-oxidant agents. However, with the *Y. lipolytica*, cells treated with H_2O_2 showed less glutathione reductase activity. A 3.3-fold enhancement in enzyme activity was observed in the treatment with PQ compared to H_2O_2 exposure. It was not surprising that the higher activity of this enzyme was found for the agents that also lead to a more pronounced induction of GSH (PQ and increased air pressure), once this enzyme participates in the reduction of GSSG to GSH in the presence of NADPH.

4.3.3 Growth under increased air pressure

Since *Y. lipolytica* and *P. pastoris* showed to be able to adapt to pressures of 3 bar and 5 bar and it was showed that antioxidant defense mechanisms are induced under these conditions, batch cultures of the yeast strains under increased air pressure were performed to validate the resistance of the yeasts under this stress condition. Typical batch growth and glucose consumption profiles are shown in Figure 4.6. Both strains were able to grow for 24 h under air pressure values 5-fold higher than the atmospheric pressure.

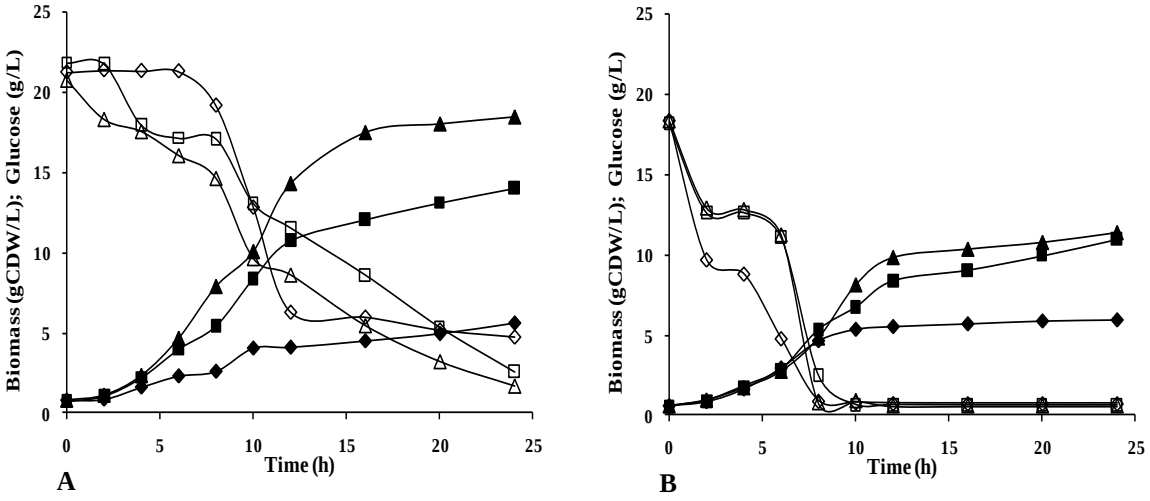

Figure 4.6 Batch growth (close symbols) and glucose consumption (open symbols) of (A) *Y. lipolytica* and (B) *P. pastoris* under pressures of 1 bar (♦), 3 bar (■) and 5 bar (▲).

Regardless of the yeast strain, the raise of total air pressure from 1 bar to 3 bar and 5 bar led to an increase in the final cell dry weight. 3.3- fold and 1.9-fold improvement in biomass production was obtained with the increase of air pressure up to 5 bar compared to 1 bar, for *Y. lipolytica* and *P. pastoris*, respectively. Among the yeast strains studied, the highest biomass yield was obtained with 5 bar of air pressure. An improvement in biomass yield of *Y. lipolytica* and *P. pastoris* cultures from 0.3 to 1.1 mass of cells per mass of glucose and from 0.3 to 0.6 mass of cells per mass of glucose, respectively, was achieved with increased air pressure up to 5 bar. The specific growth rate of *Y. lipolytica* was enhanced by the raise of air pressure. The values of specific growth rate obtained for *Y. lipolytica* at 5 bar was 36% higher than in the experiment at 1 bar. However, no significant differences were obtained on growth rates of *P. pastoris* when the pressure varies from 1 bar to 5 bar (0.27 h^{-1} – 0.29 h^{-1}). It is important to stress out that no inhibitory effects were observed in the cellular growth under high air pressures of 3 bar and 5 bar. These results confirmed the ability of the yeast to cope with oxidative stress conditions that can arise from the air pressure increase, since air pressure at to 5 bar is less leterious than other oxidant agents and cells were able to induce their antioxidant defenses.

4.4 CONCLUSIONS

Under normal physiological conditions, the toxic effects of ROS are minimized by enzymatic and non-enzymatic antioxidants. However, under stressful conditions, oxidant levels may increase to overwhelm the antioxidants, resulting in cell damage.

Our results suggest that *Y. lipolytica* have a more potent antioxidant system than *P. pastoris*, which was proved by the higher cell viability and enzymatic mechanisms induction.

Cells responses against both superoxide and peroxide stresses include enhanced expression of SOD and catalase, which are key enzymes for directly ROS scavenging. Under superoxide stress (PQ and air pressure), the SOD induction was the main observed mechanism. In contrast, and as expected, the effect of H_2O_2 treatment on antioxidant enzyme synthesis was much more pronounced for catalase than for SOD.

For the experimental conditions used in this work, an air pressure raise of up to 5 bar proved to be applicable to the batch cultivation of both *Y. lipolytica* and *P. pastoris*. The positive effects of air pressure on the growth behavior of strains, combined with the induction of antioxidant defenses against the superoxide anion, offers an opportunity to perform industrial bioprocesses based in these yeast strains, under increased air pressure, with increased biomass yields.

5 *YARROWIA LIPOLYTICA* GROWTH UNDER INCREASED AIR PRESSURE: INFLUENCE ON ENZYME PRODUCTION

Improvement of microbial cell cultures oxygenation can be achieved by the increase of total air pressure, which increases oxygen solubility in the medium. In this work, a pressurized bioreactor was used for *Yarrowia lipolytica* batch cultivation under increased air pressure from 1 bar to 6 bar. Cell growth was strongly enhanced by the pressure raise. Fivefold and 3.4-fold increases in the biomass production and in specific growth rate, respectively, were observed under 6 bar. The increase of oxygen availability caused the induction of the antioxidant enzyme superoxide dismutase, which indicates that the defensive mechanisms of the cells against oxidative stress were effective and cells could cope with increased pressure. The pregrowth of *Y. lipolytica* under increased pressure conditions did not affect the lipase production ability of the cells. Moreover, the extracellular lipase activity increased 96% using a 5-bar air pressure instead of air at 1 bar during the enzyme production phase. Thus, air pressure increase in bioreactors is an effective mean of cell mass and extracellular homologous enzyme productivity enhancement in *Y. lipolytica* cultures.

The information presented in this Chapter was published in:

Lopes M., Mota M., Belo I. (2009) *Yarrowia lipolytica* adaptation to oxidative stress induced by increased air pressure. New Biotechnol 25 (S1): S77-S78.

Lopes M., Gomes N., Mota M., Belo I. (2009) *Yarrowia lipolytica* growth under increased air pressure: influence on enzyme production. Appl Biochem Biotechnol 159: 46–53.

5.1 INTRODUCTION

Yarrowia lipolytica is a non-conventional yeast, nontoxic, that can grow to very high densities (Barth and Gaillardin, 1997). It is most used in studies of the biodegradative pathways for a variety of hydrophobic compounds including alkanes, oils, and fatty acids (Zvyagilskaya et al., 2004) and thus for its capacity to produce lipid-degrading enzymes, such as lipases.

The amount of oxygen available in culture media with *Y. lipolytica* is an important parameter since this organism is strictly aerobic. 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 (Aguedo et al., 2005; Charoenrat et al., 2006; Knoll et al., 2007). Moreover, the energy and cost efficiencies of high-pressure fermentation for industrial application have already been demonstrated (Knoll et al., 2005).

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 approximately 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, reactive oxygen species such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical are generated. The ROS may give raise to damage of enzymes, nucleic acids, or lipids (Izawa et al., 2005). To counter oxidative stress, cells constitutively express enzymes that detoxify the ROS and repair the damage caused by them. Antioxidant enzymes, such as catalase and superoxide dismutase, constitute the primary defenses of the cells because they are responsible for transforming these reactive oxygen species into non-reactive ones (Moradas-Ferreira et al., 1996).

The aim of this work is to investigate whether increasing air pressures, may be applied for the production of extracellular enzymes by *Y. lipolytica* W29, in a range of air pressure values that cells could grow without the effects of oxidative stress. Thus, the ability of the strain to induce antioxidant enzymes as a response to increased oxygen partial pressure was again monitored. Moreover, this paper reports an investigation into the influence of a pre-adaptation phase of cells to hyperbaric conditions on the lipase production by *Y. lipolytica* cells.

5.2 MATERIALS AND METHODS

5.2.1 Strain and media

Y. lipolytica W29 (ATCC 20460) was grown in yeast extract peptone dextrose (YPD) medium. The lipase production medium was composed of 6.7 g/L yeast nitrogen base (Pronadisa, 1545.1), 7 g/L olive oil, 5 g/L arabic gum, and 400 mM Tris–HCl buffer, pH 7.2.

5.2.2 Operating conditions

Yeast cells were pre-grown in 250-mL Erlenmeyer flasks filled with 100 mL of the YPD medium at 140 rpm, at 27°C of temperature, 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 medium, at 27°C, and 400 rpm in order to assess the effect of air and oxygen pressure in cellular growth and on antioxidant enzyme induction. Compressed air was continuously sparged into the culture at an aeration rate of 1 vvm. The values of air pressure studied were from 1 to 6 bar. The operating pressure was set by the manipulation of the pressure of the inlet 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. 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 investigating 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 was preceded by a 24 h growth in YPD medium at 1 bar or 5 bar of total air pressure.

5.2.3 Analytical methods

Culture samples were collected for analysis of cell concentration (optical density at 600 nm and cell number and converted to g cell dry per liter), total soluble protein, glucose consumption, and enzymatic assays. Total soluble protein was obtained by Bradford's method (Bradford, 1976). Glucose was determined using the 3,5-dinitrosalycilic acid method (Gonçalves et al., 2010).

Extracellular lipase was measured in the sample supernatant using p-nitrophenyl-butyrate in sodium acetate buffer 50 mM at pH 5.6 as a substrate, at 37°C 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 using 0.5% (w/v) azocasein in acetate buffer as substrate at pH 5.0, at 37°C for 40 min. One unit of activity was defined as the amount of enzyme that causes an increase of 0.01 of absorbance relative to the blank per minute under assay conditions. The antioxidant enzymes were measured after cell disruption and dialysis of cell extracts as described in section 4.2.4. Catalase was assayed using the method described by Beers and Sizer (1952) and SOD was quantified by the method of Marklund and Marklund (1974).

5.3 RESULTS AND DISCUSSION

5.3.1 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 5.1. The application of 6 bar air pressure stimulated cell growth compared to the atmospheric conditions.

The oxygen availability increase imposed by pressure raise had a clear positive effect on this yeast metabolism since the biomass production was enhanced 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 assay and in the bioreactor at 1 bar, respectively.

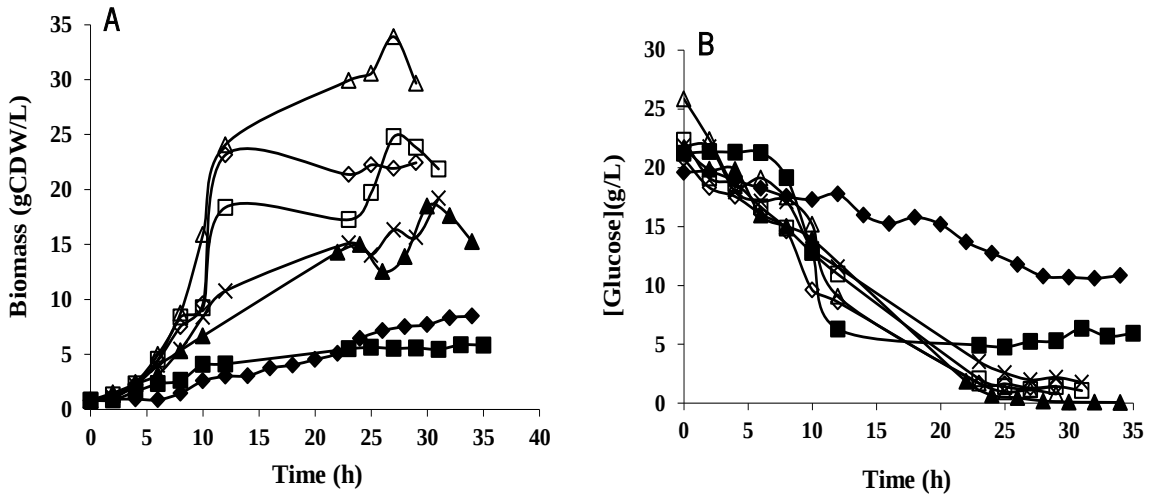

Figure 5.1 Batch growth (A) and glucose consumption (B) of *Y. lipolytica* W29 at atmospheric pressure (♦) and in hyperbaric reactor under pressures of 1 bar (■), 2 bar (▲), 3 bar (x), 4 bar (□), 5 bar (◊), and 6 bar (Δ).

The results described above are in accordance with the previous work of Lopes et al. (2008), in which no cellular activity inhibition by air pressure increase was detected in batch cultures of *Y. lipolytica*, albeit a different culture medium without glucose and a different bioreactor were used. In control experiment and 1 bar of air pressure in the bioreactor, glucose was not totally consumed. On the other hand, the raise of air pressure up to 6 bar led to a complete consumption of glucose.

Thus, the use of increased air pressure in *Y. lipolytica* W29 cultures might be exploited up to 6 bar to improve the biomass yield and productivity (Table 5.1). With 6 bar of air pressure, biomass yields increased 67.8% and 86.4% compared with the experiments under atmospheric pressure (control) and 1 bar, respectively. Also, a 4.1-fold improvement in biomass productivity was obtained with the increase of air pressure up to 6 bar compared to the control. Other non-conventional yeasts such as *Pichia pastoris* (as shown in previous chapter) and *Kluyveromyces marxianus* (Pinheiro et al., 2000) were successfully cultivated under increased air pressure with significant improvements on biomass productivity.

Table 5.1 Changes in biomass yield, specific growth rate and productivity with air pressure in batch experiments.

Pressure (bar)	$Y_{x/s}$ (g cells/g glucose)	μ (h^{-1})	P_x (g cells/L·h)
Control	0.53	0.09	0.25
1	0.35	0.18	0.17
2	0.70	0.19	0.45
3	0.97	0.23	0.62
4	1.04	0.26	0.71
5	1.18	0.28	0.77
6	1.21	0.31	1.02

However, for yeasts with respiro-fermentative metabolic pathways, like *S. cerevisiae*, the increase of air pressure led to a decrease on biomass productivity for a batch mode of operation (Pinheiro et al., 1997). In fact, for this yeast, air pressure increase showed to be useful for fed-batch mode of operation at operating conditions that allow fully respiratory metabolism and with a pressure increase program that enabled cellular adaptation to hyperbaric conditions (Belo et al., 2003). This shows that microorganisms react differently to the air pressure raise, depending also on other culture conditions (Hang and Zhong, 2003; L'italien et al., 1989; Knoll et al., 2007; Matsui et al., 2006; Onken and Liefke, 1989).

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 had more oxygen in the medium giving higher growth rates, and less time is necessary to obtain maximum cell concentration. The improvement of specific cellular growth of *Y. lipolytica* by *OTR* enhancement through other means than pressure was previously observed, as is the case of oxygen carrier use, like perfluorocarbons (Amaral et al., 2007).

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.

Through the optical microscopic observation of the cells, it was found that the cells displayed a typical oval form in all assays up to 6 bar (Figure 5.2). The results demonstrated that cell exposure to increased air pressure did not induce hyphae formation as reported by other authors as a result of oxidative stress due to chemical agents (Kawasse et al., 2003).

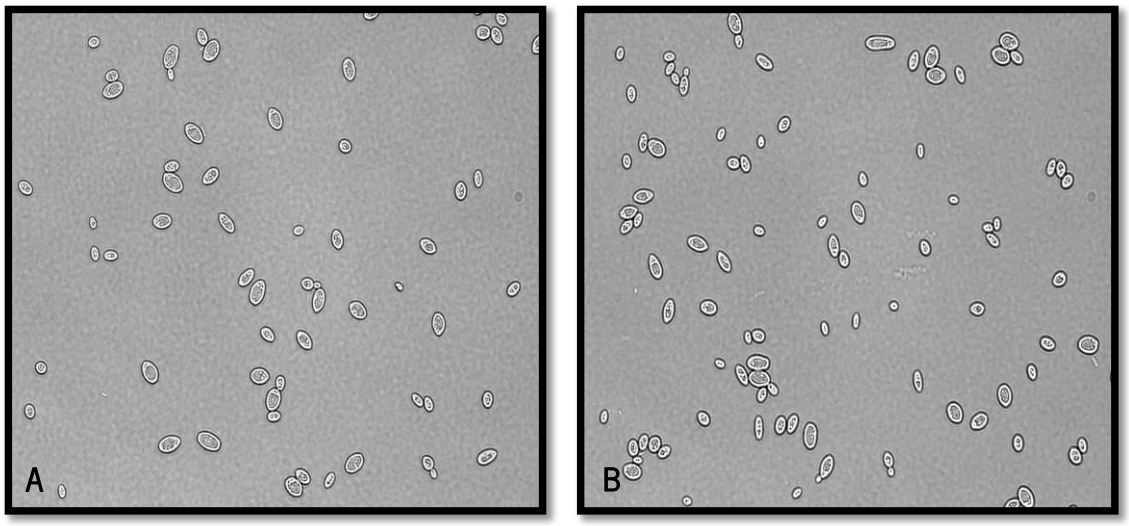

Figure 5.2 Microscopic observations (magnification 400x) of *Y. lipolytica* W29 grown at (A) 1 bar and (B) 6 bar.

5.3.2 Pressure effects on antioxidant enzyme activities

Intracellular antioxidant enzyme activities such as SOD and catalase under different air pressures were monitored since these were the antioxidant enzymes that present higher activities as a response to pressure in *Y. lipolytica* (Chapter 4). Figure 5.3 presents the data of the catalase and SOD-specific activities, measured at the end of the cell cultivation under hyperbaric conditions.

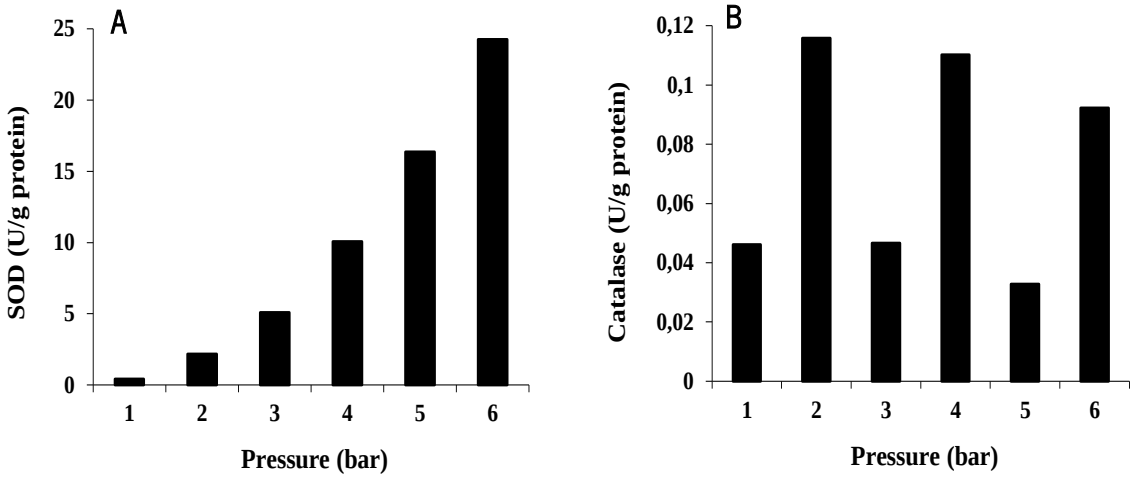

Figure 5.3 Effect of air pressure on (A) superoxide dismutase and (B) catalase specific activities, in the final batch cell cultures (approximately 30 h of growth).

Superoxide dismutase specific activity was induced by air pressure increase to a maximum at 6 bar. An increase of the SOD-specific activity at 6 bar (1.26 bar of oxygen partial pressure) of 53.4-fold was obtained compared with the experiments under 1 bar. This confirms the ability of *Y. lipolytica* cells to respond to the increase of reactive oxygen species formation caused by hyperoxygenation. The adaptive response of the yeast *Y. lipolytica* to the oxidative stress induced by the oxidants like hydrogen peroxide, menadione, and juglone has been shown to be associated with an increase in the activity of cellular superoxide dismutase and other main enzymes involved in cell defense against oxidative stress (Biryukova et al., 2006). In the herein presented work, the SOD induction showed the cell sensitivity to high dissolved 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 such high air pressure values up to 6 bar that corresponds to a 6-fold increase in oxygen solubility in the medium.

The influence of total air and oxygen pressure increase 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 caused by oxygen partial pressure increase, for *Y. lipolytica* W29. This is to our knowledge the first report on the response of SOD and catalase response to increased air–oxygen pressure in

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

5.3.3 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 pre-grown on the bioreactor at normal and increased pressure followed by a lipase production phase at normal and increased pressure.

This work shows that the increase of total air pressure influences enzymatic activity. As can be seen in Figure 5.4, 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.

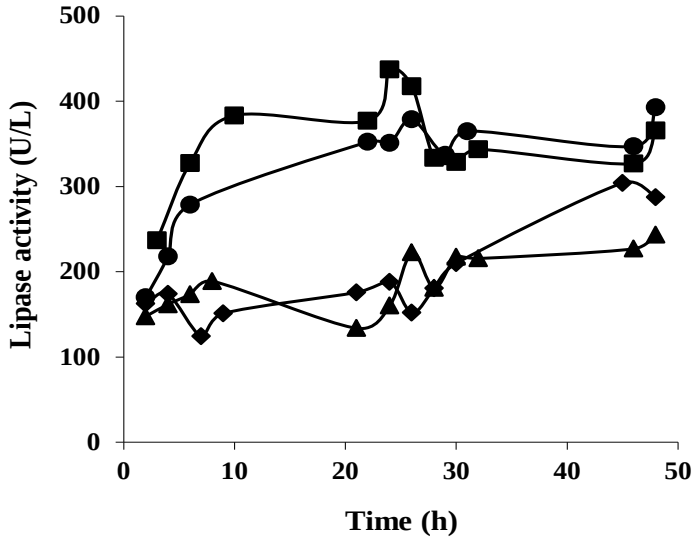

Figure 5.4 Extracellular lipase activity profiles by *Y. lipolytica* W29 during batch experiments with pre-adaptation to hyperbaric conditions: growth at 1 bar and production at 1 bar (♦), growth at 1 bar and production at 5 bar (■), growth at 5 bar and production at 1 bar (▲) and growth at 5 bar and production at 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 pre-grown at the same air pressure was similar to that obtained with cells grown under 1 bar. This indicates that responses of the cells, in what lipase production is concerned, occur irrespective of the pre-culture pressure conditions. 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. Contrary to this result, it was previously observed that pregrowth of *Y. lipolytica* W29 under 1 bar or 5 bar of air pressure had a strong influence in the activities of enzymes of the β-oxidation pathway of the methyl ricinoleate biotransformation to decalactones leading to considerable differences in metabolites production at 1 bar or 5 bar of total air pressure (Aguedo et al., 2005).

Besides lipase production, the production of other enzymes, such as proteases, by *Y. lipolytica* strains has been reported (Puthli et al., 2006). In spite of the fact that the culture medium conditions used favor lipase production, protease activity was detected in the medium. Figure 5.5 shows the results of monitoring protease secretion along time. 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) favors 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 enzyme due to its decomposition. In this work, highest value of protease production was reached at the same air pressure (5 bar) that the maximum lipase productivity was obtained but the pre-exposition of cells to increased air pressure reduced 40% the protease activity, leading to a decrease of the ratio between lipase and protease activities from 0.12 to 0.08, that may have important impact in subsequent downstream and purification processes.

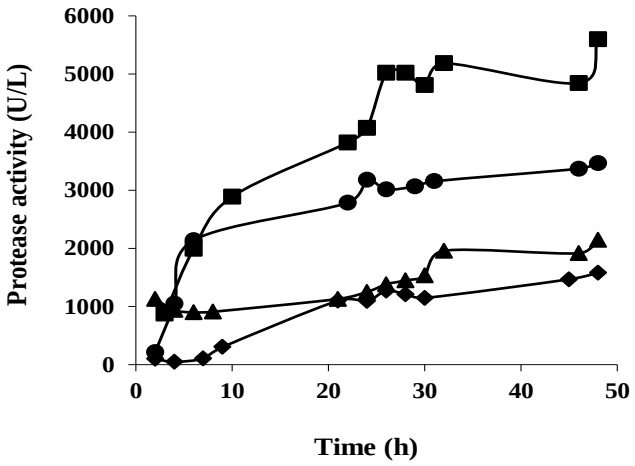

Figure 5.5 Extracellular protease activity profiles by *Y. lipolytica* W29 during batch experiments with pre-adaptation to hyperbaric conditions: growth at 1 bar and production at 1 bar (♦),growth at 1 bar and production at 5 bar (■),growth at 5 bar and production at 1 bar (▲) and growth at 5 bar and production at 5 bar (●).

5.4 CONCLUSIONS

For the experimental conditions used in this work, air pressure raise up to 6 bar proved to be applicable to the batch cultivation of *Y. lipolytica* W29. It has been demonstrated that the use of air pressure has positive effects on the growth behavior of the yeast and that air pressure may be a way of improving oxygenation and consequently 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 compared 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 at low levels during normal growth. Air pressure increase can be used for SOD induction, in the values of air pressure used in this work. *Y. lipolytica* W29 adapts rapidly to hyperbaric conditions; thus, these conditions can be imposed to cultures of this strain as a way of preventing oxygen limitation to cell growth and as a mean of enzyme production improvement such as lipases and SOD. Air pressure can be seen as an important operating parameter such as medium composition (Amaral et al., 2007) and strain selection (Destain et al., 2005) for lipase production optimization.

6 BATCH AND FED-BATCH GROWTH OF *PICHIA PASTORIS* UNDER INCREASED AIR PRESSURE

Pichia pastoris CBS 2612 behavior under air pressures of 1 bar, 3 bar 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.

The information presented in this Chapter was published in:

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Lopes M., Belo I., Mota M. (2013) Batch and fed-batch growth of *Pichia pastoris* under increased air pressure. Bioproc Biosyst Eng DOI 10.1007/s00449-012-0871-5.

6.1 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 (Cregg et al., 2000); and (2) since *P. pastoris* assimilates methanol, the expression system is linked with alcohol oxidase, which is abundantly produced in presence of methanol (Cos et al., 2006).

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* (Çelik et al., 2008). 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.

Published works have reported the use of increased air pressure as a way of improving the oxygen transfer rate that can be applied for cell cultivation with energy and capital cost efficiencies acceptable for industrial application (Knoll et al., 2005). In fact, the authors proved that the use of increased pressure can reduce the running costs when high oxygen transfer rates 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* (Lopes et al., 2008) and *Kluyveromyces marxianus* (Pinheiro et al., 2003). However, the effect of increased air and oxygen pressure is strongly dependent of the species and

strains (Coelho et al., 2004; Onken and Liefke, 1989; Pinheiro et al., 2000) 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 (Charoenrat et al., 2006). In Chapter 4, the enhancement of *P. pastoris* growth in glucose medium by air pressure increase was demonstrated.

In this study, it was intentended to extend the application of increasing air pressure (up to 5-fold above atmospheric pressure) as an alternative way of *OTR* improvement for *P. pastoris* cultures growing different carbon sources, such as methanol or glycerol (pure and crude), and in different modes of operation, like batch and fed-batch cultures.

6.2 MATERIALS AND METHODS

6.2.1 Oxygen transfer rate (*OTR*)

OTR in bioreactors was estimated in blank assays using the sulfite oxidation method (Cooper et al., 1944), as described on Chapter 3.2.1.

6.2.2 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 bar, 3 bar, 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 (Figure 6.1). The short interruption of aeration allowed the determination of the specific oxygen uptake (q_{O_2}) rate at exponential phase for each carbon source.

Figure 6.1 A 2-L bioreactor (BIOLAB, B. BRAUN, Germany) with *P. pastoris* culture growing in glycerol medium.

6.2.3 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 4563, Parr Instruments, USA) as illustrated in Figure 6.2. The values of absolute air pressure studied were 1 bar 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 h^{-1} 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 mL/min to 0.06 mL/min, according with the equation:

$$F = DV_0e^{Dt} \tag{6.1}$$

where F is the feed rate, D is the dilution rate, V_0 is 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 h or 120 h of the fed-batch phase, the process was switched to batch mode during 24 h.

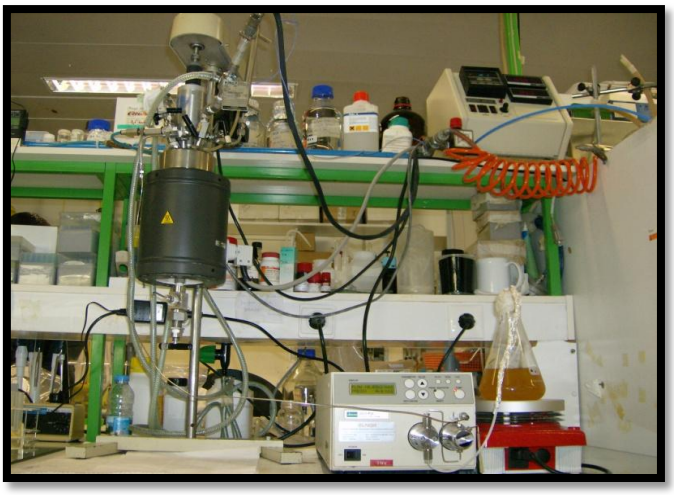

Figure 6.2 Pressurized bioreactor operating in fed-batch mode and high-pressure pump, drawing the feed medium into the reactor.

6.2.4 Analytical methods

Culture samples were collected (every 2 hours 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 M 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 (Bradford, 1976).

6.3 RESULTS AND DISCUSSION

6.3.1 Air pressure effect on batch cultures

Glycerol and methanol were used as carbon sources for *Pichia 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.

Firstly, 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 Figure 6.3.

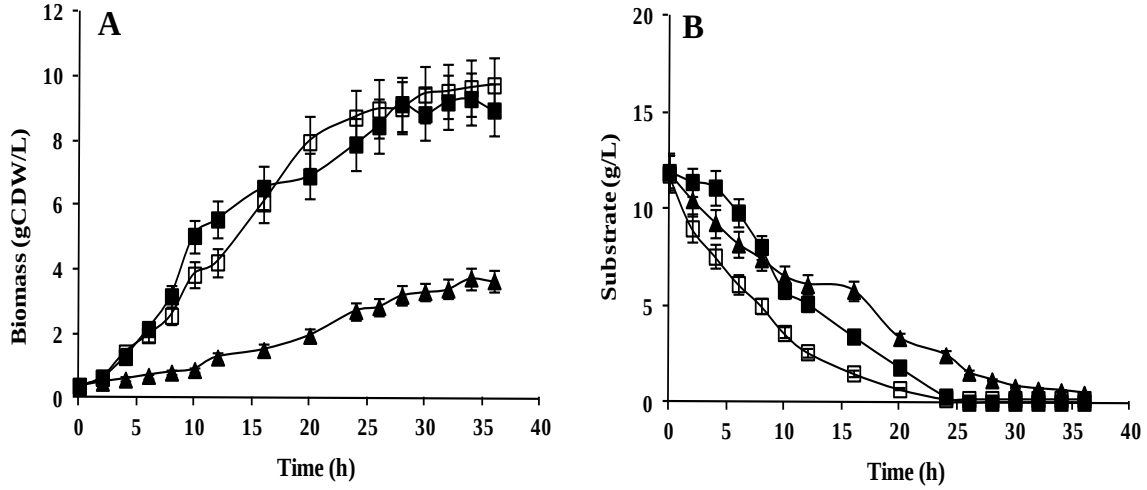

Figure 6.3 Batch growth of *P. pastoris* CBS 2612 (A) and substrate consumption (B) in 2L-Biolab bioreactor with (▲) methanol, (■) pure glycerol and (□) crude glycerol medium.

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 glycerol 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 (Figure 6.4). 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 genes (Cereghino and Cregg, 2000).

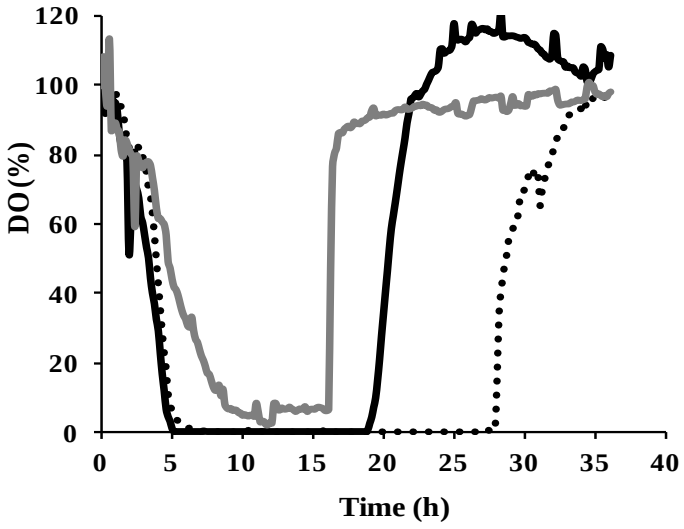

Figure 6.4 Time course of dissolved oxygen concentration at methanol (grey line), pure glycerol (black line) and crude glycerol (dotted line) medium.

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. (2007) observed a qO_2 value of 57 mg O_2/(g h) for recombinant *P. pastoris* in fed-batch with methanol. Solà et al. (2007), 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 of 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 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 metabolization of other components present in this biodiesel sub-product. In fact, in crude glycerol, oxygen depletion from the medium was observed for a longer period of time (Figure 6.4) than in the other carbon sources which indicates the need of improving oxygen transfer rate 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). *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 1152 mg O_2/(L h) to 5 bar (Chapter 3.3.1).

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 Figure 6.5.

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.

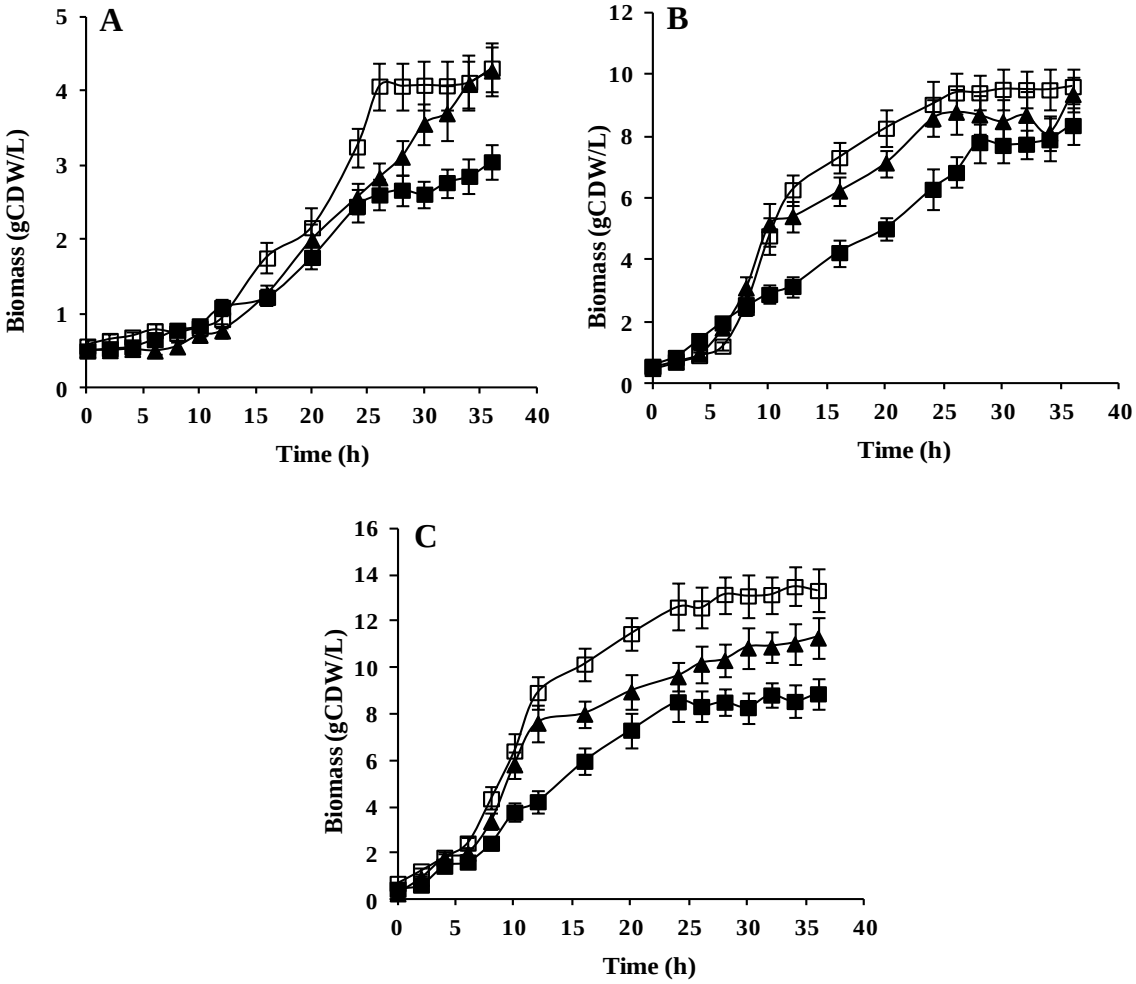

Figure 6.5 Batch growth of *P. pastoris* CBS 2612 in hyperbaric reactor under pressures of 1 bar (■), 3 bar (▲) and 5 bar (□), in (A) methanol, (B) pure glycerol and (C) crude glycerol medium.

Regardless of the carbon source, the raise of total air pressure from 1 bar to 5 bar led to an increase in the final cell dry weight. Compared to 1 bar, a 1.4-fold, 1.2-fold, 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 oxygen transfer rate from the air to the liquid phase, thus allowing the unlimited cellular growth. Similarly, Knabben et al. (2010) 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 Figure 6.6. The raise of total air pressure led to an earlier consumption of carbon sources.

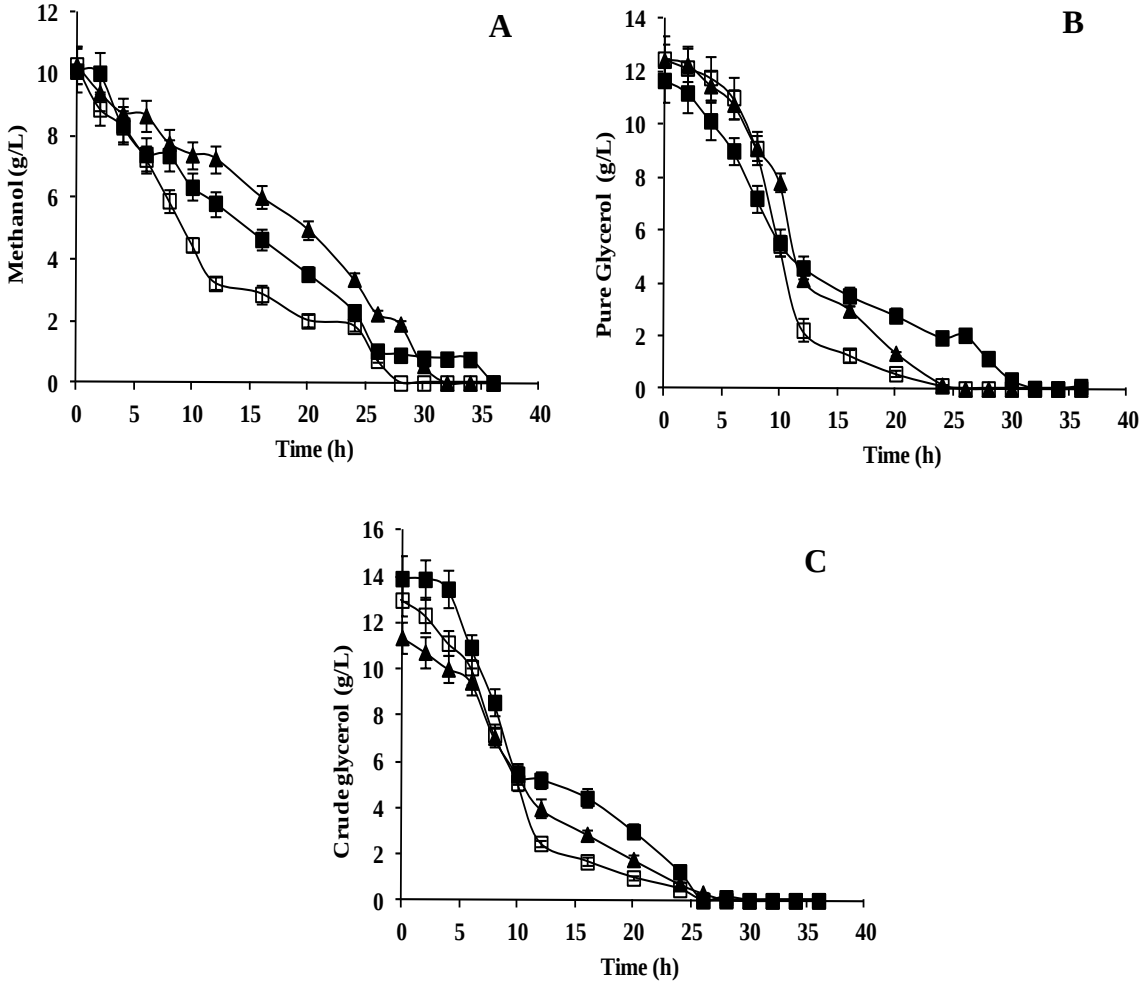

Figure 6.6 Methanol (A), pure glycerol (B) and crude glycerol (C) consumption of *P. pastoris* CBS 2612 in hyperbaric reactor under pressures of 1 bar (■), 3 bar (▲) and 5 bar (□).

Among the substrates studied, the highest biomass yield was obtained with glycerol (crude and pure), followed by methanol (Table 6.1). The raise of total air pressure to 5 bar caused a 1.6-fold and 1.4-fold improvement in biomass yield for crude glycerol and for 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.

Table 6.1 Changes in biomass yield and maximum specific growth rate in batch experiments under increased air pressure. Values are average ± standard deviation of three experiment replicates.

		1 bar	3 bar	5 bar
$Y_{x/s}$ (g cell/g 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

It is reasonable to speculate that the increase of total air pressure resulted in complete 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 (Gao and Ackman, 1995; Heinonen et al., 1997), and trace elements (Cindric et al., 2007) 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 (Walker, 1998) and increase in intracellular NAD level (Chen et al., 2007). Moreover, the yeast *P. pastoris* has the ability to use fatty acids as sole carbon and energy source (Wriessnegger et al., 2007).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 (Koleva et al., 2008) and 0.51 mass of cells per mass of substrate (Guo et al., 2007) and even 0.86 mass of cells per mass of substrate (Chiruvolu et al., 1999), 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 (2008) 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 glycerol 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) indicate 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. (2002) 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 (Xu et al., 1999)).

The specific cellular growth rate of *P. pastoris* was slightly enhanced by the increase of total air pressure for all carbon sources used (Table 6.1). 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 q_{O_2} obtained for this substrate, the increase in *OTR* by the values of pressure used, overcame the oxygen demand of the culture.

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 in different carbon sources. Thus, an increase of air pressure up to 5 bar may successfully be applied to the improvement of biomass production. Charoenrat et al. (2006) also showed that the cell mass productivity of *P. pastoris* cultures can be improved by the oxygen transfer rate 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 oxygen transfer rate 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. (2006) reported that the total activity of recombinant β-glucosidase of *P. pastoris* was enhanced by

increasing air pressure to 1.9 bar. Pinheiro et al. (2003) also demonstrated that the specific β-galactosidase production by *K. marxianus* increased 3 times using a 6-bar air pressure instead of air at atmospheric pressure. However, Belo et al. (1998) reported that the increase of air pressure from 2 bar to 4 bar showed a negative effect on cytochrome b5 heterologous expression by *E. coli* TB1 cells.

6.3.2 Air pressure effect on fed-batch cultures

As the results above demonstrated, the raise 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 glycerol 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 section.

The raise of air pressure up to 5 bar led to an increase in final cell mass for both carbon sources and feeding strategies. 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 (Figure 6.7), 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 bar and 5 bar was attained with this carbon source, compared to the other one.

With the exponential feeding rate strategy (Figure 6.8), when air pressure varied from 1 bar 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.

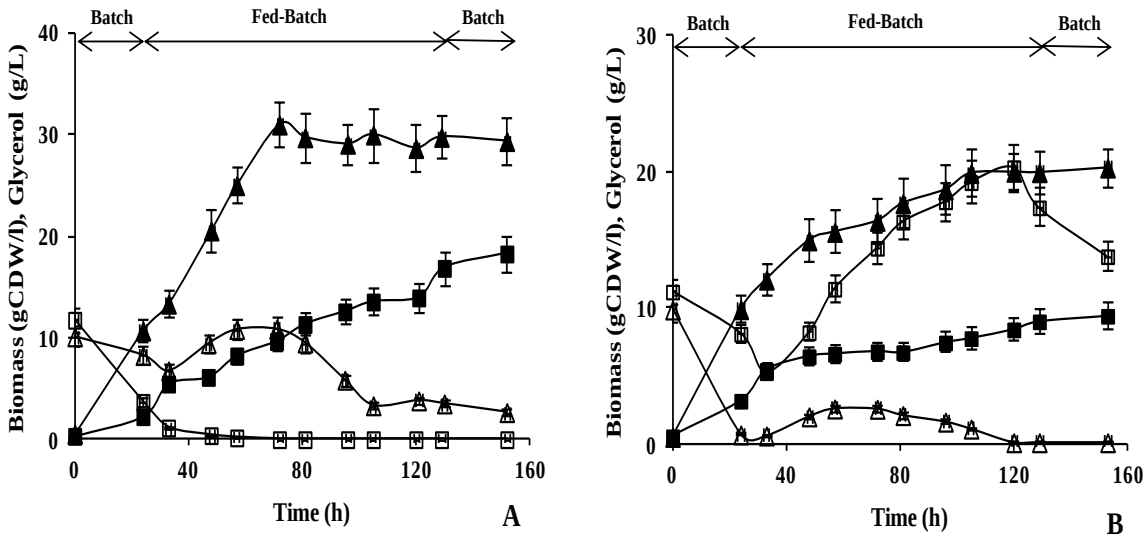

Figure 6.7 Fed-batch growth of *P. pastoris* CBS 2612 (■ 1 bar; ▲ 5 bar) and glycerol concentration (□ 1 bar; Δ 5 bar) in (A) pure glycerol and (B) crude glycerol with constant feeding rate strategy. The glycerol concentration in the medium feed was 50 g/L.

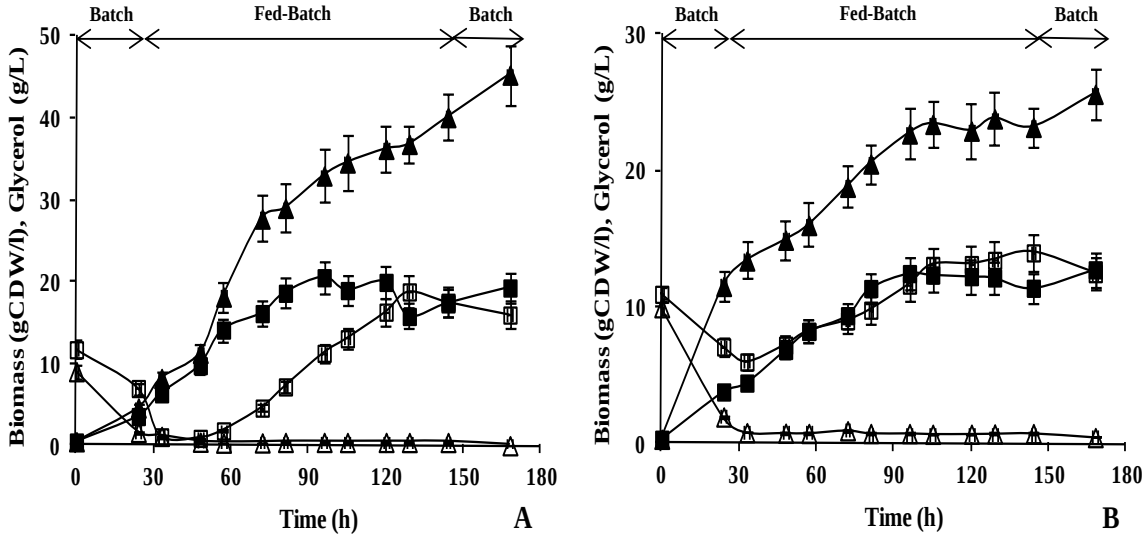

Figure 6.8 Fed-batch growth of *P. pastoris* CBS 2612 (■ 1 bar; ▲ 5 bar) and glycerol concentration (□ 1 bar; Δ 5 bar) in (A) pure glycerol and (B) crude glycerol with exponential feeding rate strategy. The glycerol concentration in the medium feed was 50 g/L.

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 6.2). With exponential feeding rate, the raise of air pressure to 5 bar caused 1.3-fold and 1.4-fold improvement in biomass yield per crude and pure glycerol, respectively. For the constant feeding rate, a 1.2-and 1.6-fold improvement in biomass yield was obtained at 5 bar compared to 1 bar, for crude and pure glycerol, respectively.

Table 6.2 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. Values are average ± standard deviation of three experiment replicates.

	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

Jahic et al. (2002) found a yield of 0.7 mass of cells per mass of substrate when *P. pastoris* cells growth 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 others researchers.

The differences on biomass yield between the two fed-batch strategies were more pronounced at 1 bar. Probably, 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% (Pyle et al., 2008). 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 ions of sodium, calcium and potassium presents could interfere with the ionic balance and affect the yeast metabolism.

6.4 CONCLUSIONS

For the experimental conditions used in this work, an air pressure raise 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.

7 HETEROLOGOUS PROTEIN EXPRESSION IN *P. PASTORIS* UNDER INCREASED AIR PRESSURE

Pichia pastoris is a widely used host for the production of heterologous proteins. However, the oxygen limitation generally has a detrimental effect on the expression of foreign genes. The increased air pressure could be used to improve the oxygen solubility in the medium and to reach the high oxygen demand of methanol metabolism.

In this study, two recombinant *P. pastoris* strains (GS115/pPICZ/*lac*Z and KM71H/ pPICZαA/frutalin) producing β-galactosidase and frutalin, respectively, were used to investigate the effect of increased air pressure on yeast growth and heterologous protein expression. Several experiments were carried out in a steel stainless bioreactor under total air pressure of 1 bar and 5 bar. The increase of air pressure up to 5 bar has a small effect on biomass production, but led to a 9-fold improvement in β-galactosidase specific activity compared to1 bar. Also, the recombinant frutalin secretion was enhanced by the increased air pressure up to 5 bar.

The protease specific activity reached at 5 bar was 2.4 times lower than that obtained at atmospheric pressure in baffled flasks. This result revealed that the use of increased air pressure up to 5 bar provided optimal conditions for reduction of the proteolysis that occurred on frutalin secretion in baffled flasks.

The information presented in this Chapter was submitted to *Enzyme Microb Tech:*

Lopes M., Oliveira C., Domingues L., Mota M., Belo I. Enhanced heterologous protein expression in *Pichia pastoris* under increased air pressure (January 2013).

7.1 INTRODUCTION

The methylotrophic yeast *Pichia pastoris* is currently one of the most effective and versatile systems for the expression of heterologous proteins. The increasing popularity of *P. pastoris* is attributed to: (1) its powerful and tightly regulated methanol-inducible alcohol oxidase 1 promoter (pAOX1) that is used to drive the expression of the foreign gene; (2) it can be easily manipulated at the molecular genetic level (e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation); (3) its ability to produce foreign proteins at high levels, intracellularly or extracellularly; (4) its capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation, and proteolytic processing; (5) the ability to grow on defined media at high cell densities; (6) its strong preference for respiratory rather than fermentative mode of growth (fermentation products include ethanol and acetic acid, which quickly reach toxic levels in the high cell density environment of a fermenter with strongly fermentative organisms); and (7) its ready availability as a commercialized expression kits (Cereghino et al., 2002; Cregg et al., 2000; Potvin et al., 2012). As a result of these characteristics, to date, more than 400 recombinant proteins have been cloned and expressed in *P. pastoris* systems (Plantz et al., 2006).

During the induction phase, *Pichia* cells utilize methanol through the oxidative pathway only when oxygen is non-limiting. The oxidation of methanol with molecular oxygen is the first step of both energy production and carbon assimilation (Baumann et al., 2008). 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 genes (Cereghino and Cregg, 2000). The dissolved oxygen levels are maintained at certain set points, typically between 15% and 20% of saturation, through agitation feedback control and by varying the oxygen content in the inlet air stream (Potvin et al., 2012). However, another strategy could be used to reach the large oxygen transfer rates (*OTR*) required at the high cell densities normally achieved in *P. pastoris* cultivation, such as the use of increased air pressure. This is particularly important for heterologous protein expression where high cell densities are reached.

Oliveira et al. (2008) reported the production of recombinant biologically active frutalin in the methylotrophic *P. pastoris* KM71H yeast strain. The recombinant frutalin was recognized by native frutalin antibody, its ability to bind galactose was maintained and may be used as histochemical

biomarkers for the prostate cancer (Oliveira et al., 2009). Due to the success of such assays, it would be interesting to study new strategies to improve the frutalin production.

Although *P. pastoris* has become a popular host for the expression and mass production of industrial proteins, few studies are available on the application of air pressure increase for the cultivation of this yeast and for the production of heterologous proteins, and only slight pressure increase has been applied, namely 1.2 bar (Woo et al., 2005), 1.5 bar (Cunha et al., 2004) and 1.9 bar (Charoenrat et al., 2006). To our knowledge, this is the first report on the study of production of recombinant proteins under increased air pressure up to 5 bar. Therefore, it was important to first understand the effect of increased air pressure on the growth of the two recombinant *P. pastoris* strains (Mut^+ and Mut^S) and then to address the effect on the expression level of two recombinant proteins (intracellular β-galactosidase and extracellular frutalin).

7.2 MATERIALS AND METHODS

7.2.1 Strains

The recombinant strains *P. pastoris* GS115/pPICZ/*lacZ* (His^-/Mut^+) (Invitrogen), expressing β-galactosidase intracellularly, and *P. pastoris* KM71H/pPICZαA/frutalin (Arg^+/Mut^S) (Oliveira et al., 2008), expressing frutalin extracellularly, were used. The expression of both proteins was under the control of alcohol oxidase 1 gene promoter by methanol induction. The construction of the *P. pastoris* strain KM71H secreting recombinant frutalin is described elsewhere (Oliveira et al., 2008).

7.2.2 Batch growth assays

P. pastoris GS115/pPICZ/*lacZ* and *P. pastoris* KM71H/pPICZαA/frutalin strains were pre-grown overnight in 250 mL Erlenmeyer flasks filled with 100 mL of BMGH (1.34% YNB, 1% glycerol, 4×10^{-5}% histidine, 400 mM potassium phosphate buffer pH 6.0) and BMG (1.34% YNB, 1% glycerol, 4×10^{-5}% biotin, 400 mM potassium phosphate buffer pH 6.0), respectively, at 30 °C and 200 rpm. Batch cultivations of each yeast strain were carried out using a 600 mL stainless steel stirred tank bioreactor (PARR 4563, Parr Instruments, USA), with 300 mL of BMGH or BMG medium, at 30 °C

and 400 rpm. The values of air absolute pressure studied were 1 bar and 5 bar and the compressed air was continuously sparged into the culture at an aeration rate of 1 vvm. An experiment in a baffled flask (500 mL) with 150 mL of each yeast culture, under atmospheric pressure and an agitation rate of 200 rpm was used as a control.

7.2.3 Induction assays

The recombinant yeasts were cultivated overnight in BMGH (*P. pastoris* GS115/pPICZ/*lac*Z) and BMG (*P. pastoris* KM71H/pPICZαA/frutalin), harvested and ressuspended in fresh BMMH (same composition as BMGH but glycerol is replaced with 0.5% (w/v) or 1% (w/v) methanol) and BMM (same composition as BMG but glycerol is replaced with 0.5% (v/v) methanol) medium, respectively. The induction assays were performed in the same stirred tank bioreactor used for batch growth assays, at 400 rpm, filled with 300 mL of medium, and 30 °C for β-galactosidase and 15 °C for frutalin. Fresh methanol (100%) was added to the medium every 12 h in order to keep the methanol concentration around 0.5% or 1% for β-galactosidase and 0.5% for frutalin. The values of air absolute pressure studied were 1 bar and 5 bar and the compressed air was continuously sparged into the culture at an aeration rate of 2 vvm. As a control, an induction experiment in a baffled flask filled with 150 mL of medium, under atmospheric pressure and an agitation rate of 200 rpm was carried out for *P. pastoris* KM71H recombinant strain.

7.2.4 Analytical methods

Culture samples were collected for analysis of cell concentration (optical density at 600 nm and converted to dry cell weight per liter), carbon source concentration and recombinant proteins production.

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).

β-galactosidase intracellular enzyme activity was measured after cell disruption and dialysis of cell extracts, as described on Chapter 3.2.1. The β-galactosidase activity was determined using 8.3 mM *o*NPG (*ortho*-nitrophenyl-β-D-galactoside) in Z buffer (100 mM potassium phosphate buffer pH 7.0 and 0.04 M β-mercaptoethanol) as the substrate. The release of *o*-NP (*o*-nitrophenol) was measured by following the increase in the absorbance at 405 nm for 16 min at 37 °C. One unit of enzyme activity was defined as the amount of enzyme that release 1 μmol of *o*-NP per minute under the conditions described. Total protein was obtained by Bradford's method (Bradford, 1976).

Protease in cell-free samples was quantified using 0.5% azocasein in acetate buffer as substrate at pH 5.0, at 37°C for 40 min. One unit of activity was defined as the amount of enzyme that causes an increase of 0.01 of absorbance relative to the blank per minute under assay conditions.

For the evaluation of the recombinant frutalin expression, supernatants from methanol-induced cultures were separated from yeast cells by centrifugation (10 min at 4000 *g* and 4 °C). The pH of the supernatants was increased to 7.5 by adding 10 M NaOH to precipitate salts, which were removed by centrifuging twice for 10 min at 4000 *g* and 4 °C. Treated supernatants were analyzed by SDS–PAGE with 12% gels, as described by Laemmli (1970). Bands were visualized by staining with Coomassie Brilliant Blue R250.

7.3 RESULTS AND DISCUSSION

7.3.1 Effect of air pressure on cellular growth

Two recombinant *P. pastoris* strains were chosen for this work: a *P. pastoris* GS115 strain, expressing intracellular *Escherichia coli* β-galactosidase, and a *P. pastoris* KM71H strain, secreting the plant lectin frutalin.

In order to understand the effect of increased air pressure on cellular growth of each yeast strain, several experiments were carried out in pressurized bioreactor under total pressure of 1 bar (equivalent to atmospheric pressure) and 5 bar. Also, an assay at atmospheric pressure in baffled flask was performed.

Typical batch growth curves and glycerol consumption profiles for the experiments under increased air pressure and atmospheric pressure are shown in Figure 7.1. The main difference between the two yeast strains was that recombinant *P. pastoris* KM71H grew better in glycerol medium than the recombinant *P. pastoris* GS115, since the final biomass concentration was higher for the first one. Also, glycerol consumption was complete for the KM71H strain, unlike for GS115 strain culture where after 24 h at least 4 g/L of glycerol still remained in the medium.

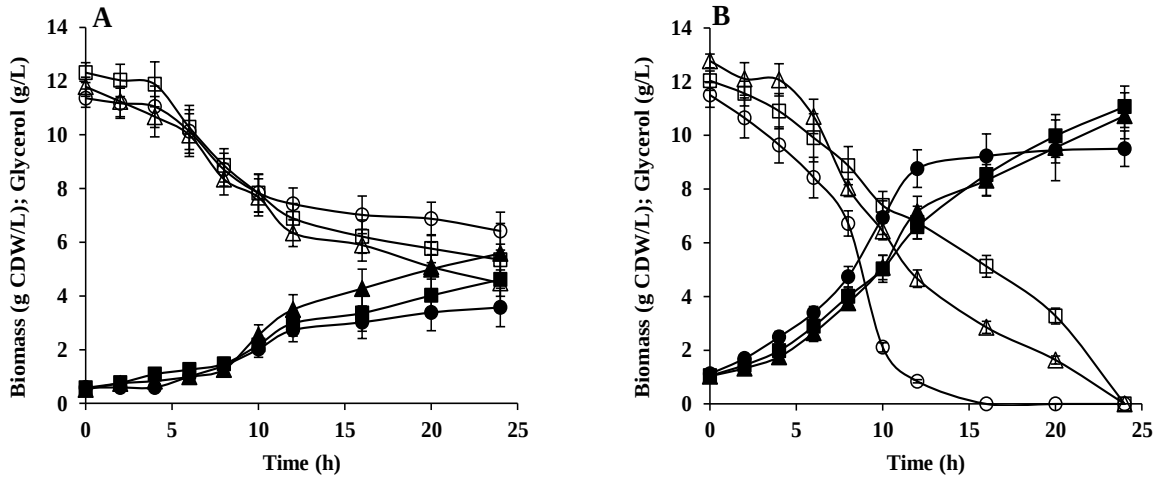

Figure 7.1 Batch growth (close symbols) and glycerol consumption (open symbols) of (A) *P. pastoris* GS115/pPICZ/*lac*Z and (B) *P. pastoris* KM71H/pPICZαA/frutalin in baffled flask (▲, Δ), and in pressurized bioreactor under 1 bar (■, □) and 5 bar (●, ○) of air pressure. Values are average ± standard deviation of two experiment replicates.

Regardless of the yeast strain, the increase of air pressure has a small effect on biomass production. Thus, no inhibitory effects were observed in the cellular growth under air pressure of 5 bar as compared to the control (baffled flasks). In fact, for all the pressure conditions used, similar values of specific cellular growth rates were obtained for both strains, 0.16 h^{-1} for *P. pastoris* KM71H and from 0.12 h^{-1} to 0.14 h^{-1} for *P. pastoris* GS115 cultures.

The biomass yield per glycerol consumed obtained for the strain KM71H was higher than that achieved for the GS115 strain. For each *Pichia* strain, no significant differences ($P < 0.05$) on the biomass yield were observed with the raise of air pressure up to 5 bar compared to 1 bar.

The effect of increased air pressure on cellular growth of different microbial cultures has been reported by various authors and is dependent on the strain. Charoenrat et al. (2006) reported a 12% enhancement on *P. pastoris* Y-11430 biomass under 1.9 bar of air pressure. Other non-recombinant microbial strains, such as *S. cerevisiae* (Coelho et al., 2004) and *Thermus* sp. RQ-1 (Belo et al., 2000) were successfully cultivated with hyperbaric air, improving the final biomass. However, above certain limits (11 bar) the increased air pressure had detrimental effects on microbial cell growth of *E. coli* K-12 (Matsui et al., 2006), *S. cerevisiae* (Belo et al., 2005) or *Pseudomonas putida* (Knoll et al., 2005).

The recombinant yeasts *P. pastoris* GS115 and *P. pastoris* KM71H were able to grown on methanol as a sole carbon and energy source. As occurred in glycerol medium, the increase of total air pressure inside the bioreactor did not led to an improvement in final biomass concentration (Figure 7.2). Nevertheless no inhibitory effects were observed in the cellular growth under air pressure of 5 bar as compared to the control, and the final biomass concentration was similar in baffled flask (operating at atmospheric pressure) and in pressurized bioreactor (operating at 1 bar and 5 bar).

Sufficient levels of methanol are required during the induction phase to ensure maximal protein production. Excessive methanol concentrations, typically ranging from 3.7 g/L to 20 g/L, are, however, cytotoxic and lead to growth inhibition. Concentrations between 2 g/L and 3.5 g/L are optimal for recombinant protein production (Cunha et al., 2004; Surribas et al., 2003; Zhang et al., 2000). The experiments in the bioreactor at both values of total air pressure led to a less accumulation of methanol in the medium than the observed in flasks, which indicates higher methanol utilization by the bioreactor cultures, since in these cases lower losses by evaporation were found. In the *P. pastoris* GS115 culture with addition of 0.5% methanol every 12 h, the accumulation of this carbon source in the baffled flask reached approximately 15 g/L. However, in the assay under 5 bar, even with addition of 1% methanol every 12 h, the methanol concentration in the medium remains lower than 3 g/L. Similar result was obtained for *P. pastoris* KM71H cultures.

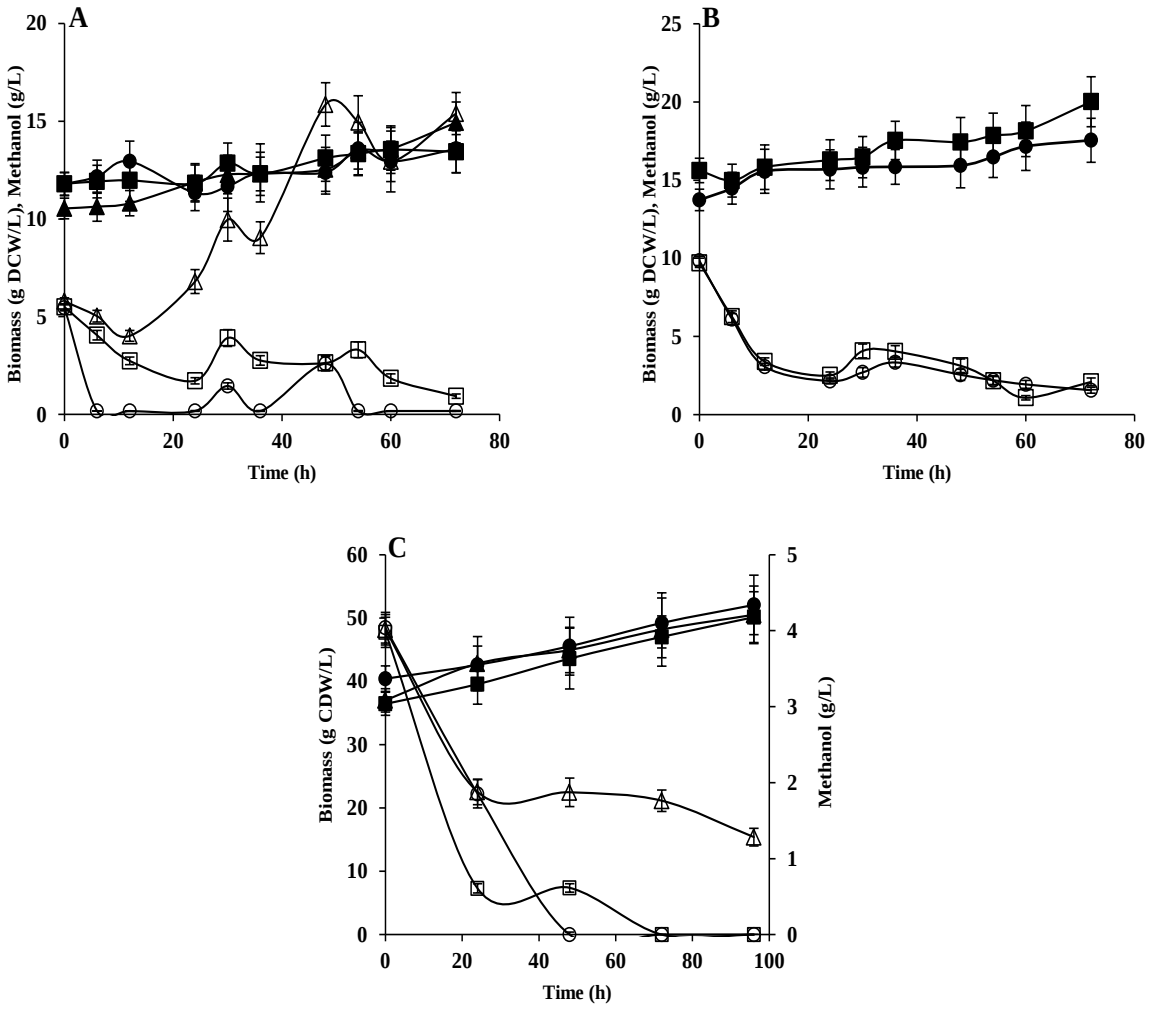

Figure 7.2 Fed-batch growth (close symbols) and methanol concentration (open symbols) of (A) *P. pastoris* GS115/pPICZ/*lac*Z with 0.5% methanol and (B) 1% methanol and (C) *P. pastoris* KM71H/pPICZαA/frutalin with 0.5% methanol in baffled flask (▲, Δ), and in pressurized bioreactor under 1 bar (■, □) and 5 bar (●, ○) of air pressure. Values are average ± standard deviation of two experiment replicates.

7.3.2 Effect of air pressure on recombinant protein production

Foreign proteins expressed in *P. pastoris* are affected by a variety of factors, such as temperature, pH, methanol feed strategy and, particularly, the dissolved oxygen in the medium. The optimization of increased air pressure as a way to improve oxygen mass transfer in *Pichia* cultures can represent an advantage in industrial recombinant protein production.

In order to study the effect of increased air pressure on intracellular (using β-galactosidase as a model) and extracellular (using frutalin as a model) recombinant protein expression, several assays were carried out in baffled flask (atmospheric pressure) and in pressurized bioreactor at 1 bar and 5 bar.

As the addition of 0.5% methanol to the baffled flask medium of GS115 strain resulted in a large accumulation of carbon source (Figure 7.2A), the induction experiment in this system was not carried out.

Firstly, the assays were performed with the addition of 0.5% (w/v) methanol every 12 h. As the accumulation of methanol in the medium remained lower than 5 g/L and 3 g/L in assays conducted at 1 bar and 5 bar, respectively, experiments with the addition of 1% (w/v) methanol every 12 h were also performed. Figure 7.3 shows the β-galactosidase specific activity profiles during the induction phase with methanol.

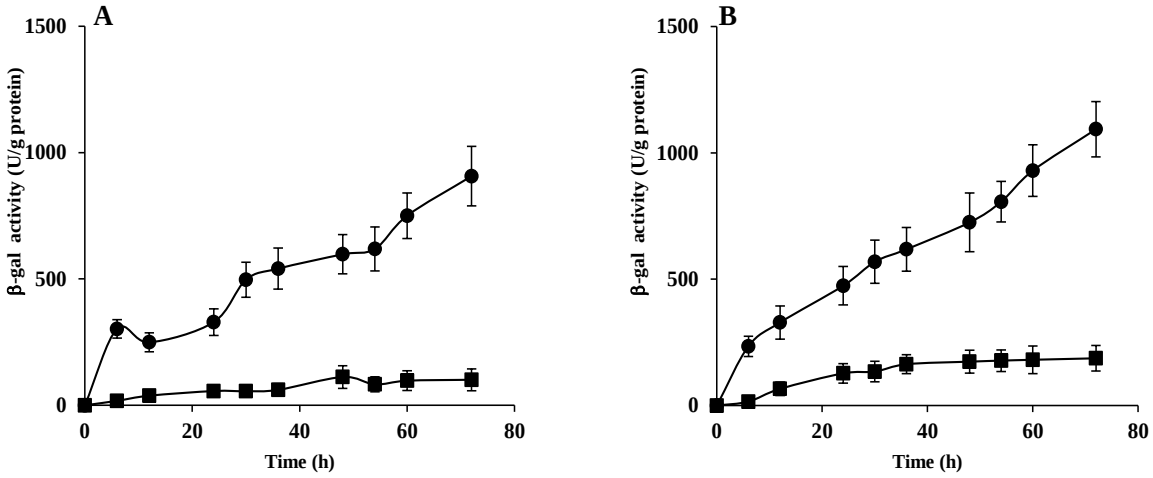

Figure 7.3 β-galactosidase specific activity profiles by *P. pastoris* GS115/pPICZ/*lac*Z inducted with (A) 0.5% methanol and (B) 1% methanol in the pressurized bioreactor at 1 bar (■) and 5 bar (●). Values are average ± standard deviation of two experiment replicates.

Even though the biomass production did not increased when the air pressure varied from 1 bar to 5 bar, an improvement of enzyme specific activity was observed with the increase of oxygen

availability in the bioreactor. Raising the total air pressure from 1 bar to 5 bar, a 9- and 5.8-fold improvement in β-galactosidase specific activity was reached, respectively in experiments with 0.5% and 1% methanol.

The methanol feed rate in pAOX1-regulated systems is one of the most important factors to control, as the residual methanol concentration directly influences the rates of production and oxygen transfer (Potvin et al., 2012). The increase of methanol addition from 0.5% to 1% to *P. pastoris* GS115 cultures led to an increment of β-galactosidase specific activity (Figure 7.3B). A 1.9- and 1.2-fold improvement in enzyme activity was achieved in the 1% methanol experiments at 1 bar and 5 bar, respectively, compared to the assays with 0.5% methanol. The higher enhancement observed at 1 bar can be due to the fact that the foreign genes can only be expressed in methanol limiting conditions.

As expected, recombinant frutalin was successfully expressed in *P. pastoris* as a secreted protein and could be observed in SDS-PAGE as a single band, having a molecular weight of about 17 kDa (Figure 7.4).

The recombinant frutalin was not detected in the first sample collected (0 h of induction; i.e., before induction) and the protein concentration increased gradually until the end of the cultivation time, reaching the maximal expression level after 96 h of methanol induction. The raise of air pressure up to 5 bar led to an improvement in frutalin secretion to the medium as show by the higher intensity of frutalin bands in SDS-PAGE analysis. For the experimental conditions used, the increased air pressure up to 5 bar proved to be applicable as a way to enhance the recombinant frutalin expression in pressurized bioreactor.

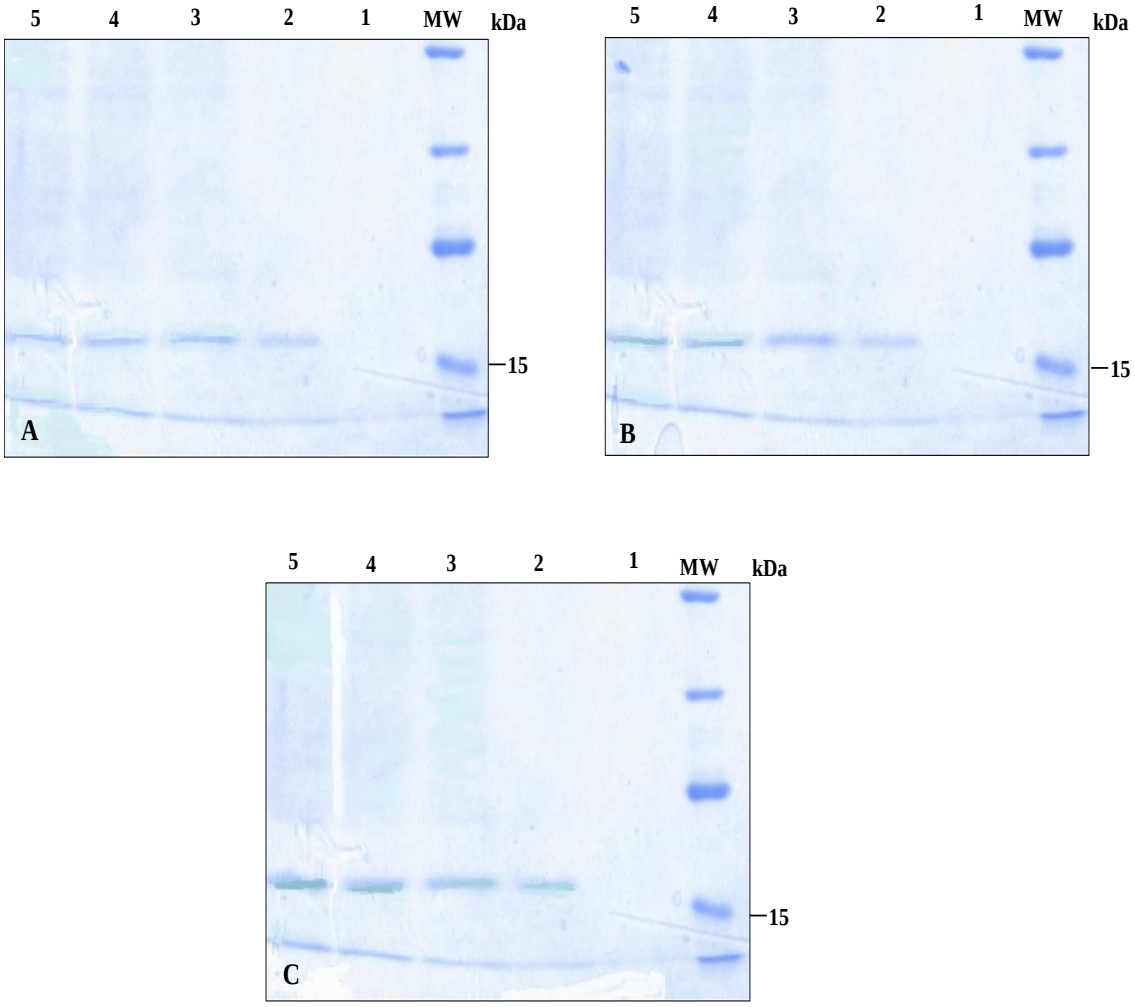

Figure 7.4 SDS-PAGE analysis of supernatants from the *P. pastoris* KM71H strain expressing recombinant frutalin in (A) baffled flask and in pressurized bioreactor under (B) 1 bar and (C) 5 bar of air pressure. Legend: 1, 0 h; 2, 24 h; 3, 48 h; 4, 72 h; 5, 96 h; MW, molecular weight standards.

Methanol metabolism utilizes oxygen at a high rate (Cregg et al., 2000) and it is well known the influence of oxygen supply on the heterologous expression by *P. pastoris*. Lee et al. (2003) indicated that in induction phase, maintaining a higher DO set point could significantly enhance elastase inhibiting peptide (EIP) expression. Jin et al. (2010) stated that with oxygen-enriched air (50% O_2), the effective pIFN-α expression period by recombinant *P. pastoris* KM71H (IFNα-pPICZαA) could be prolonged, resulting in a further enhancement in pIFN-α antiviral activity, comparatively to the strategy without DO control. However, Baumann et al. (2008) reported that

hypoxic fed-batch of recombinant *P. pastoris* led to 2-fold increased volumetric productivity of 3H6 Fab, human trypsinogen and porcine trypsinogen.

The results obtained in this work are in line with what has been previously reported in the literature for heterologous protein production under increased air pressure. Charoenrat et al. (2006) observed that for *P. pastoris* Y-11430, the recombinant β-glucosidase yield increased 50% in the moderate pressure process (1.9 bar). Belo and Mota (1998) reported that for *E. coli* TB1/pUC13 cells a 4-fold increase in the cyt.b5 final productivity was achieved by an air pressure increase to 4.8 bar. Matsui et al. (2006) reported that the production of tryptophan synthase by *E.coli/* pBR322trpAB increased in the air-pressurized culture, compared to the oxygen gas-enriched culture. The increased air pressure has been also successfully applied in homologous proteins production of other microbial strains. Lopes et al. (2008) reported that an increase of the lipase productivity at 5 bar of 3.7-fold was obtained compared with the experiments under 1 bar. Pinheiro et al. (2003) showed that it is possible to use the air pressure raise up to 6 bar as an optimization parameter of β-galactosidase production by *K. marxianus* CBS 7894.

One of the major drawbacks of *P. pastoris* expression systems is the post-secretory proteolytic degradation of recombinant products (Idiris et al., 2010). Some secreted proteins are unstable in the *P. pastoris* culture medium because they are rapidly degraded by proteases, which are over-expressed to the medium as a response of stress caused by methanol itself or by the transition from a given carbon source to methanol during the induction phase of pAOX-regulated cultures (Yamashita et al., 2009). Protease activity profiles over the course of *P. pastoris* KM71H strain induction are shown in Figure 7.5. In spite of the fact that the culture medium conditions used favors frutalin secretion, protease activity was detected in the medium. The total protease activity increased with time throughout the entire induction process. The same result was reported by Wu et al. (2008) in a recombinant *P. pastoris* expressing a human consensus interferon-α.

The highest value of protease production was found for the baffled flask assay, whereas in the experiment carried out in pressurized bioreactor under 5 bar its concentration in the medium was lower. In this work, highest value of protease production was reached in the same experiment (baffled flask) that the minimum frutalin expression was obtained. The presence of protease in culture medium can influence the stability of secreted frutalin and contributed to their higher degradation at atmospheric pressure.

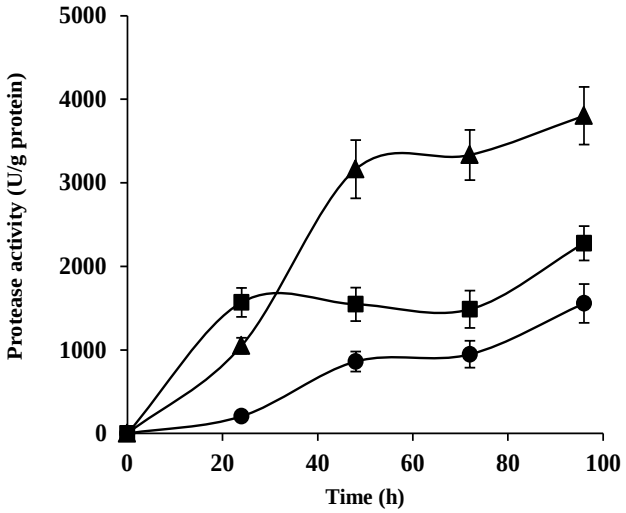

Figure 7.5 Protease specific activity profiles by *P. pastoris* KM71H/pPICZα/frutalin in baffled flask (▲) and in the pressurized bioreactor at different air pressures: 1 bar (■) and 5 bar (●). Values are average ± standard deviation of two experiment replicates.

There are reports in literature about strategies that have proven to be effective in minimizing the proteolytic degradation of recombinant proteins, namely: addition of amino acid rich supplements, such as peptone or casamino acids (Cereghino et al., 2001); adjust the pH of the culture medium to one that is not optimal for the problem protease and reduction of induction temperature (Jahic et al., 2003); and the use of a protease-deficient *P. pastoris* host strain (e.g. SMD1163, SMD1156 and SMD1168) (Cregg et al., 2000). The results reported herein revealed that the use of increased air pressure up to 5 bar provided optimal conditions for reduction of the proteolysis that occurred on frutalin secretion in baffled flasks.

7.4 CONCLUSIONS

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins, as illustrated by the large number of proteins synthesized in *P. pastoris*. Several strategies were developed in order to overcome the lower protein secretion and the high oxygen demand in methanol-induced cultures.

For the first time, air pressure up to 5 bar was applied for recombinant protein production improvement in *P. pastoris*. The production of intracellular (β-galactosidase) and extracellular (frutalin) heterologous proteins were tested in two different *Pichia* strains (Mut^+ and Mut^S). The air pressure raise had similar positive effects on the production of both recombinant proteins, which indicates that pressure can be an important factor of recombinant protein expression and can be used as a control parameter for heterologous protein production optimization.

The expression of intracellular and extracellular recombinant proteins was enhanced by increased air pressure up to 5 bar, contrarily to what happened with cellular growth, which is an indirect evidence that oxygen demand played a greater role for recombinant protein production than for cellular growth. Furthermore, the results reported herein showed that recombinant frutalin, a lectin with a high diagnostic/therapeutic potential application, can be produced under increased air pressure in the heterologous *P. pastoris* system in higher amounts than that obtained using standard culture conditions.

8 GENERAL CONCLUSIONS AND FINAL REMARKS

This chapter presents the concluding remarks and the main outcomes of this thesis.

Regarding the results obtained in this thesis, the suggestions for future work are also presented.

8.1 GENERAL CONCLUSIONS

In industrial biomass production, yeasts are required to have an efficient metabolism, with high productivities, in order to achieve an economical production process. During a biotechnological process differences in operational conditions take place, like pressure gradients, leading to changes in cell metabolism. In a typical industrial cell cultivation system, quite high cell densities are reached and oxygen is usually the major growth limiting factor. The use of increased pressure in bioreactors may be a way of improving oxygen transfer rate of aerobic cultures avoiding oxygen limitation. Although there are published works describing the use of increased air pressure on microbial cultures, their effects are strongly dependent of the species and strains. Thus, the study of other microbial strains, with relevance to industrial bioprocesses, is of great importance.

This work started with a study about the influence of increased air pressure on oxygen mass transfer rate (*OTR*). The use of increased air pressure up to 5 bar proved to be a successful way to improve *OTR*, that can be applied to avoid the shear stress to the cells caused by the increased stirring rates. An empirical correlation to predict the oxygen volumetric mass transfer coefficient (k_La) value as a function of pressure, power input and superficial gas velocity was established. It was demonstrated that the k_La increase was higher with the raise of absolute pressure than with the superficial gas velocity and specific power input. The proposed correlation for k_La prediction could be very useful for further work on the development of strategies for the optimization and scale-up of the processes where oxygen transfer is a limiting factor.

The cellular response of *Y. lipolytica* W29 and *P. pastoris* CBS 2612 (at exponential phase of growth) to the exposure (3 h) to the ROS-inducing agents paraquat (1 mM), hydrogen peroxide (50 mM) and increased air pressure (3 bar and 5 bar) was analyzed in Chapter 4. For both strains the cellular viability loss and lipid peroxidation was lower for the cells exposed to increased air pressure than for those exposed to chemical oxidants. Under superoxide stress (paraquat and air pressure), the superoxide dismutase (SOD) induction was the main observed mechanism. In contrast, and as expected, the effect of H_2O_2 treatment on antioxidant enzyme synthesis was much more pronounced for catalase than for SOD. The results suggest that *Y. lipolytica* have a more powerful antioxidant system than *P. pastoris*, which was proved by the higher cell viability and enzymatic mechanisms induction.

As the results obtained in Chapter 4 proved that *Y. lipolytica* culture underwent no cellular growth inhibition with increased air pressure, a pressurized bioreactor was used for yeast batch cultivation under air pressure up to 6 bar. The pressure raise up to 6 bar led to a 5-fold and 3.4-fold improvement in the biomass production and in specific growth rate, respectively, comparatively to atmospheric pressure. The increase of oxygen partial pressure caused the induction of the antioxidant enzyme SOD, which indicates that the defensive mechanisms of the cells against oxidative stress were effective and validate the results obtained in previous chapter.

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 pre-grown on the bioreactor at normal and increased pressure (5 bar) following by a lipase production phase at normal and increased pressure. The extracellular lipase activity increased 96% using a 5-bar air pressure instead of air at 1 bar pressure during the enzyme production phase, regardless of the pre-culture pressure conditions. However, the pre-exposition of cells to increased air pressure seems to slight reduce the protease activity. The results obtained in this study proved that air pressure increase in bioreactors is an effective way of cell mass and enzymes productivities enhancement in bioprocess based in *Y. lipolytica* cultures.

In Chapter 6, it was investigated whether increasing air pressures (5-fold above atmospheric pressure) may be applied as an alternative way of *OTR* improvement in *P. pastoris* CBS 2612 cultures growing in methanol or glycerol (pure and crude) as carbon sources, in batch and fed-batch cultures. In batch cultures, 1.4-fold, 1.2-fold, 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 raise of air pressure to 5 bar using exponential feeding rate leaded to 1.4-fold improvement in biomass yield per glycerol mass consumed, for crude and pure glycerol. The 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.

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins and several strategies were developed to overcome the lower protein secretion and the high oxygen demand in the methanol induction medium. For the first time, air pressure up to 5 bar was applied for recombinant protein production improvement in *P. pastoris*.

The production of intracellular (β-galactosidase) and extracellular (frutalin) heterologous proteins were tested in two different *Pichia* strains (Mut^+ and Mut^s). The expression of intracellular and extracellular recombinant proteins was enhanced by increased air pressure up to 5 bar, contrarily to what happened with cellular growth, which is an indirect evidence that oxygen demand played an greater role for recombinant protein production than for cellular growth. Moreover, the results revealed that the use of increased air pressure up to 5 bar provided optimal conditions for reduction of the proteolysis that occurred on frutalin secretion in baffled flasks.

8.2 SUGGESTIONS FOR FUTURE WORK

Although the present work brings new insights on the effects of increased air pressure on non-conventional yeasts growth, enzymes production and induction of antioxidant defenses, new tips for further research arose. Firstly it is necessary to extend the range of air pressure used in this work and perform experiments with other gases, namely oxygen—enriched air and pure oxygen.

It would be interesting to extend the study of the effects of increased air pressure to other biotechnological processes with industrial relevance, such as production of single cell oil or citric acid by *Y. lipolytica* (since this yeast is strictly aerobic) or to other microbial species, namely, filamentous fungi.

In Chapter 7 was reported a preliminary study of the effect of increased air pressure on production of heterologous proteins by *P. pastoris*. This production must be optimized by fed-batch mode for the glycerol phase (in order to attempt a high density culture) and a different strategy for methanol feeding.

Since the reactive oxygen species are known to mediate the damage of cellular constituents, their measurement is useful to understand the mechanisms of cellular alterations under increased air pressure. The formation of peroxides can be assayed by monitoring the oxidation of non-fluorescent dihydrorhodamine 123 (DHR 123) to fluorescent rhodamine 123 (Rh 123). The formation of superoxide radical can assessed by dihydroethidium (DHE) oxidation to fluorescent ethidium (ET).

Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins. Thus, the analysis of protein oxidative damage in cells growing in hyperbaric conditions could be

carried out spectrophotometrically as protein carbonyl content using the 2,4-dinitrophenylhydrazine (DNPH) binding assay.

As pressure may have delicate effects on the metabolism and on gene expression of cells, it would be interesting to study the proteomic profile of the cells and the identification of genes involved in oxidative stress caused by hyperbaric air.

Several modifications on bioreactor could be performed in order to overcome some limitations found along this work, namely:

- Insert an oxygen probe and a pH control system (which in the case of PARR bioreactor is only possible with a separate vessel);
- Change the "in and out" valves in order to operate in continuous mode, allowing the study of microbial physiology under hyperbaric conditions at defined dilution rates.
- Introduction of a system to measure and analyse the gases out (CO_2 and O_2), allowing the evaluation of respiratory coefficient of the cells and thus to better conclude on the metabolism.

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SCIENTIFIC OUTPUT

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